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Influence of micropropagation on the ontogenetic phases of Paulownia

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Influence of Micropropagation on the Ontogenetic Phases of *Paulownia*

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ABSTRACT

The aim of this research was to examine whether the method of micropropagation and tissue source affects the early growth and development of *Paulownia* in the first six months following transfer from tissue culture and establishment in soil. This tree species was chosen as it is a fast growing, short-rotation timber tree and able to adapt successfully to new environments. It is easily established *in vitro* and has been micropropagated using a range of different techniques. Three methods of micropropagation were chosen: callus regeneration, somatic embryogenesis and the third method was inducing root suckers *in vitro*. The third method was developed during this study and has never been documented in other research. Newly established explants and stabilised explants that had been in culture for over 6 months were used to test the efficacy of these methods. Genotype was also another important aspect to examine, as clones of the same species have shown differing response to being micropropagated. Previous studies have not compared different methods of micropropagation and rarely past the initial stages of laboratory experiments to fully determine the influence they have on the explants development *ex vitro*.

Cultures were sourced from five clones (P1, P2, P3, P4, P5) of mature *Paulownia elongata x fortunei* stock plants. P1 was first established *in vitro* and had been micropropagated for five years to induce stabilisation. Newly established explants from clones P1, P2, P3, P4 and P5 had been established in culture for three months before being utilised for micropropagation analysis experiments. Examination of these methods *in vitro* showed that tissue sources from P1 were the easiest to manipulate and propagate *in vitro*. Callus regeneration was the most successful in its ability to produce explants and in large quantities. Initial callus experiments showed a significant response in shoot regeneration from stabilised cultures. Subsequent experiments showed a greater response from greenhouse material and newly established cultures, while stabilised cultures failed to produce shoots. Root sucker induction was also successful in stabilised and newly established clones of P1, however, it took a significant amount of time to induce root suckers and the quantity of material produced was limited. Somatic embryogenesis was unsuccessful in regenerating new shoots and the complexity of current methods made it difficult to develop a full protocol in this study.

Explants produced from callus regeneration and root sucker induction were transferred to the greenhouse, along with controls from stabilised and newly established cultures. All sources readily produced adventitious roots and there was a 100% survival rate upon transfer to the greenhouse. While initial comparisons showed slight variations in growth factors such

as height and floral development, these were not statistically significant. Any slight variation became indistinguishable after two months of growth. Most importantly, after six months, plants from all sources readily produced flowers, indicating that the explants retained the mature phenology of the parent material while being maintained in culture.

While callus regeneration and root sucker induction were successful in producing new explants *in vitro*, these methods had no effect on the overall growth and development under greenhouse conditions. All explants exhibited early flowering, which indicates that they maintained the mature characteristics of the parent material. This is not necessarily an undesirable outcome if the intention is to micropropagate mature tissue while still retaining their mature phenology. Ultimately, the method of micropropagation utilised is determined by what growth characteristic is desired and the purpose for which the plants are being propagated.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

(I) *incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;*

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Michael Pezzaniti

20th September 2017

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CHAPTER 1 INTRODUCTION

1.1 Paulownia

Paulownia Siebold & Zucc. (*Paulowniaceae*) is a fast growing, short-rotation timber tree with approximately nine species and a few hybrids native to China having been described (Yadav *et al*., 2013). All species are fast growing and able to adapt successfully to new environments. *Paulownia* trees originate from the temperate climates of East Asia, specifically China, Korea and southern Japan, where they have been cultivated for many centuries (Ede, Auger, & Green, 1997; Yadav *et al*., 2013). In their native environment, *Paulownia* trees produce a distinct broad conical crown and a ten year old tree will generally have a 30-40cm trunk (Zhu, Chao, Lu, & Xiong, 1986). They are normally winter deciduous, flowering in spring (Mar-Apr) and fruiting in summer and autumn (Jul- Nov) (eFloras, 2008).

Table 1.1: List of Paulownia species based on the Flora of China (Yadav et al., 2013; Zhu et al., 1986).

Species	Distribution	Uses
Paulownia elongata S.Y, Hu.	Anhui, Hebei, Henan, Hubei, Jiangsu, Shaanxi, Shandong, Shanxi	Timber
Paulownia fortunei (Seem.) Hemsl.	Anhui, Fujian, Guangdong, Guangxi, Guizhou, Hubei, Hunan, Jiangxi, Sichuan, Taiwan, Yunnan, Zhejiang, Vietnam	Timber
Paulownia tomentosa (Thunb.) Steud.	Anhui, Gansu, Hebei, Henan, Hubei, Hunan, Jiangsu, Jiangxi, S Liaoning, Shaanxi, Shandong, Shanxi, N Sichuan	Timber/Ornamental
Paulownia taiwaniana T.W. Hu & H. J. Chang	Fujian, Guangdong, Hunan, Taiwan and Zhejiang	Timber
Paulownia kawakamii T. Ito	Fujian, Guangdong, Guangxi, Guizhou, Hubei, Hunan, Jiangxi, Taiwan, Zhejiang.	Ornamental
Paulownia fargesii (Seem.) Hemsl.	Guizhou, Hubei, Hunan, Sichuan, Yunnan (Vietnam)	Timber
Paulownia catalpifolia T. Gong	Shandong (Zou Xian)	Timber

Paulownia has a variety of uses, most frequently for furniture manufacturing, building component production and as fertilizer and fodder (Bergmann, 1998; Tang, Chen, Song, He, & Cai, 2010). Because of their fast growth and short rotation times, *Paulownia* plantations are also valuable for carbon sequestration and reducing pressure on old growth forests (Bergmann, 2003; Xu, Zhang, & Shi, 2001). Their versatility and ability to thrive in nutrient deficient soils makes them a highly valuable commodity for the timber industry (Z. Ipekci, Altinkut, Kazan, Bajrovic, & Gozukirmizi, 2001). Profitable *Paulownia* plantations have been established in China, Japan, USA, New Zealand and more recently in Western Australia (Hardie, Kundt, & Miyasaka, 1989; Z. Ipekci *et al*., 2001; Perera, Bayliss, & Jones, 2005). *Paulownia* is highly valued in Japan and China and it has the potential to become a commercially viable alternative to other conventional timbers in Australia (Beel, Davis, Murphy, & Piper, 2005; Johnson, Mitchem, & Kreh, 2003; Perera *et al*., 2005). As such, *Paulownia* could become a valuable commodity to the Australian timber industry (Johnson *et al*., 2003). While the need for increased production expands, plantations must ensure that new materials used have the most desirable qualities. This includes characteristics such as the reliability of timber, trunk yield and ease of large-scale production.

The species of *Paulownia* used for plantation production is dependent upon many variables (Bergmann, 1998). Growth and form of *Paulownia* is highly changeable which can be due to variation in environmental factors of plantation sites, the initial characteristics of the stock plants chosen for propagation and finally the method of propagation (Bergmann, 2003). *Paulownia elongata* S.Y,Hu is the commonly chosen species for timber production as it outperforms other species in terms of yield potential (Bergmann & Moon, 1997). However, other varieties of *Paulownia* are still sought after for their particular growth characteristics (Bergmann & Whetten, 1998).

Intraspecific variability and interspecific crossing is high in *Paulownia* and, because of this, hybridisation of two species is a common occurrence (Zhu *et al*., 1986). Consequently, plantations of *Paulownia* may contain stock of unknown genetic heritage or hybrid origin (Finkeldey, 1992; Zhu *et al*., 1986). Because of this variability, care must be taken when choosing the method of propagation so that time and resources are not used counter-productively (Bergmann & Whetten, 1998).

While seedling reproduction is successful and commonly used to propagate *Paulownia,* this method has some disadvantages. Firstly, it produces plants of varying genetic characteristics as plants produced from seed cannot be guaranteed to have the same features as the parent plant (Finkeldey, 1992). Secondly, seedling germination is slower than other

methods of propagation such as root or shoot cuttings, and overall plant development is comparatively longer (Bergmann, 2003). Lastly, although some hybrids of *Paulownia* have been produced from seedlings, survival rates are low and so plants must be produced asexually to provide consistent growth rates and patterns (Bergmann, 2003). *Paulownia* can be grown using a number of conventional propagation methods (Bergmann, 1998; Bergmann & Moon, 1997; Ede *et al*., 1997) or through tissue culture (Yadav *et al*., 2013). Specialised laboratories in the USA have been established to grow *Paulownia* trees using micro-propagation techniques (e.g. Carolina Pacific International, Inc).

Paulownia clones used for plantations in Western Australia have exhibited some variation in form and development and this is most likely due to the hybrid nature of the plant source (N, Malajczuck. pers comm.). In particular, there is evidence to suggest that the time of canopy formation, an indicator of plant maturity, is linked to the type of tissue that is used to produce the plants (N, Malajczuck. pers comm).

The generally positive response of *Paulownia* to tissue culture and its capacity to regenerate this species from a wide range of methods, makes it an ideal species for examining the influence of different modes of propagation on development and ontogeny. This study examined the efficacy of several different micropropagation modes, and whether the method of production of *Paulownia* clones affects the early growth and development of the micropropagated shoots in the greenhouse. The first phase explores micropropagation: how the different tissue culture methods can be used to successfully micropropagate selected clones and how explants produced from different sources respond to them. The second phase examines the transition of the micropropagated explants to the greenhouse, specifically, the morphological characteristics of the shoots and whether the various tissue culture methods or explant sources affect development.

1.2 From Juvenility to Maturity

During their lifespan, plants express a wide variety of changes in morphological and physiological characteristics (Poethig, 1990; Yang, Conway, & Poethig, 2011). The ontogeny of trees is marked by five stages: germination, the juvenile vegetative phase, the mature vegetative phase, the reproductive phase and the eventually senescence. Changes in vegetative morphology usually occur with the progression from one phase to another and include variations in leaf shape, phyllotaxis and other growth patterns (Haffner, Enjalric, Lardet, & Carron, 1991; Robinson & Wareing, 1969; Smith *et al*., 2006; Wang *et al*., 2011).

The germination phase can be the most time consuming period for plantation managers,

as embryos can be slow to germinate, have low viability, exhibit genetic variability and are subject seasonal variances (George, 2008; Mendoza de Gyves, Royani, & Rugini, 2007; Moon, Park, Kim, & Kim, 2008). For these reasons, plantations frequently use vegetative propagation, harvesting the most juvenile parts of the plant to ensure successful ramet production (Moon *et al*., 2008).

It is during the juvenile phase that cuttings are most easily propagated (Wendling, Trueman, & Xavier, 2014b). Juvenile growth is orthotropic, and characterised by rapid growth in height, and juvenile tissues have a higher rate of survival due to their successful rooting capacity (Barthélémy & Caraglio, 2007; Wendling, Trueman, & Xavier, 2014a), all characteristics that are highly desirable for timber production. However, the juvenile phase in some trees may last as long as forty years (Wendling *et al*., 2014a), and this period is of considerable importance because mature characteristics determine the quality of the resulting wood and eventually the profitably of the plantation (Greenwood, 1995; Wendling *et al*., 2014b). For this reason, it is necessary to capture the desirable characteristics of mature tissue.

As the tree reaches maturity, vegetative development decreases, growth is slower, plagiotropic and directed on increasing in trunk width as opposed to height (Barthélémy & Caraglio, 2007; Wendling *et al*., 2014a). Mature tissue is harder to propagate, as the ability of cuttings to produce adventitious roots declines, which also decreases the survival of the cuttings. There may be no indication of phase change except when the tree enters the reproduction phase and begins to produce flowers (Wendling *et al*., 2014a).

1.3 The Juvenile Zone

Different parts of the plant can enter the maturation phase at different rates (England & Attiwill, 2006), meaning juvenile characteristics can still be found in some parts of a mature tree (England & Attiwill, 2006). For example, some tree species maintain juvenile foliage on their lower branches while producing flowers and morphologically distinct mature foliage closer to the top of the canopy (Husen & Pal, 2006; Munne-Bosch, 2007). Such regions are termed the juvenile zone, and new shoots that originate within it display characteristically juvenile morphology and physiology (England & Attiwill, 2006; Heuret, Meredieu, Coudurier, Courdier, & Barthelemy, 2006; Munne-Bosch, 2007). Root suckers are also considered part of the juvenile zone in trees (George, 2008) and are commonly used as a conventional propagation method.

It is important to balance the need for juvenile tissue that enables successful propagation and establishment with that of having a final product with the most desirable mature

characteristics. This in turn leads to the manipulation of conventional methods of vegetative propagation to successfully restore juvenility in mature tissues. A variety of methods can be applied to achieve this, including coppicing, serial grafting and re-rooting.

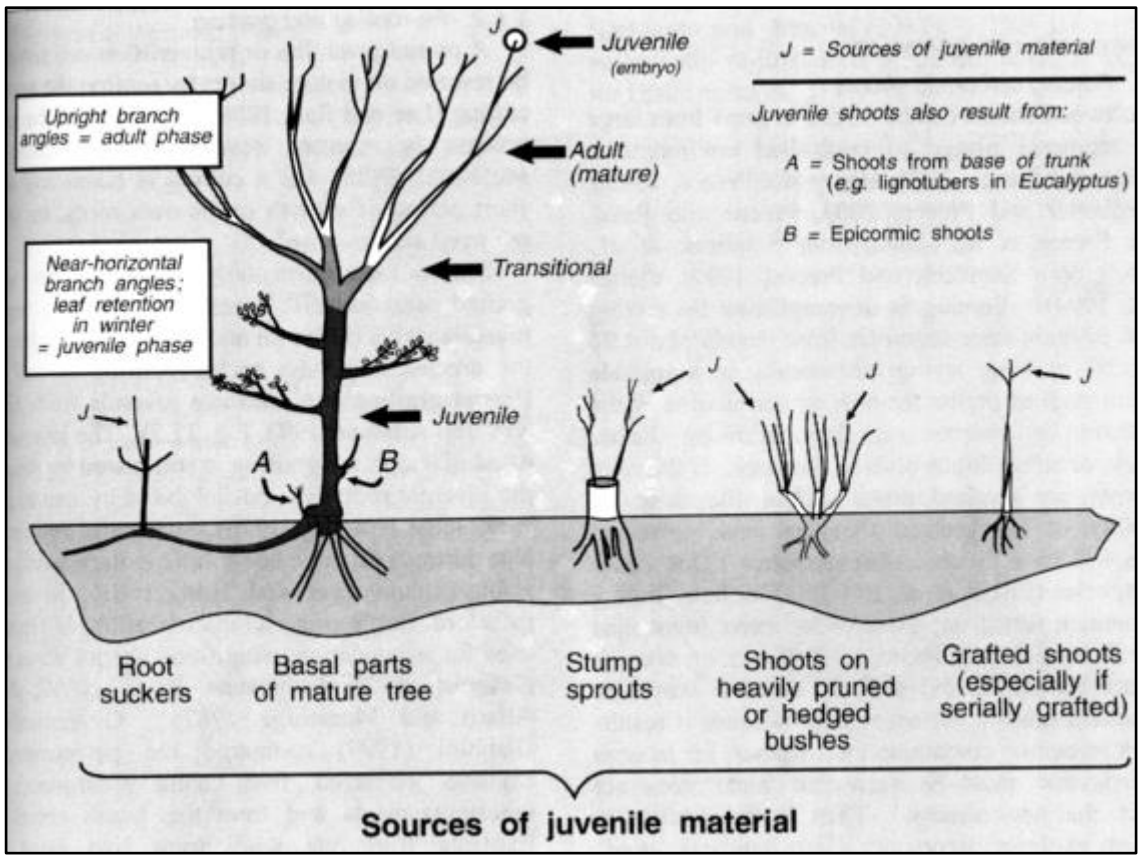

Figure 1.1: Diagram depicting the juvenile zones present on a mature tree, and juvenile zones present in stumps, hedges and grafts that have been manipulated to produce juvenile tissue (from George 2008).

1.4 Conventional Propagation

Many of the methods used in conventional propagation have the indirect effect of inducing juvenility in new shoots or growth.

1.4.1 Coppicing

Large scale coppicing, or hedging, is a common and relatively simple technique (Rosier, Frampton, Goldfarb, Blazich, & Wise, 2006), which maintains the production of juvenile shoots in the basal zone of the tree (Eldridge, Davidson, Harwood, & van Wyk, 1993; Singh, Bhandari, & Ansari, 2006). It requires the removal of the main trunk, leaving a stump that produces new shoots mainly at the base or from surface roots (Beck, Dunlop, & van Staden, 1998; Laureysens, Deraedt, & Ceulemans, 2005; Mason, Menzies, & Biggin, 2002). Coppicing increases the rooting potential of new shoots and the resulting plant is characteristically

juvenile (Eldridge *et al*., 1993; Singh *et al*., 2006). One limitation is that there is often a decrease in rooting potential of cuttings when taken from mature stumps compared to those taken from newly established seedlings (Wendling *et al*., 2014b).

1.4.2 Serial Grafting

Serial grafting is an extension of conventional grafting in which scions originally sourced from mature stock plants are repeatedly grafted onto a juvenile rootstock (Amissah & Bassuk, 2009; Danthu, Ramaroson, & Rambeloarisoa, 2008; Greenwood, Day, & Schatz, 2010; Ky-Dembele *et al*., 2011). Several cycles may be necessary before the desired shoots are completely rejuvenated, with the number needed dependent upon the maturity of the scion and the length of time it takes for the cutting to graft successfully (Zaczek, Steiner, Heuser, & Tzilkowski, 2006). Grafting juvenile scions onto juvenile rootstocks can be more successful than using mature scions, however, a limitation is that juvenile tissue does not necessarily express the characteristics that are desired for wood production (Moon *et al*., 2008). An alternative is to use juvenile root suckers from mature trees and graft them onto juvenile rootstocks (Chang, Ho, Chen, & Tsay, 2001; Danthu, Hane, Sagna, & Gassama, 2002; Zaczek *et al*., 2006). Another aspect of serial grafting is rootstock compatibility. Many factors need to be considered when selecting a rootstock, such as its ability to adapt to different soil conditions, disease resistance and its effect on the scion cultivar (Shafieizargar, Awang, Juraimi, & Othman, 2012). There is also no guarantee that rootstock will be compatible with the selected scion as genotypic variation in rootstock compatibility has commonly been observed (Schinor, Cristofani-Yaly, Bastianel, & Machado, 2013; Shafieizargar *et al*., 2012). If these conditions are not met, then the rootstock variant cannot be used and more research is required to find an alternative.

1.4.3 Re-Rooting

Re-rooting is one of the simplest techniques and can be used in conjunction with other methods such as coppicing or hedging. It also avoids problems such as rootstock incompatibility (Zaczek *et al*., 2006). Mature shoots are subjected to a rigorous process that is repeated several times, in which cuttings taken from mature tissues are exposed to a rooting compound and then transplanted to new substrate (Krakowski, Benowicz, Russell, & El-Kassaby, 2005; Mitchell & Jones, 2006). Once the cutting has begun rooting, it is left to grow new meristematic tissue, which is later excised and placed on the same rooting compound. The number of rooting cycles needed to ensure complete rejuvenation is dependent upon the

maturity of the original cutting and the ease of rooting in the species (Krakowski *et al*., 2005). This process can restore material to a more juvenile state, but can be time-consuming and the re-rooting capacity of mature tissue declines as the stock plant ages (Chang *et al*., 2001; Haapala, Pakkanen, & Pulkkinen, 2004; Husen & Pal, 2003).

1.5 Micropropagation

While conventional methods of propagation in *Paulownia* have been highly effective, the source of the cutting material can often be limiting (Bergmann, 1998). A viable alternative in this circumstance is micropropagation or tissue culture, the process of growing plant cells, tissues or organs in an artificial medium (George, 2008). When successful, micropropagation of forestry trees has several advantages over conventional methods (Pierik, 1997), such as rapid rates of multiplication, and independence from season of the year. (McComb, Bennett, & Tonkin, 1996; Riemenschneider & Bauer, 1997; Vettori *et al*., 2010). Most importantly, micropropagation can be successful for species that do not respond to conventional means of asexual propagation (George, 2008; Gomes & Canhoto, 2009). Factors that sometimes limit the utility of micropropagation include variation between genotypes in response e.g. shoot induction from adventitious root production and the success of transfer to soil (McComb *et al*., 1996; Thomson & Deering, 2011; Vettori *et al*., 2010). This limitation can easily be overcome by choosing genotypes that respond well to micropropagation in the lab and conventional propagation in the field.

Paulownia species have been produced using a number of micropropagation techniques including shoot multiplication, regeneration from callus and somatic embryogenesis (Bergmann & Whetten, 1998; Guo-qiang Fan, Zhai, Zhai, & Bi, 2001; Z. Ipekci & Gozukirmizi, 2003; Sha Valli Khan, Kozai, Nguyen, Kubota, & Dhawan, 2003). The most successful programs for the production of large numbers of these plants usually integrate tissue culture with conventional means of propagation (Pierik, 1997).

Micropropagation generally consists of five stages (Fig 1.2). Stage 0 is the selection and preparation of stock plants, which ensures that there is an adequate supply of healthy material ready to be used. Preparation can include using environmental and chemical pre-treatment and taking precautionary steps such as disease indexing and elimination (Leifert & Cassells, 2001). Once the tissue has been prepared it enters Stage 1 (Fig 1.2), where it is surface-sterilised and established in an aseptic culture. There is a short incubation time when explants are grown on a specific medium and any that become contaminated or begin to senesce

are discarded (Leifert & Cassells, 2001). This leads into Stage 2, where manipulations that increase the amount of propagating material can be applied (Auge *et al*., 1995) (Fig 1.2). Multiplication can be achieved using a number of methods, such as shoot multiplication, regeneration from callus, somatic embryogenesis or a combination of any or all of these (Auge *et al*., 1995). The method incorporated is dependent upon its efficacy and the desired outcome - for example, where genetic variability is undesirable the callus stage is usually avoided because of possibility of inducing somaclonal variation (Auge *et al*., 1995; Bairu, Aremu, & Van Staden, 2011; George, 2008). During this phase explants are generally grown on a medium containing cytokinins (George, 2008) plant growth regulators (PGR's) which induce the production of new shoots. At the end of Stage 2, explants are either transferred to Stage 3 for root induction or sub-cultured back into Stage 2 medium and further multiplied (Davies, Hartmann, Geneve, & Kester, 1997; George, 2008).

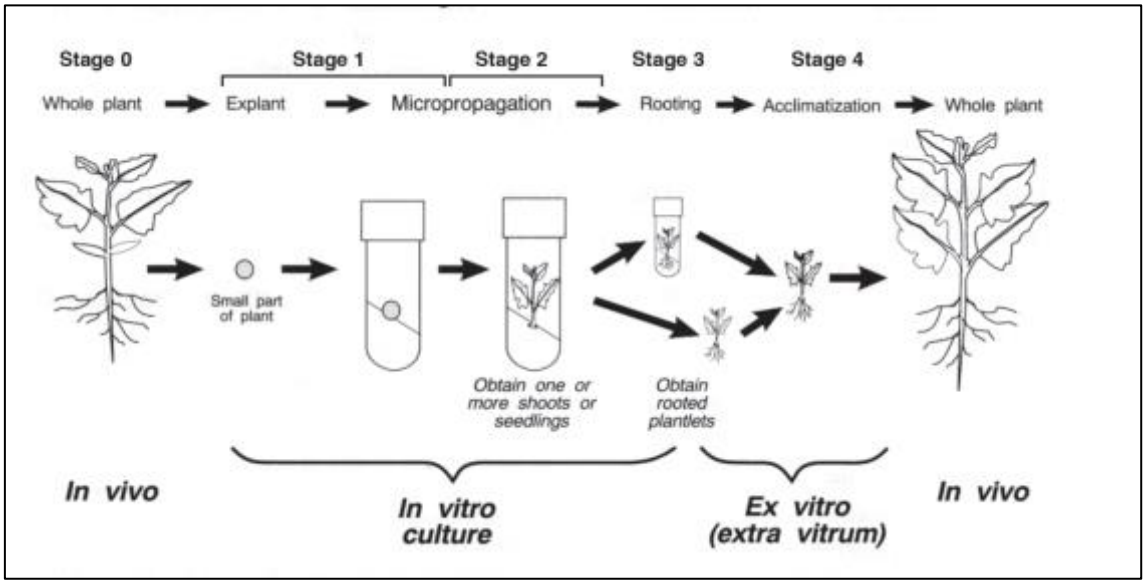

Figure 1.2. General representation of the stages of micro-propagation: Stage 0-preparation; Stage 1-initiation/establishment in vitro; Stage 2-multiplication; Stage 3-rooting or plantlet production; Stage 4-acclimatisation ex vitro; (adapted from George 2008).

Stage 3 generally includes exposure to auxins (Davies *et al*., 1997), which promote the growth of roots and elongation of shoots and hence increase the chances of survival when explants are transferred *ex vitro* (Davies *et al*., 1997). In Stage 4, the explants are transferred to new substrate and placed in humid conditions to acclimatise to the external environment (Davies *et al*., 1997).

Stage 2 is of most significance to the present study as this is where the restoration of juvenile characteristics takes place. There are a number of methods available and these include

plant growth regulator treatments, callus regeneration, somatic embryogenesis, etiolation, heat treatments, co-culture and culture stabilisation (George, 2008).

1.6 Methods of Rejuvenation in Micropropagation

Micropropagation (particularly Stage 2) allows for the capacity to manipulate the juvenility of tissue through utilising chemical and/or physical manipulations (Burn, Bagnall, Metzger, Dennis, & Peacock, 1993; Jain & Babbar, 2003).

1.6.1 Chemical Methods

Exposing *in vitro* cultures to cytokinins and auxins has long been used to induce partial rejuvenation through the production of adventitious shooting (Corredoira, Ballester, & Vieitez, 2008; George, 2008). Full or partial rejuvenation may require exposure to multiple plant growth regulators, which can be combined with other micropropagation methods (Husen & Pal, 2003; Ma, 2008).

Callus regeneration is another common method used to induce partial rejuvenation. Callus tissue is primarily amorphous parenchyma cells and occurs naturally within many species, often as a response to wounding or physical stresses e.g. limb loss or bark removal (Delvaux, Sinsin, Van Damme, & Beeckman, 2010; Stobbe, Schmitt, Eckstein, & Dujesiefken, 2002). Small pieces of non-meristematic tissue are placed in culture on medium containing various cytokinins, auxins or a combination of the two (Magyar-Tábori, Dobránszki, Teixeira da Silva, Bulley, & Hudák, 2010). Tissues are then able to form callus cells, which are composed primarily of de-differentiated and unspecialised cells (Naik & Chand, 2011; Rumyantseva, Sal'nikov, & Lebedeva, 2005). Typically, two types of callus tissue can be produced with the first (Type 1) being friable, yellowish to white in colour and more likely to produce embryos; and the second (Type 2) being green and compact and more likely to give rise to new shoots (Naik & Chand, 2011), however, these can vary depending on species. Callus can be used to produce shoots or embryos based on time or cost restraints (Naik & Chand, 2011).

Somatic embryogenesis is a micropropagation technique that theoretically has the potential to rejuvenate mature tissue. Somatic embryos are produced from diploid tissue (von Aderkas & Bonga, 2000) and are reportedly the most juvenile form of a plant (Bonga, Klimaszewska, & von Aderkas, 2010). However, its efficacy has never been demonstrated extensively, and some research suggests it only induces partial rejuvenation (Martínez, Vidal, Ballester, & Vieitez, 2012). Somatic embryos can be induced directly or indirectly using an

extensive variety of PGR combinations (Leljak-Levanić, Mihaljević, & Bauer, 2015). In the direct method, embryos form without the induction of a separate callus phase while the indirect method involves an intermediumte callus phase, followed by the embryogenesis phase (von Arnold, Sabala, Bozhkov, Dyachok, & Filonova, 2002). Somatic embryos can be directly grown into mature plants (Bonga *et al*., 2010) or used as a source of tissue for further rounds of somatic embryogenesis (von Aderkas & Bonga, 2000).

1.6.2 Physical Methods

Manipulation of the physical environment can also have the effect of inducing juvenility in plant tissues. For example, partial or full removal of light results in etiolation, which has been shown to improve rooting capacity of *in vitro* propagated shoots derived from mature tissues (Chory, Reinecke, Sim, Washburn, & Brenner, 1994). The stem that is to be propagated is placed in an area with low light or complete darkness (Chory *et al*., 1994; Husen & Pal, 2003), which induces the formation of pale, elongated shoots and small unexpanded leaves (Husen & Pal, 2003). The rooting capacity of such shoots can be increased when compared with that of mature, light-grown cuttings (Haapala *et al*., 2004). The application of high temperatures to the bottom of a mature plant also induces juvenile growth in adult plant material in some species (Adams, Pearson, Hadley, & Patefield, 1999; Burn *et al*., 1993; George, 2008).

Co-culture is a common practice, which involves placing the mature adult shoots in the same containers as juvenile shoots (George, 2008). Endogenous plant growth regulators (PGR's) exuded into the medium by the juvenile shoots are absorbed by the adult shoots, causing either partial of full rejuvenation (George, 2008).

Culture stabilisation refers to physiological changes that occur while cultures are maintained and subcultured over long periods of time (McCown, 2000; McCown & McCown, 1987; von Aderkas & Bonga, 2000) and has been reported for many tree species. Such cultures may become partially rejuvenated and the resulting explants often express juvenile characteristics, such as juvenile foliage, growth form and adventitious rooting (George, 2008; Mankessi, Saya, Baptiste, Nourissier, & Monteuuis, 2009; Wendling *et al*., 2014b). Stabilised cultures can revert back to mature characteristics when removed from *in vitro,* suggesting that while this method offers at least partial rejuvenation its effects may not be long-lasting (George, 2008; Wendling *et al*., 2014b).

1.7 Aims

This research aimed to examine whether the method of micropropagation and the tissue source used affects the early growth and development of *Paulownia* plantlets. Previous studies have focussed on either developing a single method of micropropagation or comparing one method against conventional methods of propagation (Bergmann, 1998; Bergmann & Moon, 1997; Bergmann & Whetten, 1998; Corredoira *et al*., 2008; Guo-qiang. Fan, Zhai, Jiang, & Liu, 2002; Guo-qiang Fan *et al*., 2001; Ipekci & Gozukirmizi, 2003, 2005; Sha Valli Khan *et al*., 2003; Taha, Ibrahim, & Farahat, 2008) but none have compared multiple methods of micropropagation against each other. Furthermore, micropropagation studies in *Paulownia* rarely extend to comprehensive observations of long term phenological development in the greenhouse (Dimps Rao, Goh, & Kumar, 1996; Ipekci & Gozukirmizi, 2003, 2005).

To answer these questions, the project was divided into two distinct and interlinked phases. In the Micropropagation phase (Chapter 3) several methods were examined to establish how successfully they produced juvenile shoots or somatic embryos from different sources. Two of the micropropagation methods - callus regeneration (Guo-qiang. Fan *et al*., 2002; Guo-qiang Fan *et al*., 2001) and somatic embryogenesis (Ipekci & Gozukirmizi, 2003, 2005) - have previously been shown to be effective in micropropagating *Paulownia.* The third method, that of root sucker induction, was developed during the study, and has not been documented previously. In the Greenhouse phase (Chapter 4) explants grown *in vitro* from the different micropropagation methods and tissue sources were transferred to a greenhouse where they were monitored and later harvested for comparison of growth patterns.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Stock Plant Material and Tissue Sources

Mature stock plants were established from five elite clones of *Paulownia* (P1, P2, P3, P4 and P5) and were selected from a plantation source, based on individual growth characteristics such as height and trunk diameter. Clones were all hybrids between *Paulownia elongata* and *Paulownia fortunei*. Cuttings were established in the greenhouse at Edith Cowan University and mature stock plants were used as a source of material for the micropropagation and greenhouses phases (Fig 2.1).

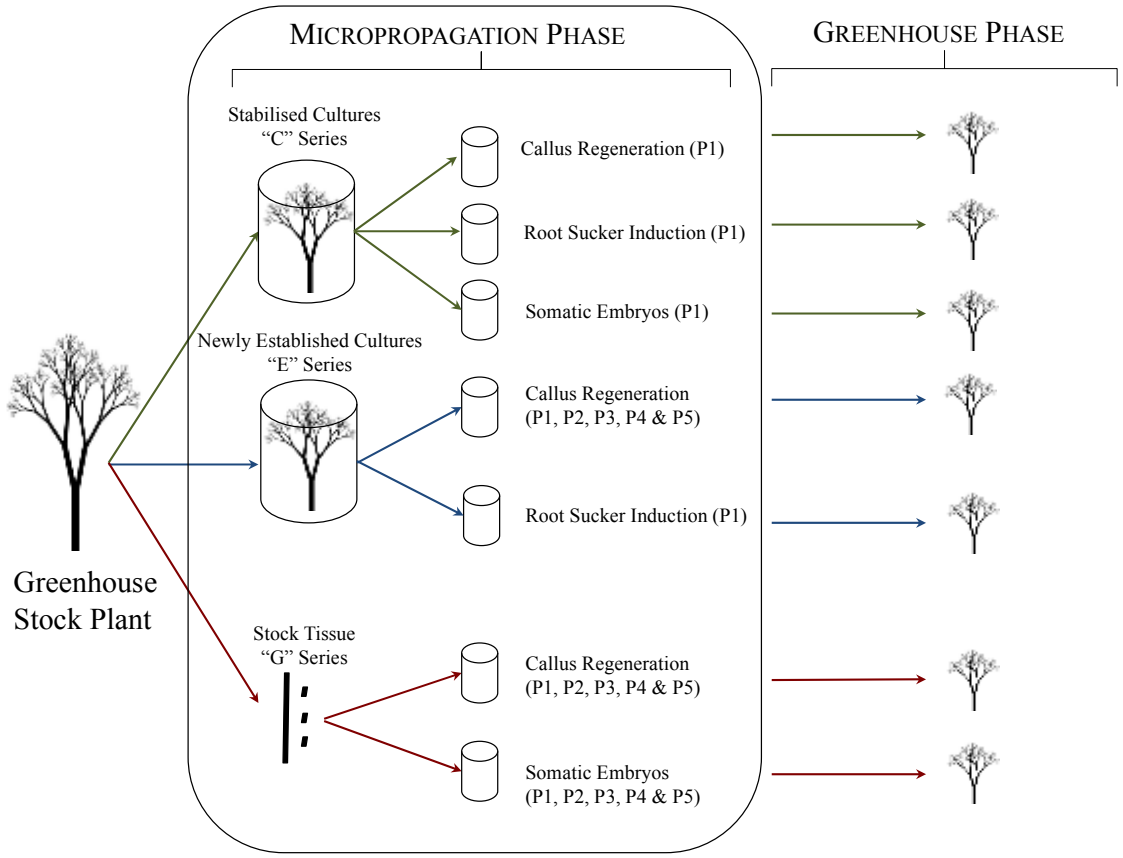

Figure 2.1 Experimental designs for the Micropropagation and Greenhouse phases showing source of clones, source of explant material and tissue culture techniques to be applied.

2.2 Labelling and Identification Method

To identify these different sources *in vitro* and in the greenhouse, stabilised explants were denoted with the letter C followed by the *Paulownia* clones utilised i.e. CP1. The same identification method was used for newly established explants; however, the C was replaced

with the letter E, and for explants obtained from greenhouse material the letter G was used i.e. EP1 and GP1, respectively.

2.3 Basal Medium and Culture Room Conditions

Basal medium (BM) was used in culture establishment, culture and explant subculturing, callus regeneration experiments and some somatic embryogenesis experiments. BM contained full strength Murashige and Skoog's (1962) (M&S), with $30gL^{-1}$ of sucrose and solidified with $2.5gL^{-1}$ of agar (Sigma-Aldrich plant cell culture tested) and $2.5gL^{-1}$Gelrite™ adjusted to pH 5.8. Base Rooting medium (1/2BM) consisted of half strength M&S with $20gL^{-1}$ of sucrose, $2.5gL^{-1}$ of agar and $2.5gL^{-1}$ Gelrite™ adjusted to pH 5.8 was used in rooting and root sucker induction experiments.

When subculturing explants 40mLs of BM was placed into 250mL polycarbonate containers. When establishing new cultures *in vitro,* 5mL of multiplication medium (MM) was used and placed in 30mL polycarbonate containers. All medium was autoclaved at 121°C for 20 minutes and stored at 4°C in a cool room prior to use, when necessary. *In vitro* propagation and experimental medium were developed from BM and RM. All cultures and experiments were conducted under the same laboratory conditions and maintained at 23±1°C with a photoperiod of 16h light (90µmoles $m^{-2}s^{-1}$) from fluorescent lamps, 8h dark.

2.4 Multiplication Medium.

A standard multiplication medium (MM) was used in culture establishment, culture and explant maintenance and subculturing newly developed explants utilised from micropropagation experiments (Ch 3). Stabilised cultures were maintained on BM supplemented with 5µM of kinetin (Kn), 5µM of benzylaminopurine (BAP) plus 0.5µM of naphthalene acetic acid (NAA). Newly established cultures were maintained on the same BM, however, the concentration of Kn was increased to 10µM. All stabilised, newly established and explants used for greenhouse experiments (Ch 4) were maintained on this medium for 4 weeks before being subcultured.

2.5 Rooting Medium

Rooting medium was used for root induction and root sucker induction. To induce roots, five shoots were place into a 250mL polycarbonate container with 40mL of 1/2BM supplemented with 2.5µM IBA for one week. Shoots to be used for *in vitro* experimentation

(Ch 3) were transplanted into RM with no IBA, while those used in greenhouse misting chambers (Ch 4) were transferred to soil.

2.6 Stock Plant Harvesting and Sterilisation Procedure

Greenhouse material was sourced from either the internodes of stems, axillary buds or anthers and ovaries from flower buds. Freshly collected tissues were surface sterilised in 2% benzylkonium chloride with 10% ethanol and 88% sterile distilled water for five minutes before rinsing in sterile distilled water (x3) and being placed into culture.

2.7 Stabilised and Newly Established Cultures

Stabilised and newly established cultures were established *in vitro* as described above. The stabilised cultures used in this study had been *in vitro* for five years before being used, while newly established cultures had been *in vitro* for three months (Fig 2.1).

To establish new cultures *in vitro*, axillary buds were taken from clones P1 and P2, P3, P4 and P5, surface sterilised and placed into 30mL polycarbonate containers with 5mL of MM. Once axillary buds produced new growth, shoots were removed, positioned into 250mL polycarbonate containers with 40mL of MM and placed in the tissue culture room. The explants were subcultured every four weeks, by removing old tissue and placing new shoots into fresh medium.

2.8 Acclimatisation and Greenhouse Transfer.

For greenhouse experiments (Ch 4) *in vitro* shoots were cut to a length of 3cm and placed in RM for one week, before being transferred to individual 40mm x 88mm crack pots containing a mixture of 1:1 pasteurised white sand to pasteurised potting mix (Baileys Premium Potting Mix). Crack pots were placed in a shaded misting chamber for two weeks under varying conditions. For the first seven days, plants were placed under 70% shade and misted for 20 seconds every minute. To allow shoots to harden off, shade was reduced to 50% and misting frequency adjusted to 20 seconds every two minutes from 8 to 14 days, after which the shade was removed and misting frequency reduced to ten seconds every five minutes for a further seven days. After 28 days shoots were transferred to the greenhouse where they were watered by sprinklers for ten minutes once every 24 hours. They were maintained between 20-30^{o}C and grown thereafter under ambient light.

CHAPTER 3 DEVELOPMENT OF *IN VITRO* PROTOCOLS

3.1 Introduction

Numerous studies have shown that *Paulownia* species can be readily propagated *in vitro* using a variety of methods that include adventitious shoot induction, organogenesis, callus regeneration, embryogenesis and somatic embryogenesis (Bergmann, 1998; Bergmann & Moon, 1997; Bergmann & Whetten, 1998; Corredoira *et al*., 2008; Guo-qiang. Fan *et al*., 2002; Guo-qiang Fan *et al*., 2001; Z. Ipekci & Gozukirmizi, 2003, 2005; Sha Valli Khan *et al*., 2003; Taha *et al*., 2008). Of the methods described, the simplest and most commonly utilised technique is adventitious shooting (Yadav *et al*., 2013) while callus regeneration (Guo-qiang. Fan *et al*., 2002; Guo-qiang Fan *et al*., 2001) and somatic embryogenesis (Ipekci & Gozukirmizi, 2003, 2005) have been reported twice.

Callus regeneration has been successfully used in tissue culture for many species (George, 2008) but to date only one study has reported regenerating explants from callus in juvenile *Paulownia* seedlings (Guo-qiang Fan *et al*., 2001). Callus regeneration involves the manipulation of plant growth regulators (usually cytokinins and auxins) to induce de-differentiation of tissue and its reorganisation through organogenesis but is limited by the potential to induce somaclonal variations in micropropagated shoots (Dimps Rao *et al*., 1996). However, its simplicity and ability to produce large quantities of new shoots makes it an important method to evaluate.

Somatic embryogenesis has been documented for many species although there are few reports of successful somatic embryogenesis with *Paulownia* (Ipekci & Gozukirmizi, 2003, 2005). The methods developed vary greatly between species, genotypes and explant source and method development including PGR combinations, culture conditions, light exposure, basal medium composition and biochemical compounds. While this method can lead to significant increases in budget and time constraints, if successful it has the greatest potential for tissue rejuvenation. Theoretically, somatic embryogenesis has the potential to completely rejuvenate tissue, though this has never been demonstrated conclusively (Martínez *et al*., 2012). It can potentially also produce thousands of somatic embryos in relatively short periods of time. Two somatic embryogenesis methods were developed for this study, based on optimal methods produced by Ipekci and Gozukirmizi (2003, 2005) for three month old *Paulownia elongata* seedlings. Should these methods be unsuccessful, further invesitigation into developing new methods would need to be explored.

This study also adopts a novel technique, the induction of root suckers *in vitro*. In

Paulownia, preliminary experiments (I, Bennett. pers. comm) have shown that root suckers can be successfully produced *in vitro* which has never been reported in micropropagation studies. Explants that were left in medium began to regenerate roots, and if left long enough will sometimes produce root suckers (I, Bennett. pers. comm) (Fig 3). Auxin influence was also a key factor to examine, as there is a significant link between auxin production and root sucker suppression so the use of an auxin inhibitor was also assessed (Wan, Landhausser, Lieffers, & Zwiazek, 2006). This technique does not involve complex methods of induction and could be used alone or in combination with other *in vitro* methods.

Figure 3.1 In vitro roots from stabilised Paulownia cultures showing root sucker growth after 10 weeks in culture with no rooting PGR's applied.

The objective of this research was to develop a tissue culture protocol for three methods of explant production in *Paulownia*: callus regeneration, somatic embryogenesis and root sucker induction. The efficacy of each method was assessed on its ability to produce large quantities of explants with minimal difficulty.

3.2 Materials and Methods

3.2.1 Callus Regeneration

Previous studies have shown (Dimps Rao *et al*., 1996; Guo-qiang Fan *et al*., 2001) that the most successful combinations of PGR's for inducing callus induction in *Paulownia* were Benzylaminopurine (BAP) and Naphthaleneacetic acid (NAA) and were used as a point of reference for developing a callus regeneration protocol. Preliminary experiments showed that optimal concentrations of BAP were between 5µM and10µM and optimal concentrations of NAA were between 0.25µM and 0.5µM (I, Bennett. pers. comm).

Five experiments were developed; three experiments manipulated the concentrations and range of PGRs, one examined the effect of explant source and clone physiology on the efficacy of the developed method, and a fifth experiment determined the effect that season of source collection and internode position had on the production of shoots. BM (Chapter 2) were across all experiments to ensure tissue samples were exposed to the same concentration of PGR's and nutrients.

All callus regeneration experiments used a minimum of 15 internodal segments per treatment. Internodal segments used for callus initiation measured 2-3mm diameter (*in vitro* sourced) or 5-10mm diameter (greenhouse sourced) and were randomised across treatments, except in the fifth experiment, which examined the effect of internode position on shoot regeneration. The cumulative percentage of callus clumps producing shoots and mean number of shoots produced were scored each week for six weeks. To use greenhouse material in culture, the tissue was surface sterilised (Ch 2) and then used immediately in experiments with no period of establishment.

Effect of BAP and NAA Concentrations on Shoot Production

This experiment was to determine which of several combinations of BAP and NAA induced the highest shoot regeneration. Stabilised cultures from clone P1 and greenhouse material sourced from clones P1 and clone P2 were used. Explants were subject to a complete factorial design of three concentrations of BAP (5µM, 10µM and 15µM) and three concentrations of NAA (0.25µM, 0.5µM and 1µM).

Effect of BAP Concentrations on Shoot Production

The effect of increasing concentrations of BAP on shoot production was examined in stabilised, newly established and greenhouse material sourced from clone P1. Twenty internode

segments were used per treatment and explants exposed to one of five concentrations of BAP (10µM, 12.25µM, 14.5µM, 16.75µM and 19µM) at a constant rate of NAA (0.5µM).

Effect of Different Cytokinins on Regeneration

The effect of five different cytokinins was studied in stabilised, newly established and greenhouse material of clone P1 with twenty-five internode segments used per treatment. Explants were exposed to 5µM of either BAP, Kn, Zeatin (Z), Zeatin riboside (ZR) and Isopentenyl adenine (2iP) combined with NAA at 0.5µM.

Effect of Combined BAP and NAA on Different Tissue Sources

This experiment was designed to study the response of tissues from each clone to different concentrations of BAP and NAA. Based on results of experiments 1-3, two optimal concentrations of BAP (10µM and 15µM) were applied in combination with three optimal concentrations of NAA (0.25µM, 0.5µM and 1µM). Twenty-five internode segments were obtained from stabilised cultures (P1), newly established cultures from clone P1 and P4, internodes harvested from greenhouse grown plants from clone P1 and internodes, anthers and carpels of greenhouse grown plants from clones P2, P3, P4 and P5.

The Influence of Season, Source Material and Internode Position on Shoot Induction

Due to the variability of responses from greenhouse material and *in vitro* cultures, this experiment was designed to determine whether the node from which the sample was taken and the month of sampling (from July to June) had any influence on the response of greenhouse materials and *in vitro* cultures. Internodes were taken from all clones and treated with a single combination of 10µM BAP and 0.5µM NAA. The number of shoots produced was then counted after four weeks in culture. Greenhouse material from GP1 was collected every month for 12 months while all other sources (GP2, GP3, GP4, GP5, GR1, EP1, EP2, EP3) were analysed every month for three months.

3.2.2 Regeneration by Root Suckers

Preliminary experiments demonstrated that stabilised explants (P1) left in culture for extended periods of time would initiate root suckers and based on this observation a new set of methods was developed.

To induce root sucker production, *in vitro* shoots were exposed to stresses similar to

ones a plantation tree might experience e.g. coppicing, root damage. Two techniques mimicked trunk damage from coppicing; the stem was either removed below the first node (Below) or alternatively above the first node (Above). The third technique involved leaving the main stem intact and slicing through the roots (Roots). These techniques were compared against stems that had no manipulation applied to them (Control). Finalised experiments utilised both manipulation techniques and applying various concentrations of the auxin inhibitor Naphthylphthalamic acid (NPA), which has been shown to induce root suckers in other species in studies conducted in the field (Wan *et al*., 2006). The number of root suckers produced and the percentage of shoots producing root suckers were scored each week for 18 weeks after the physical manipulation was applied.

Effect of Induction Technique on Root Sucker Production

This experiment examined the rate of production of root suckers using the three physical induction techniques (Above, Below and Roots). Shoots were sourced from newly established and stabilised cultures from P1. Each shoot was trimmed to approximately 3cm in length before being placed in RM to induce roots (Ch2). After four weeks, each sample had one of the three physical manipulations applied or were left unmarred.

Effect of Auxin Suppression

The effect of supressing auxin production on root sucker production was evaluated using a complete factorial design with four levels of NPA (0µM, 2.5µM, 5µM and 10µM). Shoots were sourced from newly established cultures (P1). To induce root suckers, shoots were placed in RM for one week (Ch 2), then placed in 1/2BM with one of the various concentrations of NPA. Four weeks after being transferred to 1/2BM each shoot was subjected to one of two physical manipulations (Below or Roots) or left unmarred. The Above treatment was not used again as it showed no effect in the previous experiment.

3.2.3 Somatic Embryogenesis

Tissue from clone 1 showed the best response in the callus experiments and was selected for attempts to induce somatic embryos. Tissue was sourced from either internode segments, from the first node, or leaf segments above the second node. Internode segments were measured between 2-5mm diameter and leaf segments measured 5 mm in diameter. Both indirect and direct somatic embryogenesis methods were utilised and based on optimal PGR's

(Thidiazuron (TDZ) 10µM and Kn 0.5µM for direct method, and TDZ 0.05µM and Kn 0.5µM for indirect method), developed by Ipekci and Gozukirmizi (2003, 2005). The number of somatic embryos induced was scored every week over a period of 12 weeks.

Induction of Somatic Embryogenesis on Solid Medium

To induce direct somatic embryogenesis a complete factorial design of six treatments was employed, using base medium supplemented with a combination of TDZ (5µM, 10µM, 15µM) and Kn (0.25µM, 0.5µM, 1µM).

Additionally, the possible effect of auxin was tested using with two concentrations of IAA (0.5µM, 2µM) in combination with 5µM TDZ (Ipekci, 2003). Internode and leaf segments (CP1) were placed on this medium for four weeks before being transferred onto fresh medium with their respective combinations of PGR's.

For indirect somatic embryogenesis, the first phase was to initiate callus from the internode and leaf segments (CP1). Initial callus was induced using varying concentrations of 2-4D (0.5µM) or NAA (0.5µM) in combination with 5µM BAP. Both leaves and internodes were used to initiate callus and left on this medium for four weeks. During the second phase, callused segments were placed on medium containing varying combinations of TDZ (0.005µM, 0.05µM and 0.5µM) with Kn (0.25µM, 0.5µM and 1µM). Callus was left on these media for four weeks to induce somatic embryos.

Induction of Somatic Embryogenesis in Suspension

Callus induced from greenhouse material (P1, Experiment 4) was used, avoiding the need to design a new indirect method. The callus tissue used was green and friable and varied in size but on average measured 20mm x 10mm (w x h). A minimum of four callus segments were ground into suspension medium supplemented with TDZ (5µM, 10µM or 15µM). The suspension medium was further supplemented with 500mg of casein hydrolysate as a source of amino acids to ensure appropriate embryo initiation (Z. Ipekci & Gozukirmizi, 2003). Suspension cultures were maintained for up to six weeks on an orbital shaker at 100rpm at standard incubation settings (Ch 2). The number of somatic embryos induced was scored every week over a period of 8 weeks.

Suppression of Somatic Embryogenesis by PGR's

This experiment was designed to determine if PGR's used for callus induction were

suppressing the formation of somatic embryos (Naing, Kim, Yun, Jin, & Lim, 2013). An indirect method was used to induce callus, in which 25 internodes used per treatment were placed on solid medium supplemented with either 2,4D (5µM, 10µM, 15µM) or NAA (5µM, 10µM, 15µM) in combination with Kn (0.25 µM, 0.5µM, 1µM). After 4 weeks, the initiated callus was transferred to either solid or suspension medium that contained no PGR's.

3.2.4 Formation of Adventitious Roots and Media Optimisation

In preparation for transfer to the greenhouse (Ch 4) the rooting competence of selected sources and medium optimisation was tested. Both rooting experiments used *in vitro* shoots from clone P1. For each experiment 30 shoots (3cm length) were used per treatment before being put into a RM.

Effect of IBA on Adventitious Root Formation

To examine how the rooting hormone IBA influences the production of adventitious roots, shoots from stabilised cultures were placed five to a container (Ch 2) in RM supplemented with one concentration of IBA (0µM 1.25µM, 2.5µM and 5µM) for one week. Each shoot was then placed in individual vessels containing IBA free, 1/2BM. The mean number of roots produced was scored each week for three weeks after being placed on hormone free medium.

Rooting Response of In Vitro Shoots from Various Explants

This experiment was designed to determine the effect shoot source had on the ability to produce roots. Shoots from stabilised cultures, newly established cultures and greenhouse explants were placed on the 1/2 BM was supplemented with 2.5µM IBA. Shoots were left on this medium for a week before being placed into 1/2BM for three weeks. The mean number of roots produced was scored at the end of week four.

3.2.5 Statistical Analysis

Levenes test for homogeneity was applied for all data sets to determine normal distribution. For data that was not normally distributed, a pairwise comparison was conducted using the Kruskal-Wallis H test to determine if there was any significant effect of treatment, explant source and clone. All percentage data was Arc sin transformed before being analysed. This was conducted using statistics package SPSS v19 and all results were tested at $P \leq 0.05$

significance level.

3.3 Results

3.3.1 Callus Regeneration

Effect of BAP and NAA Concentrations on Shoot Production

Callus induction occurred in all explants during the first week. P1 explants showed the highest rate of callus induction (100% of replicates) while P2 explants showed the lowest rate. Regeneration was observed between weeks three and five with stabilised explants achieving significantly higher percentages of callus inducing shoots than greenhouse initiated callus (P1 and P2). Callus induced from stabilised cultures were larger and greener than that produced by greenhouse material.

Stabilised explants showed the highest rate of shoot (4.2 ±1.42) regeneration at concentrations of 10µM BAP plus 1µM NAA. The lowest rate of shoot regeneration (0.13±0.13) was recorded at concentrations of 5µM BAP plus 0.25µM NAA (Table 3.1). Shoots induced from greenhouse callus produced significantly poorer results (P1 0.13 ±0.13, P2 0.07 ±0.57). The highest percentage of stabilised shoots and callus produced was at concentrations of 5µM BAP plus 0.5µM NAA and 10µM BAP plus 1µM NAA (40% and 46% respectively). The lowest percentage occurred in medium supplemented with 5µM BAP plus 0.25µM NAA (6%). A Kruskal-Wallis H test indicated there were no significant differences indicating no effect of BAP or NAA concentration (P=0.457). A pairwise comparison showed stabilised explants performed significantly better than both greenhouse sources (P<0.001).

Table 3.1: Effect of varying concentrations of BAP and NAA on shoot regeneration of Paulownia fortunei x elongata internodes (CP1, GP1 and GP2) after six weeks.

		Stabilised (P1)		Greenhouse (P1)		Greenhouse (P2)	
BAP(µM)	NAA(µM)	% Callus	#Shoots	% Callus	#Shoots	%Callus	#Shoots
5	0.25	6±0.06[a*]	0.13±0.13	0	0	0	0
	0.5	40±0.13[a]	1.9±1.03	0	0	0	0
	1	33±0.12[a]	2.6±1.21	0	0	0	0
10	0.25	27±0.11[a]	0.6±0.27	0	0	0	0
	0.5	20±0.10[a]	0.5±0.33	0	0	0	0
	1	46±0.13[a]	4.2±1.42	6±0.06[b]	0.13±0.13	0	0
15	0.25	27±0.11[a]	1.13±0.60	0	0	0	0
	0.5	27±0.11[a]	1.7±0.87	0	0	0	0
	1	27±0.11[a]	1.13±0.28	0	0	6±0.06[b]	0.07±0.57

**Percentage values in each column followed by different lower case letters are significantly different at P≤0.05 according to the Kruskal-Wallis test.*

% Percentage of callus producing shoots.

 # Mean number of shoots per callus.

Effect of BAP Concentrations on Shoot Production

Stabilised explants showed no significant difference across any of the treatments (P=0.177), whereas newly established explants showed a significant difference between treatments (P<0.001). A pairwise comparison showed that 10µM BAP had a significant effect on shoot regeneration compared to concentrations 12.25µM, 16.75µM and 19µM of BAP (P=0.02, P=0.011 and P=0.011). Among the three sources there was a significant positive response to callus inducing shoots (P<0.001), however, a pairwise comparison showed there was a significant difference only between newly established material and greenhouse material (P<0.001). No difference was found between newly established explants and stabilised explants (P=0.052) or stabilised explants and greenhouse material (P=0.412).

Results varied in relation to increasing concentration of BAP (Table 3.2) where the highest number of shoots regenerating for stabilised cultures was 16.75µM BAP. The percentage of callus producing shoots was highest at 14.5µM BAP (15%) and lowest was at 16.75µM BAP and 19µM BAP (5%). Newly established cultures produced the highest number

of shoots (0.9 ±0.31) and highest percentage of callus producing shoots (40%) when exposed to relatively low levels of BAP (10µM).

Callus induction was observed again for all explants during the first week. In general, the response to callus induction was positive, with stabilised, newly established and green house material all producing substantial amounts. Shoot regeneration did not occur until week four in stabilised and newly established explants while greenhouse material did not produce shoots across any of the treatments (Table 3.2).

Table 3.2: Effect of increasing levels of BAP on shoot regeneration of Paulownia fortunei x elongata internodes (CP1, GP1 and EP1) after six weeks.

		Stabilised (P1)		Greenhouse (P1)		New Established (P1)	
NAA(µM)	BAP(µM)	% Callus	#Shoots	%Callus	#Shoots	% Callus	#Shoots
0.25	10	0	0	0	0	40±0.11^{ac}	0.9±0.31
	12.25	0	0	0	0	0	0
	14.5	15±0.08^{ab*}	0.15±0.81	0	0	15±0.08^{ad}	0.5±1.94
	16.75	5±0.05^{ab}	0.35±0.35	0	0	5±0.05^{ad}	0.1±0.20
	19	5±0.05^{ab}	0.05±0.05	0	0	5±0.05^{ad}	0.05±0.05

**Percentage values in each column followed by different lower case letters are significantly different at P≤0.05 according to the Kruskal-Wallis test.*
% percentage of callus producing shoots
 # mean number of shoots per callus

Effect of Different Cytokinins on Regeneration

Green, healthy callus was successfully produced by all five cytokinins tested (BAP, Kn, Z, ZR and 2iP) though no shoots were produced after six weeks in any of the treatments.

Effect of Combined BAP and NAA on Different Tissue Sources

Callus production occurred within one week in all treatments, while shoot regeneration was visible during week three in newly established and greenhouse explants, and week 4 in stabilised explants.

The effect of PGR concentrations varied with tissue source (Table 3.3) and clone. Stabilised sources produced the largest number of shoots at high concentrations of BAP (15µM) and NAA (1µM) (Table 3.3), however, greenhouse sources responded better to lower concentrations of BAP (10µM) and NAA (0.25µM). Newly established explants responded positively across most treatments, though most notably at 10µM BAP and 0.5µM of NAA. P4

newly established explants also produced shoots across the various concentrations, however, they responded to higher concentrations of BAP (15µM) and NAA (0.5µM).

In P1 explants, there was a positive reaction to the varying hormone concentrations with both stabilised and greenhouse explants showing a significant difference across treatments ($P<0.001$ and $P=0.042$, respectively). There was no effect of treatment in newly established explants of either clone P1 ($P=0.245$) or P4 ($P=0.112$). P4 also showed no significant difference across treatments. A pairwise comparison of P1 explants showed a significant difference between stabilised and newly established cultures, and greenhouse material ($P=0.023$ and $P<0.001$). Comparisons between newly established explants indicated there was no significant difference between clone P1 and P4 ($P=0.642$).

Table 3.3: Effect of concentrations of BAP and NAA on shoot formation of Paulownia fortunei x elongata (CP1, GP1, EP1 and EP4) after six weeks.

		Stabilised (P1)		Greenhouse (P1)		New Established (P1)		New Established (P4)	
BAP(µM)	NAA(µM)	% Callus	#Shoots	%Callus	#Shoots	% Callus	#Shoots	% Callus	#Shoots
10	0.25	0	0	40±0.10^{c}	1.4±0.43	8±0.05^{ef}	0.1±0.08	8±0.05^{ef}	0.12±0.05
	0.5	0	0	36±0.09^{c}	1.4±0.45	24±0.08^{ef}	0.5±0.18	8±0.05^{ef}	0.16±0.06
	1	4±0.04^{a*}	0.04±0.04	8±0.05^{d}	0.3±0.19	16±0.07^{ef}	0.6±0.26	20±0.08^{ef}	0.28±0.07
15	0.25	0	0	0	0	20±0.08^{ef}	0.8±0.41	8±0.05^{ef}	0.16±0.06
	0.5	8±0.05^{ab}	0.2±0.16	4±0.04^{d}	0.08±0.08	8±0.05^{ef}	0.08±0.05	32±0.09^{ef}	0.56±0.09
	1	16±0.07^{b}	0.32±0.18	28±0.08^{c}	0.6±0.29	4±0.04^{ef}	0.1±0.12	20±0.08^{ef}	0.48±0.10

**Percentage values in each column followed by different lower case letters are significantly different at $P \leq 0.05$ according to the Kruskal-Wallis test.*

% percentage of callus producing shoots

 # mean number of shoots per callus

*The Influence of Season, Source Material and Internode Position on Shoot Induction***.**

There was a significant effect of explant source on the production of shoots ($p<0.0001$). Callus was produced by all clones, however only three (P1, P2 and P4) produced shoots (Table 3.4). Clone P1 outperformed other clones, producing the highest average number of shoots and percentage of callus-producing shoots in both greenhouse material and newly established cultures. Greenhouse material from P2 and P4 had comparatively lower percentages of callus-producing shoots, while newly established sources consistently had lower rates of shoot production in comparison to greenhouse material (Table 3.4).

Internode position had a significant effect on the ability of callus to regenerate explants. Mean number of shoots was significantly higher from callus induced from the first internode than the second internode in GP1, GP2 and GP 4 ($P<0.0001$, $P=0.031$ and $P=0.002$ respectively). Newly established shoots (EP1 and EP2) showed a similar trend, but in EP3 the opposite trend was evident i.e. there was a higher average of shoots produced from the second internode. There was, however, no significant effect of the internode position on the number of shoots regenerated (EP1 $P=0.59$, EP2 $P=0.663$, EP3 $P=1.0$).

The month when greenhouse material was collected had a significant influence on the ability of selected greenhouse material to be micropropagated *in vitro*. GP1 showed no significant difference between shoot regeneration in the months of July and August, however, when compared to the month of September there was a significant decline in the number of shoots regenerating ($P<0.001$). This pattern was observed for other explants with GP4 and GR1, showing no interaction between shoot regeneration and month ($P=0.168$ and $P=0.057$). An independent samples Kruskal-Wallis test showed that there was significant difference between the production of shoots and the different seasons of the year ($P<0.001$) i.e. Summer (December-February), Autumn (March-May), Winter (June-August) and Spring (September-November). The highest response of shoots regenerating was in July with the average declining each month with a slight increase in December (Fig 3.2). January showed no shoot regeneration and from February onwards there was a plateau in shoot regeneration (Fig 3.2).

Table 3.4: The influence of season, source material and internode position on Paulownia fortunei x elongata (GP1, GP2, GP3, GP4, GP5, GR1, EP1, EP2 and EP3) shoot induction over at 4 week intervals over 12 weeks. BM was used supplemented with 10µM BAP plus 0.5µM NAA.

		July		August		September	
Explant Source	Internode	% Callus	#Shoots	%Callus	#Shoots	% Callus	#Shoots
GP1	1	83±0.06[a]	4.9±0.63	80±0.06[a]	2.3±0.34	33±0.05[h]	0.6±0.36
	2	8±0.02[b]	0.1±0.10	8±0.02[b]	0.1±0.1	0	0
GP2	1	20±0.03[c]	0.20±0.11	0	0	0	0
	2	0	0	0	0	0	0
GP3	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
GP4	1	33±0.05[e]	0.4±0.11	16±0.03[e]	0.2±0.11	0	0
	2	0	0	0	0	0	0
GP5	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
GR1	1	3±0.01[f]	0.03±0.03	27±0.04[f]	0.4±0.12	16±0.03[f]	0.4±0.16
	2	0	0	0	0	0	0
EP1	1	20±0.04[g]	0.32±0.14	0	0	0	0
	2	0	0	0	0	0	0
EP2	1	16±0.07[g]	0.16±0.07	0	0	0	0
	2	8±0.05[g]	0.08±0.05	0	0	0	0
EP3	1	8±0.05[g]	0.08±0.05	0	0	0	0
	2	12±0.06[g]	0.2±0.12	0	0	0	0

**Percentage values in each column followed by different lower case letters are significantly different at P≤0.05 according to the Kruskal-Wallis test.*

% percentage of callus producing shoots
 # mean number of shoots per callus
GP Greenhouse tissue
EP Newly established cultures

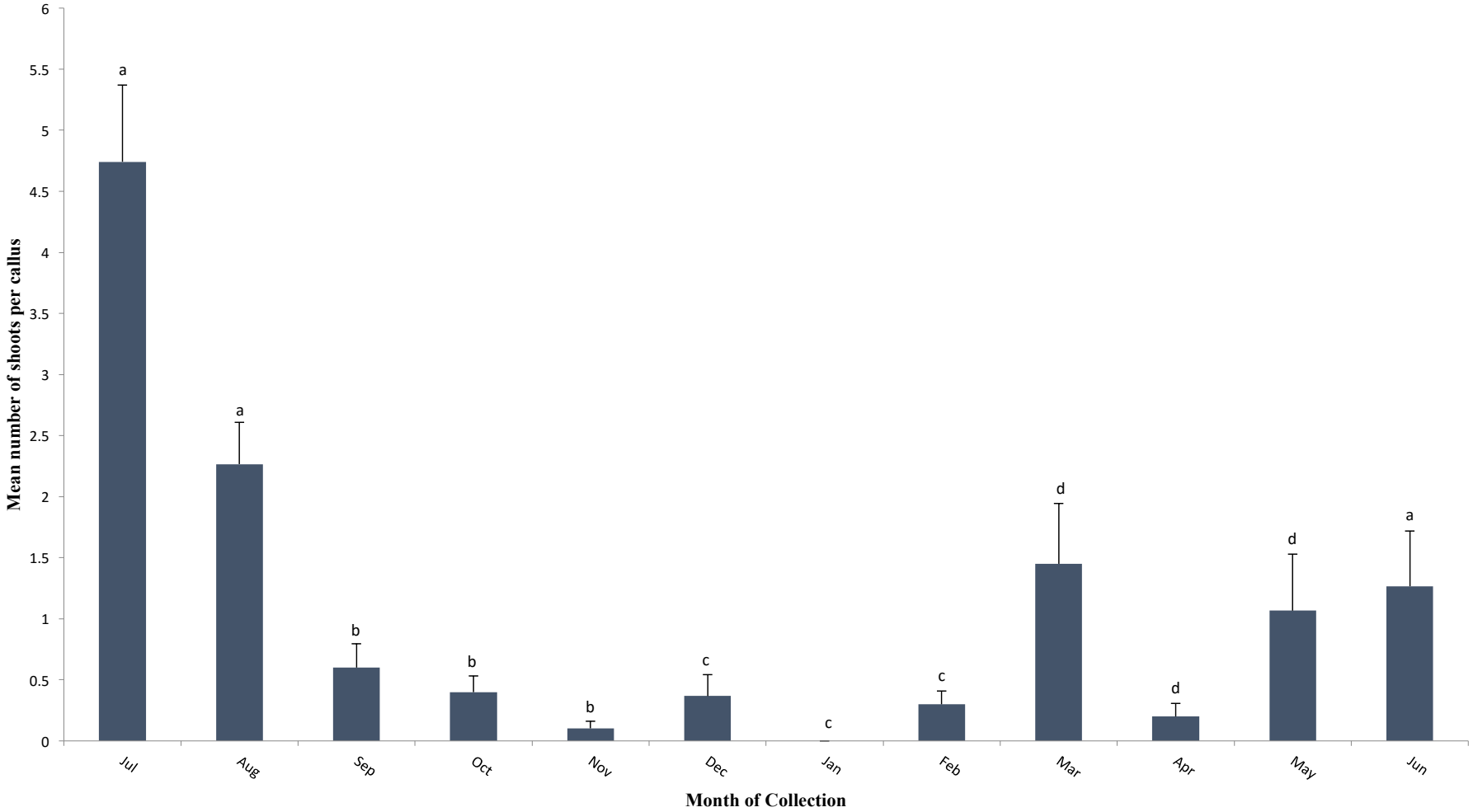

Figure 3.2 Effect of season on shoot induction of Paulownia fortunei x elongata greenhouse tissue (GP1) with standard error bars. Different lower case letters are significantly different at P≤0.05 according to the Kruskal-Wallis test. BM was used supplemented with 10µM BAP plus 0.5µM NAA.

3.3.2 Regeneration by Root Suckers

Preliminary experiments demonstrated that stabilised explants (P1) left in culture for extended periods of time would initiate roots suckers and based on this observation a new set of methods were developed.

Effect of Induction Technique on Root Sucker Production in Stabilised and Newly Established Cultures.

The rates of callus and shoot production by root suckers treated using excision above or below the node, and slicing through the roots, was generally low (Table 3.5). Control plants in both tissue sources did produce shoots at a low rate (10%, Table 3.5), however, treated stabilised explants produced none, and newly established explants responded only marginally better. The difference between control and treatments was not statistically significant (P=0.365), and there was also no significant difference the treatments (P=0.515). The average number of shoots produced was not significantly different between stabilised or newly established cultures (P=0.113) (Table 3.5). Root suckers were first evident at week 7 and final results were scored at the end of week 9 as there was no further production of root suckers after this time.

Table 3.5: Effect of induction technique on root sucker production after 9 weeks.

	Stabilised (P1)		Newly Established (P1)	
Treatment	% Shoots	# Shoots	% Shoots	#Shoots
Above	0	0	0	0
Below	0	0	20±0.13[a]	0.2±0.13
Root	0	0	20±0.13[a]	0.2±0.13
Control	10±0.1[a]	0.1±0.1	20±0.13[a]	0.2±0.13

Above – shoot tissue excised above node 1, Below - shoot tissue excised below node 1, Roots - roots sliced, Control – no physical manipulation.
**Percentage values in each column followed by different lower case letters are significantly different at P≤0.05 according to the Kruskal-Wallis test.*
% percentage of root suckers producing shoots
 # mean number of shoots per root sucker

Effect of Auxin Suppressant and Excision Position on Root Sucker Induction.

A pairwise comparison showed that there was a significant effect of the physical treatments on the proportion of shoots (P=0.015). There was no induction of root suckers in

the below treatment, while the root treatment showed the highest regeneration. However, production in the root treatment and the control was not significantly different (P=0.769)

Within physical treatment groups, the addition of NPA had no significant effect. Although the control treatments showed a varied response, with the highest percentage of roots sucker regenerating at 0µM and 10µM of NPA, no significant results were obtained from the use of NPA (P=0.29 and P=0.15). Root suckers were first evident in week 15 and results were scored at the end of week 17 due to time constraints.

Table 3.6: Effect of induction technique and auxin suppression on production of shoots by root suckers after 17 weeks.

		Newly Established (P1)	
	NPA (µM)	% Shoots	# Shoots
Below	0	0	0
	2.5	0	0
	5	0	0
	10	0	0
Roots	0	40±0.16[a*]	0.8±0.48
	2.5	20±0.13[a]	0.5±0.34
	5	10±0.10[a]	0.2±0.2
	10	10±0.10[a]	0.2±0.2
Control	0	20±0.13[b]	0.4±0.3
	2.5	10±0.10[b]	0.3±0.3
	5	10±0.10[b]	0.5±0.5
	10	20±0.13[b]	0.5±0.34

**Percentage values in each column followed by different lower case letters are significantly different at P≤0.05 according to the Kruskal-Wallis test.*
Below - shoot tissue excised below node 1, Roots - roots sliced, Control - no physical manipulation.
% percentage of root suckers producing shoots
 # mean number of shoots per root sucker

3.3.3 Somatic Embryogenesis

While tissue and callus segments appeared healthy during the initial weeks of all experiments, somatic embryos were not induced. In solid culture, prolonged exposure to PGR supplemented medium eventually lead to senescence after 6 weeks and in suspension cultures

after 8 weeks. Suspension cultures supplemented with casein hydrolysate became cloudy within a week of experiment initialisation, probably through contamination or from precipitation of the casein hydrolysate. There was also the potential that incubation conditions could have been factor.

3.3.4 Formation of Adventitious Roots and Rooting Medium Optimisation

Effect of IBA on Adventitious Root Formation

Rooting competence of clone CP1 was improved with the addition of IBA and the increase in concentration caused a direct increase in root production (Table 3.7). The highest mean numbers of roots were produced at a concentration of 5µM IBA at (week four) and the lowest at 0µM IBA (week two), representing a significant difference. There was also shown to be a significant difference between week 2 and weeks 3 and 4 ($P<0.001$) although there was no difference between weeks 3 and 4 ($P=0.622$). A two-way ANOVA indicated no interaction between week or treatment ($P=0.180$).

Table 3.7: Effect of auxin treatments on the production of roots of Paulownia fortunei x elongata (CP1) measured at weekly intervals over four weeks.

	# Roots (CP1)		
IBA(µM)	Week 2	Week 3	Week 4
0	8.5±0.33[a*]	9.4±0.46[e]	10.0±0.56[e]
1.25	12.7±0.68[b]	13.3±0.60[f]	13.9±0.60[f]
2.5	17.7±0.98[c]	21.1±1.07[g]	22.6±1.29[g]
5	25.6±1.77[d]	30.6±1.14[h]	33.5±1.46[h]

**Mean values in each column followed by different lower case letters are significantly different at p≤0.05 according to the Kruskal-Wallis test.*
 # mean number of roots per shoot

Rooting Response of In Vitro Shoots from Various Sources

While stabilised explants and newly established explants produced higher averages of roots than greenhouse explants there was no significant difference among explant sources. ($P=0.146$) (Table 3.8).

Table 3.8: Effect of standard auxin treatment on production of roots of Paulownia fortunei x elongata (CP1, EP1 and GP1) measured after 4 weeks.

	# Roots		
IBA(µM)	Stabilised (P1)	Newly Established (P1)	Greenhouse (P1)
2.5	17.5±1.26[a*]	18.2±0.82[a]	15.8±1.15[a]

**Mean values in each column followed by different lower case letters are significantly different at P≤0.05 according to the Kruskal-Wallis test.*
 # mean number of roots per shoot

3.4 Discussion

Callus regeneration was the most efficient method at inducing new explants. Due to the relative simplicity of the callus regeneration method and its ability to produce a significant quantity of explants from a wide range of sources, this method would be considered the most successful out of the three methods. Medium supplemented with higher levels of BAP (10µM-15µM) and NAA (0.25µM-0.5µM) produced the best response in shoot regeneration across different explants. Other studies (Yadav *et al*., 2013) also suggest that medium supplemented with BAP (8µM to 53µM) and NAA (0.5µM to 6µM) was effective for inducing callus and subsequent production of shoots.

The possibility of optimising a single callus regeneration method that works for all explants and clones of *Paulownia* was unlikely as, (Bergmann & Moon, 1997; Yadav *et al*., 2013) it has been demonstrated that that responses of different clones and species are highly variable even when exposed to the similar tissue culture methods and conditions (Bergmann & Moon, 1997; Yadav *et al*., 2013). Most importantly, mature tissue taken directly from the greenhouse readily induced callus and new shoots within several weeks. This eliminates lengthy periods of waiting for shoots to be established *in vitro* from axillary buds which can take up to several months.

Interestingly, shoot production from GP1 was highest in callus induced from the internode closest to shoot apical meristem (SAM), and declined when tissue was taken further away from the apex. This is most likely a physiological response to the maturation gradient, as

tissue further away from the apex becomes more recalcitrant to propagation (Wendling *et al*., 2014a). Corredoira (2008) demonstrated a similar effect in *Paulownia*, as leaves taken closest to the apex of the stem had higher rates of callus induction and shoot production than leaves harvested further away from the apex. This could explain some of the variation found in Experiments 1 and 2 as internode position was randomised among containers.

It is also important to consider the effect that continuous growth of the SAM has on the overall maturity of the plant. As previously stated, the further the meristems grow from the juvenile zone the more difficult it becomes to establish these tissues successfully *in vitro*. This was particularly evident in the response of greenhouse material to micropropagation over time (Fig 3.2). Because internodes were harvested each subsequent month, as the SAM grew further away from the base of the main trunk the production of callus and shoots decreased. This could also be linked to the changes in season as the mature stock plants would enter the flowering stages in spring and the fruiting periods in summer. A greenhouse study on the rooting competence of mature cuttings of the tropical species *Aphloia theifromi* showed greater rooting capacity during the hot months (October to March in Madagascar, where the study was conducted) (Danthu *et al*., 2008). Rooting percentage significantly decreased during the cold months, with cuttings producing no roots during the month of August (Danthu *et al*., 2008). This demonstrates the impact seasonal variability may have on plant phenology and consequently micropropagation. The regenerative capacity of *Paulownia* explants taken from adult trees and their response to *in vitro* propagation would most likely decline during the summer months, due to the phenological change from mature vegetative phase to flowering and fruiting. This could possibly explain some of the variability in the response of greenhouse material in the current study, and the low rates of shoot regeneration during the callus experiments. Future experiments exploring seasonal influence could be undertaken using juvenile stock plants as a comparison against mature stock plants observed in this study.

The success of root sucker induction was an interesting aspect of this study, as root suckers from *Paulownia* have never been successfully produced *in vitro* before. The process of inducing root suckers was relatively simple: it did not involve any complex PGR combinations and occurred spontaneously regardless of physical manipulation technique. Although it was successful, the method used took from 8 to 9 weeks to first induce roots suckers, and from 15 weeks to produce enough material for use in greenhouse experimentation. This is far longer than callus experiments (one week to initiate callus and then one week to induce shoots). The long time to root sucker induction could be due to the physiological age of the tissue that was used to initiate the stabilised and newly established cultures *in vitro*. Exploring the use of other

auxin inhibitors and their method of application maybe beneficial in decreasing the time it takes for regeneration to begin and the possible development of other physical manipulation methods should be considered. Wan *et al.,* (2006) explored these ideas, although with the notable difference that their research was conducted in nurseries, not *in vitro*. They (Wan *et al*., 2006) showed that the most significant effect of the auxin inhibitor NPA occurred was when it was applied directly to exposed xylem, whereas application to the bark of the tree produced no effect. This suggests that the method of application used in the current study could have had an impact on the effectiveness of the NPA: here it was incorporated into the medium rather than being directly applied to the explant tissue *in vitro,* which could have made it difficult for the explants to take up the NPA into the root tissue and rendering it ineffective. Other methods of NPA application could be explored, such as direct application to the exposed wound of the explant. If root sucker induction could be optimised to become more time efficient then it could be considered a practical micropropagation method. Investigation into whether this method could successfully reinvigorate mature tissue was analysed further during the greenhouse phase.

While explants were successfully generated from root suckers, somatic embryogenesis was not successful. The inability to produce new explants through indirect and direct somatic embryogenesis was anticipated, as the process of induction is complex and varied (Deo, Tyagi, Taylor, Harding, & Becker, 2011; Jiménez, 2005; Leljak-Levanić *et al*., 2015). While the methods developed in this study were based on methods that had been successful (Ipekci & Gozukirmizi, 2003, 2005), the original methods were optimised for juvenile plants of *Paulownia elongata,* while the species used in this research were a hybrid of mature *Paulownia elongata x fortunei*. Both these factors would be expected to have influenced how the explants responded and whether they could produce somatic embryos.

Furthermore, Ipecki & Gozukirmizi (2003, 2005) used a single genotype of *Paulownia elongata*, and the method may only work for the selected genotype and not in other seedlings or clones of the same species. As noted earlier, genotypic differences are common among conventionally propagated and micropropagated *Paulownia* species (Bergmann, 2003; Bergmann & Moon, 1997; Corredoira *et al*., 2008). Bergman and Moon (1997), confirmed genotypic variability was most noticeable in micropropagation and cannot necessarily be extrapolated to other clones and species. Explants produced from four different clones of the hybrid *Paulownia* 'Henan 1' showed significant differences in the number of shoots produced per explant (Bergmann & Moon, 1997). The response of shoot production was also significantly affected by the concentrations of PGR's used, with some clones responding to

higher or lower concentrations of BAP (Bergmann & Moon, 1997). The explants they used were derived from juvenile one-year-old stock plants grown from seed, indicating that genotypic variability is consistent between plants micropropagated from juvenile or mature sources (Bergmann & Moon, 1997). Such variation is common when evaluating micropropagation methods and differences in *Paulownia* genotypes have been reported in numerous studies (Bergmann & Moon, 1997; Bergmann & Whetten, 1998; Corredoira *et al*., 2008).

When somatic embryogenesis is successful, the ability to produce large amounts of new explants is significantly greater than other methods (Deo *et al*., 2011), however, unless the embryos outperform explants propagated from other methods in the field then the need to produce somatic embryos for plantation propagation becomes impractical.

The successful rooting competence of the P1 explants was not unexpected, as stock cultures readily produced roots on multiplication medium without the addition of root inducing auxins. The ease of adventitious rooting would indicate that these explants may have undergone some form of partial rejuvenation. Interestingly, rooting competence can be restored easily in other mature tree species, e.g. *Eucalyptus grandis,* after 7-12 rounds of subculturing *in vitro* (Titon, Xavier, & Otoni, 2006). This suggests that the partial rejuvenation of some characteristics can be manipulated not only by the method of micropropagation used but also the process of being introduced and subcultured *in vitro*. Although adventitious rooting is commonly used as a marker of juvenility, it is sometimes mistaken as a sign of full rejuvenation (Wendling *et al*., 2014b). It is more likely that some form of partial rejuvenation was occurring and that other characteristics such as vegetative growth and early flowering may still be physiologically mature. Although the two methods of callus regeneration and root sucker induction used here were successful in their ability to regenerate explants *in vitro,* it was important to explore whether these methods affected the overall growth and development of the explants. These characteristics can only be observed if explants are removed from culture and placed into a greenhouse or plantation environment.

CHAPTER 4: EFFECT OF MICROPROPAGATION TECHNIQUE ON PLANTLET GROWTH AND DEVELOPMENT

4.1 Introduction

Micropropagation studies in *Paulownia* have predominantly focussed on using different tissue culture methods to successfully micropropagate various species, and rarely extend to comprehensive observations in the field (Dimps Rao *et al*., 1996; Ipekci & Gozukirmizi, 2003, 2005). The long-term phenological responses of micropropagated *Paulownia* when transitioning new explants from the laboratory to the greenhouse have not been researched extensively (Dimps Rao *et al*., 1996; Ipekci & Gozukirmizi, 2005). The research that does exist focuses on development of micropropagation techniques and survival rates and/or rooting percentage of explants when transferred to the greenhouse but do not consider the long-term changes on phenology (Corredoira *et al*., 2008). A notable exception was a study conducted by Bergman *et al*. (1997, 1998 and 2003), who observed both conventional and micropropagated *Paulownia* over a period of five years. However, their studies did not compare the efficacy of multiple micropropagation methods. Bergman *et al*. (1997, 1998 and 2003) also utilised juvenile plantlets initiated from seed or juvenile explants micropropagated *in vitro*. Therefore, it is important to identify whether micropropagation technique does have a significant impact on the long-term phenology of the plants produced.

Most studies focus on survival rates or rooting percentage as a key indication of successful rejuvenation through micropropagation, and do not consider other characteristics, such as floral induction, to measure the extent of explant rejuvenation (Bergmann & Whetten, 1998; Corredoira *et al*., 2008). It is possible to assess what extent these methods have on the rejuvenation of explants by observing these traits over an extended period of time. This experiment aimed to evaluate how the different propagation methods and explant sources described earlier (Ch 3) impact growth and phenology in the long term.

4.2 Materials and Methods

Plantlets produced in previous experiments (Ch 3) were observed for a period of 6 months. This length of time was chosen based on stock plant growth and development under the same greenhouse conditions (Ch 2). This was enough time to give some indication of phenological development while still adhering to time and cost constraints.

4.2.1 Source of Treatments

Shoots were sourced from clone P1 only, as other clones did not produce sufficient material to be utilised for greenhouse experiments. A total of eight shoot/explant types were sourced from *in vitro* experiments and stock cultures (Fig 4.1): three from stabilised cultures, four from newly established cultures and one from stock tissue (Fig 4.1). Stabilised shoots were sourced from callus regeneration, one root sucker induction experiment and existing stock cultures. Newly established shoots were sourced from one callus regeneration experiment, two root sucker induction experiments and from stock cultures maintained *in vitro*. Greenhouse explants were sourced from one callus regeneration experiment. A total of 36 replicates were obtained from each source except for NE-RS2 (n = 15).

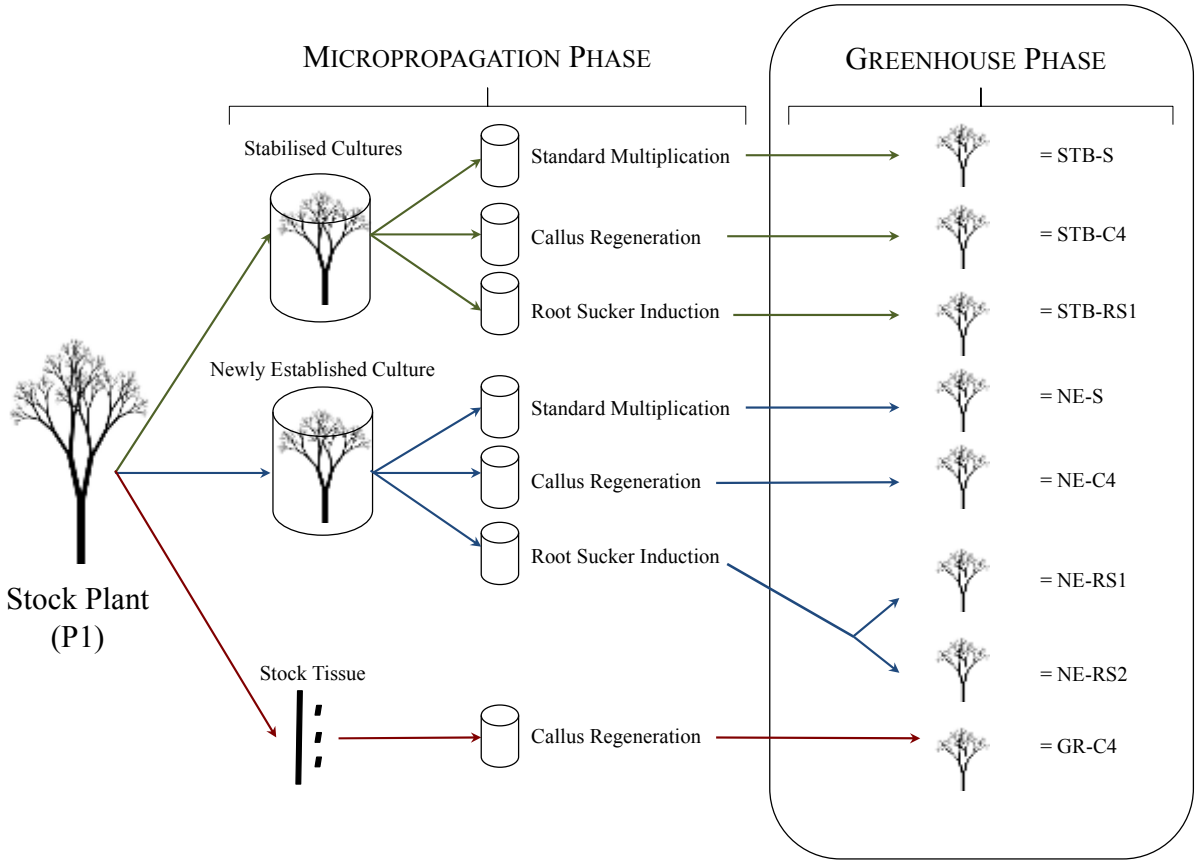

Figure 4.1. Experimental design for greenhouse phase. STB= Stabilised Cultures, NE= Newly Established Cultures, GR= Greenhouse Tissue, S= Standard Multiplication, C4= Callus Regeneration Experiment 4, RS1= Root Sucker Induction Experiment 1 and RS2= Root Sucker Experiment Induction 2.

4.2.2 Shoot Preparation

Shoots were cut to a length of 3cm and placed in rooting medium for one week to respond to auxin treatment, before being transferred to individual pots and only those that developed adventitious rooting were used. Adventitious rooting occurred in all explants and there was a 100% survival rate when transferred from the laboratory to the misting house. Shoots placed in the misting house were left to acclimatise for four weeks (Ch 2) before being transferred to the greenhouse.

All explants were between 8cm to 10cm at the time of transfer (beginning of week 1). Two weeks after transferral to the greenhouse, shoots were transplanted to 1.5L Rocket®POTS containing a mixture of 1:1 pasteurised white sand to pasteurised potting mix. Trays were set up in a randomized block design, with 8 pots per tray, and randomly repositioned at weekly intervals to ensure even exposure to all conditions. A mixture of commercial complete water-soluble fertiliser (Thrive®, 1g L^{-1}) was applied every seven days until the end of the experiment. Starting at week 12, shoots were watered twice every 24 hours for 10 minutes until they were harvested at 12 and 24 weeks. All other conditions were as outlined in in the general materials and methods (Ch 2).

4.2.3 Plant Growth and Phenology

Measurements of stem height, leaf length and width and number of flowers were taken at 8, 12 and 24 weeks. Stem height was measured from the base of the stem to the top of the first internode. The longest and widest point of the lowest two leaves was measured for leaf length and width.

Stem, leaf and flower biomass were measured by harvesting at two intervals - 12 and 24 weeks. At 12 weeks, half the replicates from each explant source were harvested at the base of the trunk. The leaves and flowers were excised from the stem and weighed separately, before being placed together into paper bags, and oven dried (90°C) for seven days. Dried samples were re-weighed and the numbers of flowers recorded.

The remaining root balls were rinsed, placed in plastic zip lock bags and stored in a cold room at 4°C until they were measured. Using the Newman Line Intercept Method to determine the total length (cm) of individual root samples (Smit *et al*., 2000). The same process was then repeated at 24 weeks with the remaining replicates.

Data were analysed using PRIMER package 6 (Primer-E, 2009). A correlation matrix was constructed for all variables to determine which were highly correlated and should be

excluded from analysis. A Principal Component Analysis (PCA) was used to determine if the source of the explant affected its overall growth and an Analysis of Similarity (ANOSIM) to determine any statistically significant differences among explant sources.

4.3 Results

4.3.1 General Growth Characteristics

There was no effect of shoot source or micropropagation method on the rate of growth and development in *Paulownia*. Adventitious rooting occurred in all explants and there was a 100% survival rate when transferred from the laboratory to the greenhouse.

Differences in height were evident at week 8 and 12 while floral development was more apparent between week 12 and 24; however, there was no statistically significant effect of time or explant source on stem height and biomass, leaf length, width and biomass, number of flowers, biomass and root length. Increases in stem height and biomass were most noticeable between 8 to 12 weeks (Fig 4.2 - Fig 4.5) with treatment NE-RS1 showing the greatest change in height (36.7cm in week 8 to 63.1cm in week 12).

Growth slowed between weeks 12 and 24 weeks (Fig 4.2), with NE-RS2 showing the lowest increase in height (from 62.8cm in week 12 to 63.1cm in week 24). There was an overall increase in stem biomass for each explant source from 12 to 24 weeks (Fig 4.3). Leaf length increased between 8 to 12 weeks and decreased between 12 to 24 weeks (Fig 4.7) and was closely correlated with a decrease in leaf biomass in the same period (Fig 4.4). It was also evident that leaf width and length decreased from week 12 to week 24 with larger leaves towards the base of the stem sensing and smaller leaves closest to the SAM remaining. There was a clear progression from leaf production at 12 weeks to flower production at 24 weeks, evidenced by the decrease in leaf biomass and the increase in flower number and biomass from weeks 12 to 24 (Fig 4.4 and Fig 4.5). It should be noted that there was no production of flowers from explants STB-C4 and NE-RS1 at 12 weeks and they also produced the least flowers at 24 weeks (Fig 4.6). Root length increased only slightly from 12 to 24 weeks, although there was a more noticeable increase in root length in NE-RS2 (Fig 4.8). Height, stem biomass, leaf biomass, root length, leaf length and leaf width were strongly correlated with one another at 12 weeks, however, while flower number and biomass were strongly correlated to each other they were not related to the other variables. At 24 weeks, there was a strong correlation between height, leaf biomass, flower biomass and flower number but none between root length and any other variable.

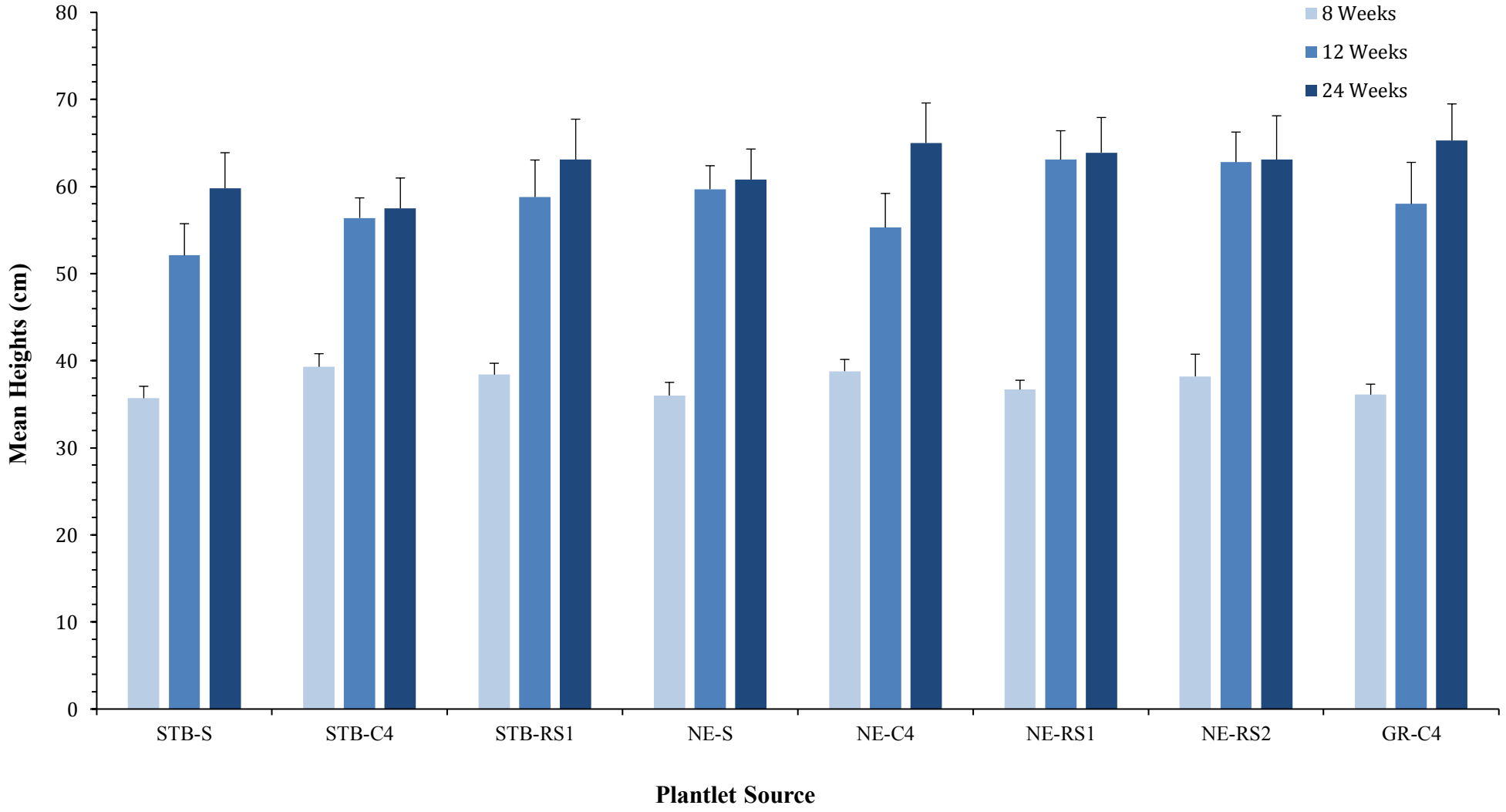

Figure 4.2 Effect of plantlet source on average height of Paulownia at 8, 12 and 24 weeks.
STB= Stabilised Cultures, NE= Newly Established Cultures, GR= Greenhouse Tissue, S= Standard Multiplication, C4= Callus Regeneration Experiment 4, RS1= Root Sucker Induction Experiment 1 and RS2= Root Sucker Experiment Induction 2.

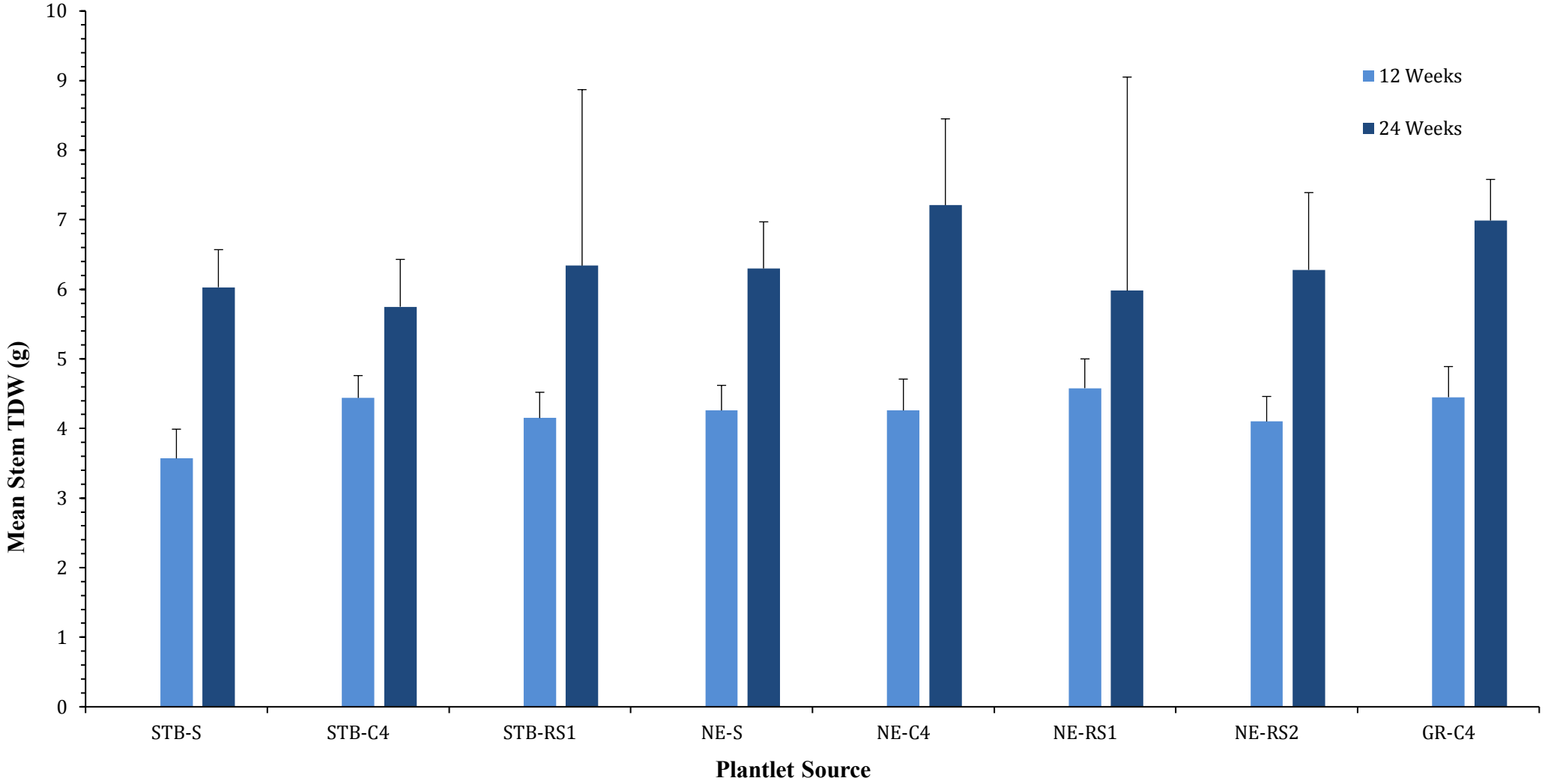

Figure 4.3 Effect of plantlet source on mean stem TDW of Paulownia at 12 and 24 weeks.
STB= Stabilised Cultures, NE= Newly Established Cultures, GR= Greenhouse Tissue, S= Standard Multiplication, C4= Callus Regeneration Experiment 4, RS1= Root Sucker Induction Experiment 1 and RS2= Root Sucker Experiment Induction 2.

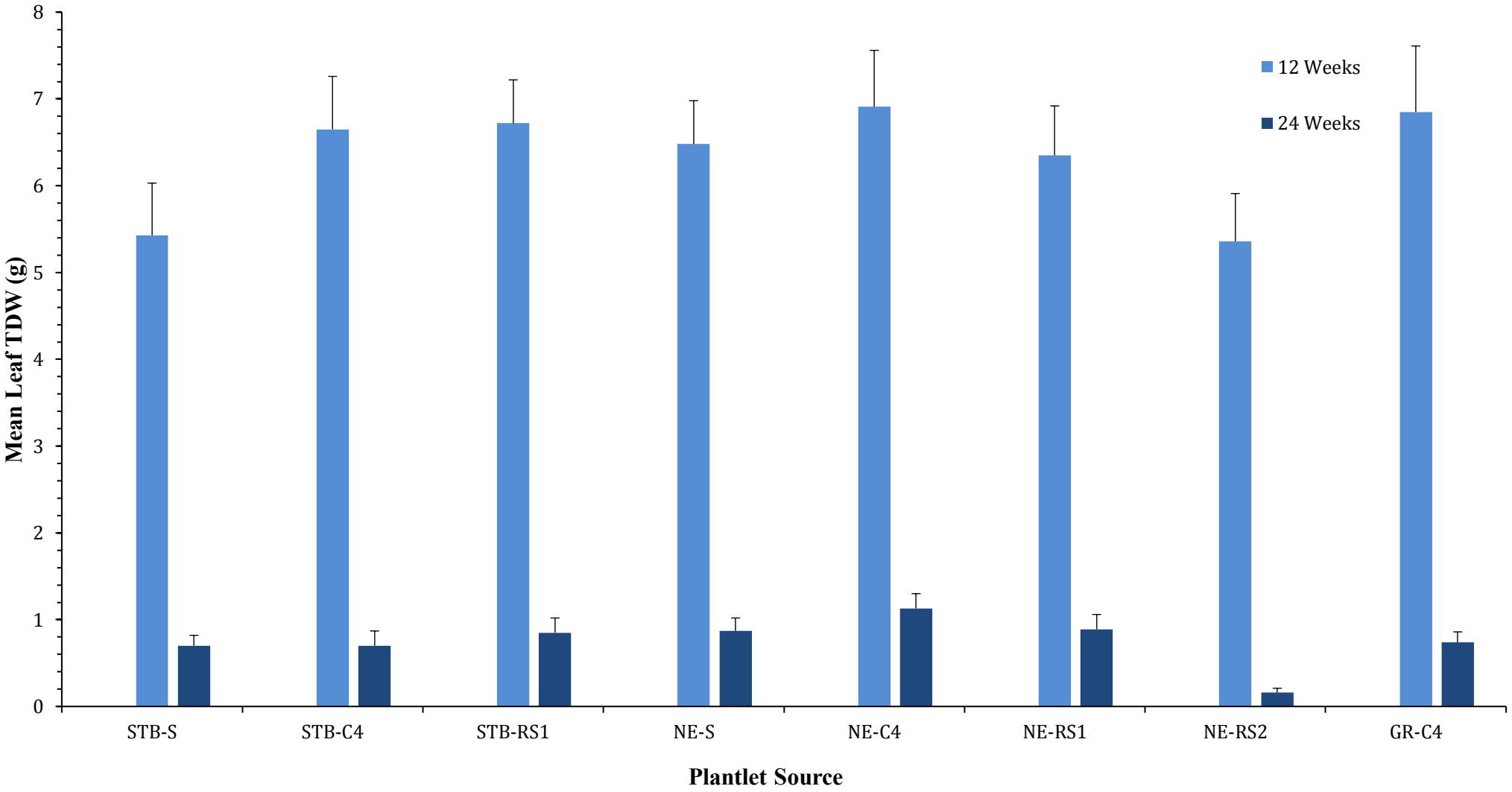

Figure 4.4 Effect of plantlet source on mean leaf TDW of Paulownia at 12 and 24 weeks.
STB= Stabilised Cultures, NE= Newly Established Cultures, GR= Greenhouse Tissue, S= Standard Multiplication, C4= Callus Regeneration Experiment 4, RS1= Root Sucker Induction Experiment 1 and RS2= Root Sucker Experiment Induction 2.

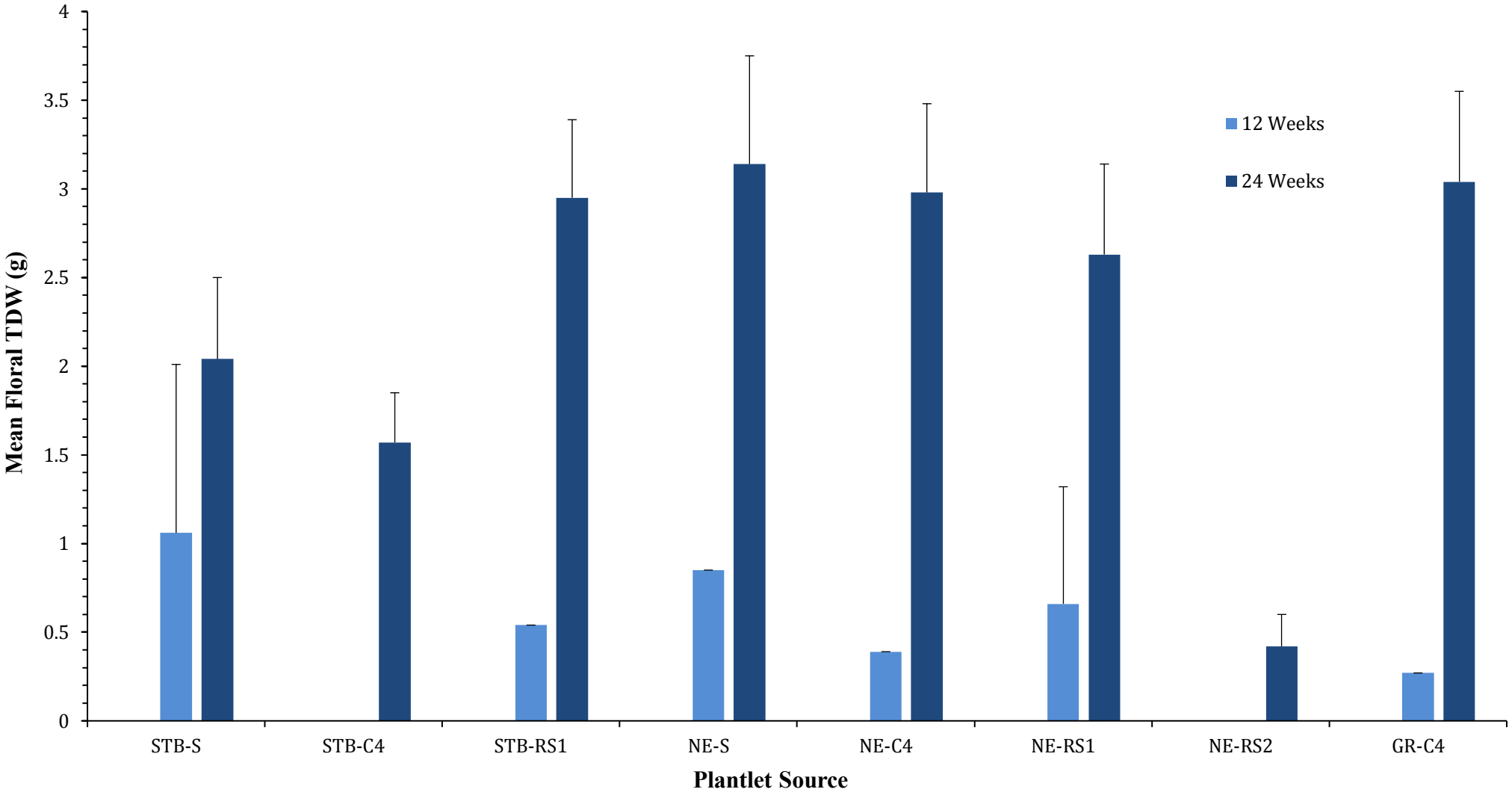

Figure 4.5 Effect of plantlet source on mean TDW of flowers of Paulownia at 12 and 24 weeks.
STB= Stabilised Cultures, NE= Newly Established Cultures, GR= Greenhouse Tissue, S= Standard Multiplication, C4= Callus Regeneration Experiment 4, RS1= Root Sucker Induction Experiment 1 and RS2= Root Sucker Experiment Induction 2.

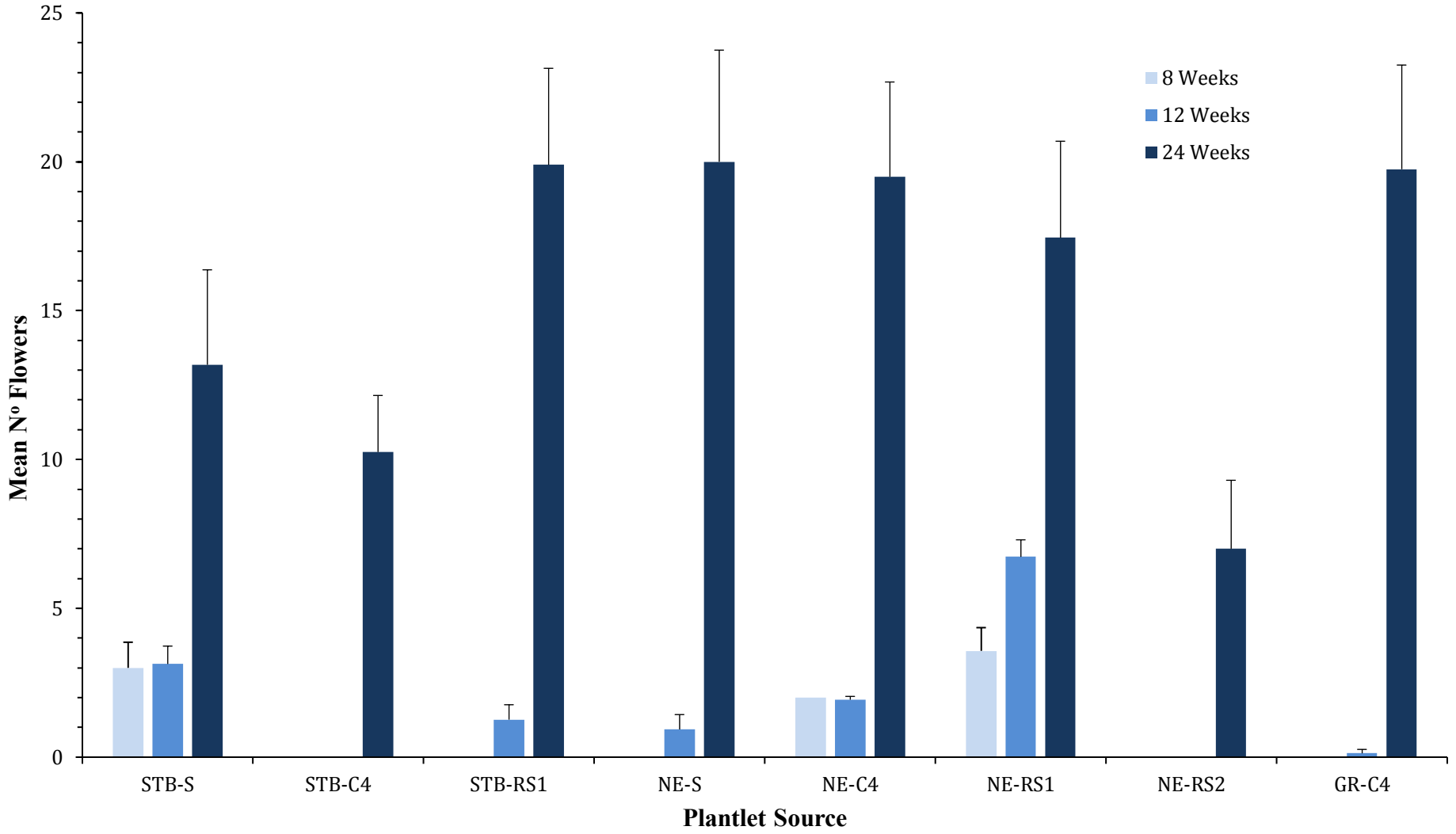

Figure 4.6 Effect of plantlet source on mean flower number of Paulownia at 12 and 24 weeks.
STB= Stabilised Cultures, NE= Newly Established Cultures, GR= Greenhouse Tissue, S= Standard Multiplication, C4= Callus Regeneration Experiment 4, RS1= Root Sucker Induction Experiment 1 and RS2= Root Sucker Experiment Induction 2.

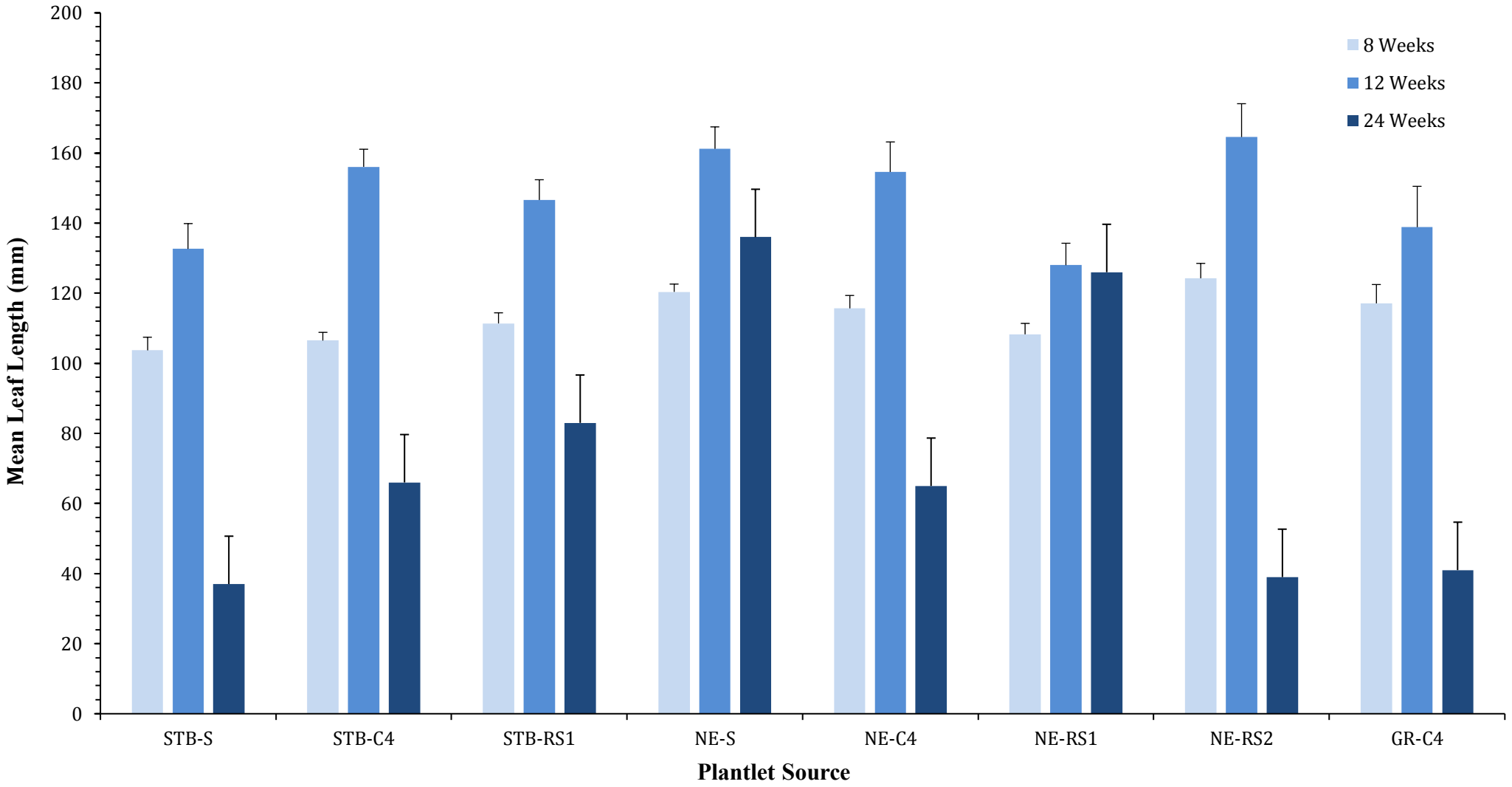

Figure 4.7 Effect of plantlet source on mean leaf length of Paulownia at 8, 12 and 24 weeks.
STB= Stabilised Cultures, NE= Newly Established Cultures, GR= Greenhouse Tissue, S= Standard Multiplication, C4= Callus Regeneration Experiment 4, RS1= Root Sucker Induction Experiment 1 and RS2= Root Sucker Experiment Induction 2.

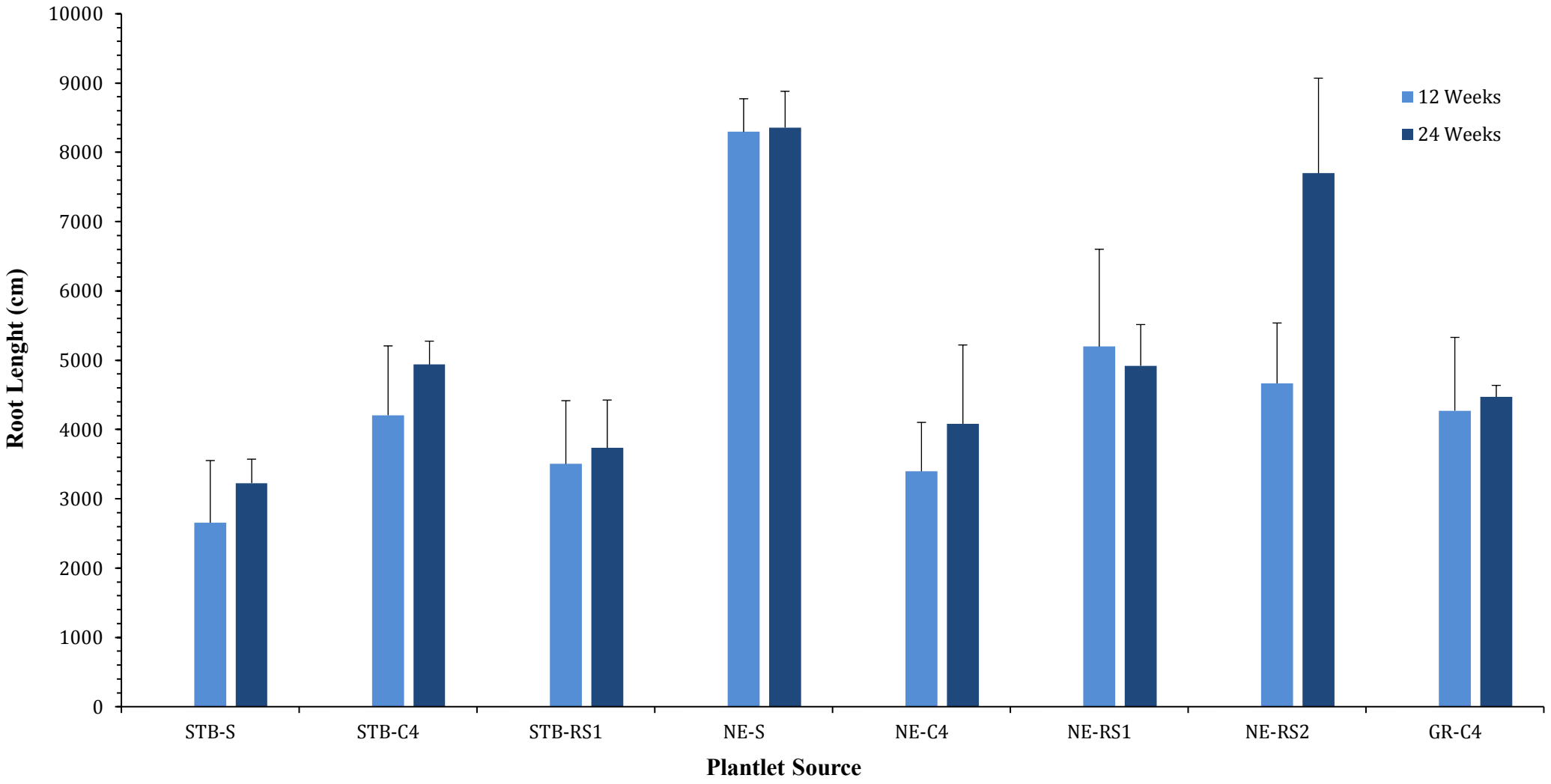

Figure 4.8 Effect of plantlet source on root length of Paulownia at 12 and 24 weeks.
STB= Stabilised Cultures, NE= Newly Established Cultures, GR= Greenhouse Tissue, S= Standard Multiplication, C4= Callus Regeneration Experiment 4, RS1= Root Sucker Induction Experiment 1 and RS2= Root Sucker Experiment Induction 2.

4.3.2 Effect of Source on Growth Characteristics

No groupings were evident among the explant sources indicating that there was no effect on the growth variables measured at each time interval. (Figs 4.9, 4.10, 4.11; Tables 4.1, 4.2, 4.3)). A principal components analysis for week 8 showed that PC1 and PC2 explained 40.7% and 32% of the variation respectively (Fig 4.9). The remainder of the variation was explained by PC3 (27.4 %). At 8 weeks, there was a cluster of individual explants grouping together that had begun to flower earlier than expected, however, this could not be attributed to explant source as there was no clear separation evident (Fig 4.9). Plotting PC3 against PC1 and PC2 showed a similar result (Appendix). PCA's for week 12 and 24 showed a greater percentage of the variation explained by PC1 and PC2 (71.5, 22.5 and 75.6, 17.1), and minimal variation being explained by PC3 (6.0 and 7.3 respectively). ANOSIM showed no significant difference between explant sources and week, as detected at the 5% significance level (Appendix).

Table 4.1 Eigenvalues and percentage variation explained by three principal component axes at 8 weeks. Eigenvectors for three growth measurements of each principal component also presented.

	Principal Component		
	1	2	3
Eigenvalues	1.22	0.959	0.821
Variation Explained (%)	40.7	32.0	27.4
Eigenvectors			
Leaf Length	-0.670	0.013	0.743
Height	-0.515	-0.729	-0.451
Flowers	0.536	-0.684	0.495

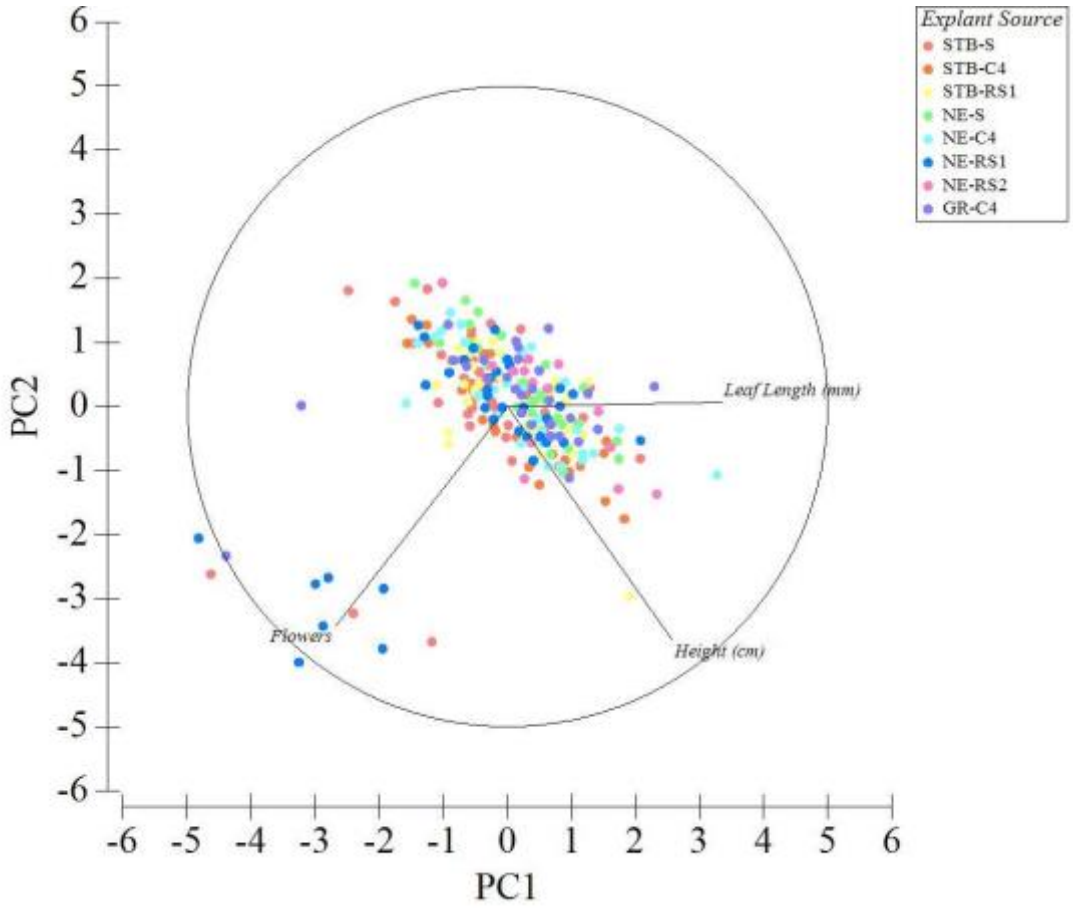

Figure 4.9 Plot of axes 1 vs 2 of principal components analysis of height, leaf length and number of flowers at 8 weeks.

Table 4.2 Eigenvalues and percentage variation explained by three principal component axes at 12 weeks. Eigenvectors for three growth measurements of each principal component also presented.

	Principal component axis		
	1	2	3
Eigenvalues	2.14	0.676	0.18
Variation Explained (%)	71.5	22.5	6.0
Eigenvectors			
Height	0.590	-0.524	0.615
Stem	0.641	-0.159	-0.751
Leaf Length	0.491	0.837	0.242

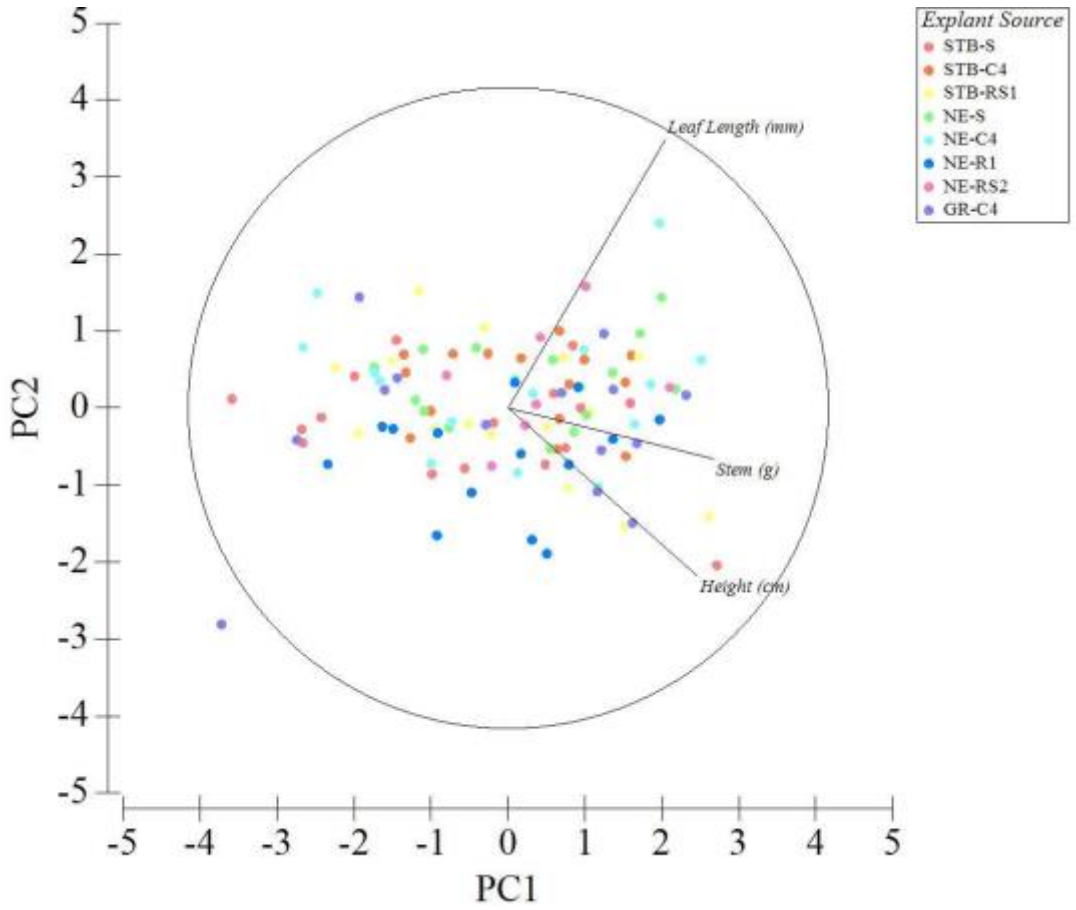

Figure 4.10 Plot of axes 1 vs 2 of principal components analysis of height, leaf length and stem biomass at 12 weeks.

Table 4.3 Eigenvalues and percentage variation explained by three principal component axes at 24 weeks. Eigenvectors for three growth measurements of each principal component also presented.

	Principal component axis		
	1	2	3
Eigenvalues	2.27	0.514	0.218
Variation Explained (%)	75.6	17.1	7.3
Eigenvectors			
Height	-0.606	0.312	0.732
Stem	-0.595	0.432	-0.677
Leaf biomass	-0.527	-0.846	-0.077

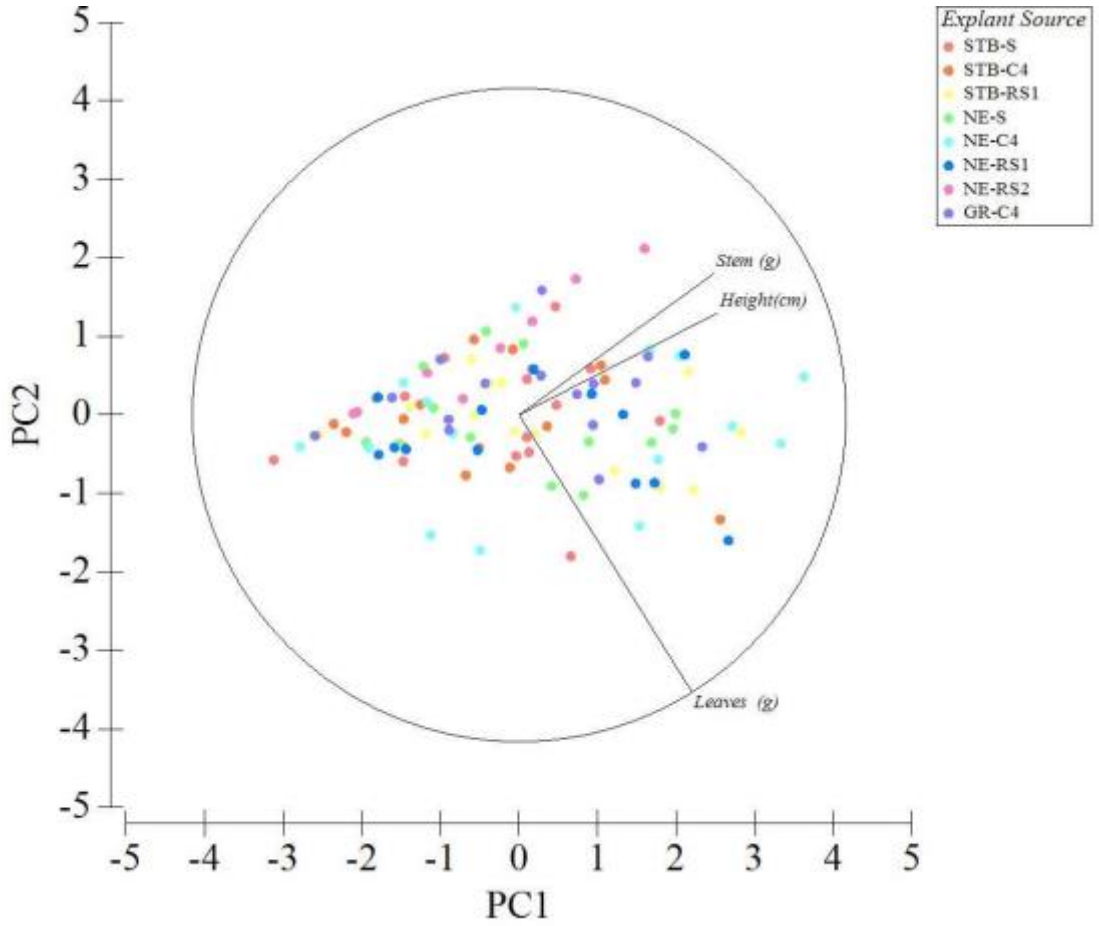

Figure 4.11 Plot of axes 1 vs 2 of principal components analysis of height, leaf and stem biomass at 24 weeks.

4.4 Discussion

It was clear from the results obtained that there was no significant effect of tissue source on the phenological variables measured. The lack of any significant variation among the heights, associated biomass and flowers produced is most likely a consequence of clone 1 being used for the majority *in vitro* experiments and greenhouse trials. As the plantlets in this study were from the same source it would be expected that they would also express the same growth rates and patterns of development. A lack of variation is not necessarily an undesirable outcome if the aim was to produce plantlets with the same phenological attributes. Often it is these desirable traits that encourage the development of various micropropagation protocols as plants grown from seedling can display different growth characteristics to the parent plant. Though clone 4 was also successfully established in culture and even used in callus regeneration experiments the resulting explants demonstrated poor growth *in vitro*.

Minor initial variation in flowering was evident between sources at week 8, however,

this was not evident after week 12 and could be attributed to the lack of genotypic variation. The production of flowers on the plant marks the progression from the juvenile phase to the mature phase. Early production of flowers here (6 months after establishment compared to 2 years from seed) appears to suggest that the materials sourced *in vitro* maintained their level of maturity while in culture, and these characteristics were expressed upon transfer to the greenhouse. In *Paulownia*, the time to production of flowers is different according to species, with some flowering during their second year and other species flowering during their fifth or even sixth year after planting (Zhu *et al*., 1986). Flower bud formation occurs over the late summer, early autumn months, with flowering following during spring and typically lasting around a month (Zhu *et al*., 1986). However, this pattern occurs in species of *Paulownia* and does not give a clear indication to the flowering pattern of *Paulownia* hybrids like those used in this research.

Early flowering was also observed in mature explants of micropropagated *Corylus* sp (Nas, Read, Miller, & Rutter, 2003), where explants that were continuously subcultured *in vitro* for more than three years produced male flowers within 12 months and nuts after three years after transfer to the field (Nas *et al*., 2003). Explants transferred to the greenhouse produced male flowers and nuts 15 to 18 months after being taken out of culture (Nas *et al*., 2003). This was premature in comparison to stock plants grown from seed, which normally produce nuts in their fifth year of growth (Nas *et al*., 2003). It would appear that mature explants retain certain physiologically mature characteristics *in vitro,* or regain them after being transferred from culture. Interestingly, Dimps *et al.* (1996) demonstrated a similar response in *Paulownia tomentosa* with juvenile explants. Micropropagated shoots were produced *in vitro* from excised juvenile leaves and transferred to a greenhouse for further analysis (Dimps Rao *et al*., 1996). A year after the transplantation date some of the micropropagated plants began to form complete flowers, a year earlier than when this species is propagated from seed (Dimps Rao *et al*., 1996; Zhu *et al*., 1986). They provide no data on the proportion of shoots that produced flowers, number of flowers and or time of flowering so a comprehensive comparison cannot be made, but this does suggest that micropropagated plantlets that are transferred to the greenhouse tend to produce flowers earlier than plantlets grown from seed.

The results from the current study are consistent with others that have shown it is possible to successfully restore rooting competence in mature *Paulownia* e.g. in mature explants of *Paulownia tomentosa* after being micropropagated *in vitro* and grown in root inducing medium containing IBA (Corredoira *et al*., 2008). It should be noted that rooting percentage was significantly reduced with the absence of IBA, a decrease from 90% to 65%

indicating the presence of auxin is a contributing factor to rooting competence (Corredoira *et al*., 2008). These effects are also evident in other species, such as mature explants of *Eucalyptus grandis* which readily produced roots after 7-12 sub-cultures *in vitro* (Titon *et al*., 2006). Micropropagation can produce at least partial rejuvenation in mature explants, however, it is unlikely that complete rejuvenation took place as the early onset of flowering indicates that plantlets maintained physiologically mature characteristics.

It would be beneficial to explore how these same methods used (Ch 3) affect the growth and development of explants sourced from juvenile stock plants. Juvenile material may respond differently and this would also provide a valuable comparison for the results obtained in the greenhouse. It is difficult to determine the extent to which these methods (Ch 3) have had an effect, as all plantlets were of the same ontogenetic age and from the same genetic material. This study does indicate that mature characteristics can be maintained *in vitro*, whilst also providing improved rooting competence necessary for plantlet survival. This could be advantageous in industries where large-scale propagation is needed, while still maintaining the physiological make-up of the selected mature tissue.

CHAPTER 5: SYNTHESIS

This study has designed and tested a set of protocols for the micropropagation of *Paulownia,* which could be used for plantation production. Many variables, such as ease of regeneration and the ability to rejuvenate tissue, were considered when determining which method would be the most applicable and efficient in terms of time and cost. Based on the outcomes of this research, several conclusions can be made about which micropropagation method was the most successful within the scope of this study.

5.1 The Micropropagation Phase

To properly evaluate the different micropropagation methods, it is first important to assess whether the clones chosen will respond to tissue culture. It has repeatedly been shown that there are differences in how clones of the same species respond to various micropropagation techniques. In this research Clone P1 was the only one to successfully respond to all micropropagation treatments, making it the most suitable choice for greenhouse studies. Clone P4 was successfully established in culture but failed to produce callus, while Clones 2, 3 and 5 performed poorly *in vitro*. Often, one or even two clones can be easily cultivated and manipulated while the rest tend to produce little to no response (Bergmann, 2003; Gitonga *et al*., 2010). In addition, the source of material to be used for cultivation *in vitro* should ideally come from the juvenile zones of the plant, especially if rejuvenation is not achieved *in vitro* and from elite clones (such as those examined here). While these clones may be from the same plantation, genotypic variation is always a possibility and will influence how well a method performs.

Of the methods examined, callus regeneration was the most successful in terms of the criteria discussed. After developing an ideal callus regeneration medium, it took on average around four weeks to produce a substantial quantity of new explants. Initial callus regeneration experiments showed a good response from stabilised and newly established cultures, but overall greenhouse material was the most consistent. However, greenhouse material is limited by seasonality and the difficulty of obtaining juvenile material suitable for micropropagation. The act of inducing callus has also been shown to induce genetic variation of the explant through the process of somaclonal variation (Bairu *et al*., 2011). Genetic testing of callus could be used to see if there are changes in the genome of *in vitro* cultures over time. This may also be a source of some of the variability observed in the other methods developed in this study.

Stabilised and newly established explants were both successful during root sucker

induction experiments. While the induction of root suckers was explored, the method as it stands is complex, labour-intensive and could be considered unviable for commercial timber production. Root sucker induction on average took 12 weeks to produce new explants and the number of new explants it yielded was comparatively small. Nevertheless, the method was successful and there is potential for further development. The very low rate of root sucker induction could be overcome by utilising standard culture multiplication. It should be noted that root sucker induction was only successful in tissue sourced from clone P1, and the ability to induce root suckers in other clones or species of *Paulownia* is not guaranteed.

Methods developed here for somatic embryogenesis were also complex and time consuming, and failed to produce any explants. It would be still worthwhile examining the potential effect somatic embryogenesis may have on the growth and development of mature *Paulownia* explants. Reports in the literature suggest it may induce only partial rejuvenation in mature tissue. Research conducted on other hardwood species (*Quercus robur)* has shown that somatic embryogenesis may only offer partial rejuvenation of particular growth characteristics (Martínez *et al*., 2012). Mature and juvenile explants of *Quercus robur* were micropropagated using standard multiplication techniques and somatic embryogenesis (Martínez *et al*., 2012). Shoots derived from somatic embryos of mature explants expressed a greater degree of shoot and root regeneration compared to mature explants that had been micropropagated through subculturing (Martínez *et al*., 2012). Shoots derived from somatic embryos of juvenile explants showed no difference in shoot growth or development compared to those produced from subculturing. Although, shoots produced from somatic embryos had a greater degree of root regeneration than those produced from subculturing (Martínez *et al*., 2012). When shoots produced from somatic embryos of both mature and juvenile explants were transferred to the greenhouse significant differences were observed in plant height (Martínez *et al*., 2012). After four months juvenile plantlets had grown on average 21.7cm while mature plantlets grew on average 10.9cm (Martínez *et al*., 2012). After 12 months the average had increased significantly from 40.4cm and 23.0cm respectively (Martínez *et al*., 2012). Again, this supports the idea that when explants are removed from culture and placed in the greenhouse or field they quickly regain their mature characteristics. Although improved rooting competence can be readily achieved by using the methods examined elsewhere in this current study, it would still be beneficial to observe the effects somatic embryogenesis has on other growth characteristics in *Paulownia*.

5.2 The Greenhouse Phase

The greenhouse experiment (Chapter 4) demonstrated there was no clear relationship between the method of micropropagation and overall plant growth and development. As the plantlets used in this greenhouse phase were sourced from the same clone, it is not unexpected that they would also express the same growth rates and patterns of development. Both callus induction and root sucker induction appear to maintain the mature characteristics of the original tissue, while improving the ability of this tissue to produce adventitious roots. As previously described, adventitious rooting is considered a juvenile characteristic and it was likely that the cultured explants have undergone a form of partial rejuvenation.

5.3 Implications for Future Research

It is evident that the method of micropropagation is not likely to have any effect on the growth and development on the resulting plantlets. While callus regeneration and root sucker induction differed in their ability to produce new shoots, once the first generation of explants have been obtained, multiplication methods can be used to overcome the limitations of quantity. However, it would be beneficial to determine how these methods affect the development of additional genotypes which was not addressed by this research. It was also evident that explants produced from both these methods were easily multiplied in culture, induce adventitious roots when required and have higher survival rates upon transfer to the greenhouse. Though Ipecki & Gozukirmizi's (2003, 2005) success in somatic embryogenesis could not be replicated however, it is still worthy of further exploration.

The method of micropropagation selected ultimately comes down to the discretion of the user and what specific result they are trying to achieve. If the outcome is to produce large quantities of either juvenile or mature shoots for production, this can be achieved by establishing new explants in culture and utilising the appropriate multiplication techniques to meet production yields. Therefore, it becomes unnecessary to subject *in vitro* cultures or greenhouse material to multiple complex micropropagation techniques, as this ultimately has no impact on their subsequent growth and development when transferred to the greenhouse.

REFERENCES

Adams, S. R., Pearson, S., Hadley, P., & Patefield, W. M. (1999). The effects of temperature and light integral on the phases of photoperiod sensitivity in *Petunia* x hybrida. *Annals of Botany, 83*(3), 263-269. doi:10.1006/anbo.1998.0817

Amissah, J. N., & Bassuk, N. (2009). Cutting back stock plants promotes adventitious rooting of stems of *Quercus bicolor* and *Quercus macrocarpa*. *Journal of Environmental Horticulture, 27*(3), 159-165.

Auge, R., Beauchedne, G., Boccon-Gibod, J., Decourtye, L., Digat, R., Jalouzot, R., . . . Vidalie, H. (1995). *In vitro culture and its applications in horticulture*. United States of America: Science Publishers.

Bairu, M., Aremu, A., & Van Staden, J. (2011). Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulation, 63*(2), 147-173. doi:10.1007/s10725-010-9554-x

Barthélémy, D., & Caraglio, Y. (2007). Plant Architecture: A Dynamic, Multilevel and Comprehensive Approach to Plant Form, Structure and Ontogeny. *Annals of Botany, 99*(3), 375-407. doi:10.1093/aob/mcl260

Beck, S. L., Dunlop, R., & van Staden, J. (1998). Rejuvenation and micropropagation of adult *Acacia mearnsii* using coppice material. *Plant Growth Regulation, 26*(3), 149-153. doi:10.1023/a:1006179620554

Beel, M., Davis, S., Murphy, J., & Piper, P. (2005). Product potential of *Paulownia* timber. *Australian Forestry, 68*(1), 3-8.

Bergmann, B. A. (1998). Propagation method influences first year field survival and growth of *Paulownia*. *New Forests, 16*(3), 251-264.

Bergmann, B. A. (2003). Five years of *Paulownia* field trials in North Carolina. *New Forests, 25*(3), 185-199.

Bergmann, B. A., & Moon, H. (1997). *In vitro* adventitious shoot production in *Paulownia*. *Plant Cell Reports, 16*(5), 315-319.

Bergmann, B. A., & Whetten, R. (1998). *In vitro* rooting and early greenhouse growth of micropropagated *Paulownia elongata* shoots. *New Forests, 15*(2), 127-138.

Bonga, J. M., Klimaszewska, K. K., & von Aderkas, P. (2010). Recalcitrance in clonal propagation, in particular of conifers. *Plant Cell, Tissue and Organ Culture (PCTOC), 100*(3), 241-254. doi:10.1007/s11240-009-9647-2

Burn, J. E., Bagnall, D. J., Metzger, J. D., Dennis, E. S., & Peacock, W. J. (1993). DNA methylation, vernalisation, and the initiation of flowering. *Proceedings of the National Academy of Sciences of the United States of America, 90*(1), 287-291.

Chang, S. H., Ho, C. K., Chen, Z. Z., & Tsay, J. Y. (2001). Micropropagation of *Taxus mairei* from mature trees. *Plant Cell Reports, 20*(6), 496-502.

Chory, J., Reinecke, D., Sim, S., Washburn, T., & Brenner, M. (1994). A role for cytokinins in de-etiolation in *Arabidopsis* (det mutants have an altered response to cytokinins). *Plant Physiology, 104*(2), 339-347. doi:10.1104/pp.104.2.339

Corredoira, E., Ballester, A., & Vieitez, A. (2008). Thidiazuron induced high frequency plant regeneration from leaf explants of *Paulownia tomentosa* mature trees. *Plant Cell, Tissue and Organ Culture, 95*(2), 197-208.

Danthu, P., Hane, B., Sagna, P., & Gassama, Y. K. (2002). Restoration of rooting competence in mature *Faidherbia albida*; a Sahelian leguminous tree, through serial root sucker micrografting. *New Forests, 24*(3), 239-244. doi:10.1023/a:1021396814374

Danthu, P., Ramaroson, N., & Rambeloarisoa, G. (2008). Seasonal dependence of rooting success in cuttings from natural forest trees in Madagascar. *Agroforestry Systems, 73*(1), 47-53. doi:10.1007/s10457-008-9116-7

Davies, F. T., Hartmann, H. T., Geneve, R. L., & Kester, D. E. (1997). *Plant propagation: Principles and practices* (6th ed.). Upper Saddle River, N.J.: Prentice Hall.

Delvaux, C., Sinsin, B., Van Damme, P., & Beeckman, H. (2010). Wound reaction after bark harvesting: microscopic and macroscopic phenomena in ten medicinal tree species (Benin). *Trees - Structure and Function, 24*(5), 941-951. doi:10.1007/s00468-010-0465-2

Deo, P. C., Tyagi, A. P., Taylor, M., Harding, R., & Becker, D. (2011). Factors affecting somatic embryogenesis and transformation in modern plant breeding. *The South Pacific Journal of Natural and Applied Sciences, 28*(1), 27-40.

Dimps Rao, C., Goh, C., & Kumar, P. (1996). High frequency adventitious shoot regeneration from excised leaves of *Paulownia* spp. cultured *in vitro*. *Plant Cell Reports, 16*(3), 204-209.

Ede, F. J., Auger, M., & Green, T. G. A. (1997). Optimizing root cutting success in *Paulownia* sp. *Journal of Horticultural Science, 72*(2), 179-185.

eFloras. (2008). Flora of China. *Published on the Internet* http://www.efloras.org/ *[accessed 22 October 2016]*. Retrieved from http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=124177

Eldridge, K., Davidson, J., Harwood, C., & van Wyk, G. (1993). *Eucalypt domestication and breeding*. New York: Oxford University Press Inc.

England, J., & Attiwill, P. (2006). Changes in leaf morphology and anatomy with tree age and height in the broadleaved evergreen species, *Eucalyptus regnans* F. Muell. *Trees - Structure and Function, 20*(1), 79-90.

Fan, G.-q., Zhai, X.-q., Jiang, J., & Liu, X. (2002). Callus induction from *Paulownia* plant leaves and their plantlet regeneration. *Scientia Silvae Sinicae, 38*, 29-35.

Fan, G.-q., Zhai, X.-q., Zhai, C.-j., & Bi, H.-t. (2001). Callus induction from leaves of different *Paulownia* species and its plantlet regeneration. *Journal of Forestry Research, 12*(4), 209-214.

Finkeldey, R. (1992). The hybrid origin of *Paulownia taiwaniana* Hu and Chang evidence from isozyme gene markers. *Silvae Genetica, 41*(4-5), 278-282.

George, E. F. (2008). *Plant propagation by tissue culture: The Background* (3rd ed.). Netherlands: Springer.

Gitonga, L., N., Gichuki, S., T., Ngamau, K., Muigai, A., W, T., Kahangi, E., M., Wasilwa, L., A., . . . Njogu, N. (2010). Effect of explant type, source and genotype on *in vitro* shoot regeneration in macadamia (*Macadamia* spp.). *Journal of Agricultural Biotechnology and Sustainable Developement, 2*(7), 129-135.

Gomes, F., & Canhoto, J. (2009). Micropropagation of strawberry tree (*Arbutus unedo*) from adult plants. *In vitro Cellular & Developmental Biology - Plant, 45*(1), 72-82.

Greenwood, M. S. (1995). Juvenility and maturation in conifers: current concepts. *Tree Physiology, 15*(7-8), 433-438. doi:10.1093/treephys/15.7-8.433

Greenwood, M. S., Day, M. E., & Schatz, J. (2010). Separating the effects of tree size and meristem maturation on shoot development of grafted scions of red spruce (*Picea rubens* Sarg.). *Tree Physiology, 30*(4), 459-468. doi:10.1093/treephys/tpq004

Haapala, T., Pakkanen, A., & Pulkkinen, P. (2004). Variation in survival and growth of cuttings in two clonal propagation methods for hybrid aspen (*Populus tremula*×*Populus tremuloides*). *Forest Ecology and Management, 193*(3), 345-354.

Haffner, V., Enjalric, F., Lardet, L., & Carron, M. P. (1991). Maturation of woody plants: A review of metabolic and genomic aspects. *Annals of Forest Science, 48*(6), 615-630.

Hardie, I., Kundt, J., & Miyasaka, E. (1989). Economic feasibility of U.S. *Paulownia* plantations. *Journal of Forestry Research, 87*, 19-24.

Heuret, P., Meredieu, C., Coudurier, T., Courdier, F., & Barthelemy, D. (2006). Ontogenetic trends in the morphological features of main stem annual shoots of *Pinus pinaster* (Pinaceae). *American Journal of Botany, 93*(11), 1577-1587. doi:10.3732/ajb.93.11.1577

Husen, A., & Pal, M. (2003). Effect of serial bud grafting and etiolation on rejuvenation and rooting cuttings of mature trees of *Tectona grandis* Linn. f. *Silvae Genetica, 52*(2), 84-88.

Husen, A., & Pal, M. (2006). Variation in shoot anatomy and rooting behaviour of stem cuttings in relation to age of donor plants in teak (*Tectona grandis* Linn. f.). *New Forests, 31*(1), 57-73.

Ipekci, Z., Altinkut, A., Kazan, K., Bajrovic, K., & Gozukirmizi, N. (2001). High frequency plant regeneration from nodal explants of *Paulownia elongata*. *Plant Biology, 3*(2), 113-115.

Ipekci, Z., & Gozukirmizi, N. (2003). Direct somatic embryogenesis and synthetic seed production from *Paulownia elongata*. *Plant Cell Reports, 22*(1), 16-24.

Ipekci, Z., & Gozukirmizi, N. (2005). Indirect somatic embryogenesis and plant regeneration from leaf and internode explants of *Paulownia elongata*. *Plant Cell, Tissue and Organ Culture, 79*(3), 341-345.

Jain, N., & Babbar, S. B. (2003). Regeneration of juvenile plants of black plum, *Syzygium cuminii* Skeels, from nodes of mature trees. *Plant Cell, Tissue and Organ Culture, 73*(3), 257-263.

Jiménez, V. M. (2005). Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. *Plant Growth Regulation, 47*(2-3), 91-110. doi:10.1007/s10725-005-3478-x

Johnson, J. E., Mitchem, D. O., & Kreh, R. E. (2003). Establishing royal *Paulownia* on the Virginia Piedmont. *New Forests, 25*(1), 11-23.

Krakowski, J., Benowicz, A., Russell, J. H., & El-Kassaby, Y. A. (2005). Effects of serial propagation, donor age, and genotype on *Chamaecyparis nootkatensis* physiology and growth traits. *Canadian Journal of Forest Research, 35*(3), 623-632. doi:10.1139/x04-207

Ky-Dembele, C., Tigabu, M., Bayala, J., Savadogo, P., Boussim, I. J., & Odén, P. C. (2011). Clonal propagation of *khaya senegalensis*: The effects of stem length, leaf area, auxins, smoke solution, and stockplant age. *International Journal of Forestry Research, 2011*.

Laureysens, I., Deraedt, W., & Ceulemans, R. (2005). Population dynamics in a 6-year-old coppice culture of poplar: II. Size variability and one-sided competition of shoots and stools. *Forest Ecology and Management, 218*(1-3), 115-128.

Leifert, C., & Cassells, A. (2001). Microbial hazards in plant tissue and cell cultures. *In vitro Cellular & Developmental Biology - Plant, 37*(2), 133-138.

Leljak-Levanić, D., Mihaljević, S., & Bauer, N. (2015). Somatic and zygotic embryos share common developmental features at the onset of plant embryogenesis. *Acta Physiologiae Plantarum, 37*(7), 1-14. doi:10.1007/s11738-015-1875-y

Ma, Q. (2008). Genetic engineering of cytokinins and their application to agriculture. *Critical Reviews in Biotechnology, 28*(3), 213-232. doi:doi:10.1080/07388550802262205

Magyar-Tábori, K., Dobránszki, J., Teixeira da Silva, J., Bulley, S., & Hudák, I. (2010). The role of cytokinins in shoot organogenesis in apple. *Plant Cell, Tissue and Organ Culture (PCTOC), 101*(3), 251-267. doi:10.1007/s11240-010-9696-6

Mankessi, F., Saya, A., Baptiste, C., Nourissier, S., & Monteuuis, O. (2009). *In vitro* rooting of genetically related *Eucalyptus urophylla* & *Eucalyptus grandis* clones in relation to the time spent in culture. *Trees - Structure and Function, 23*(5), 931-940. doi:10.1007/s00468-009-0335-y

Martínez, T., Vidal, N., Ballester, A., & Vieitez, A. (2012). Improved organogenic capacity of shoot cultures from mature pedunculate oak trees through somatic embryogenesis as rejuvenation technique. *Trees, 26*(2), 321-330. doi:10.1007/s00468-011-0594-2

Mason, W. L., Menzies, M. I., & Biggin, P. (2002). A comparison of hedging and repeated cutting cycles for propagating clones of Sitka spruce. *Forestry, 75*(2), 149-162. doi:10.1093/forestry/75.2.149

McComb, J. A., Bennett, I. J., & Tonkin, C. M. (1996). *In vitro* propagation of *Eucalyptus species*. *Tissue Culture of Australian Plants.* (pp. 112-156). Armidale: University of New England Press.

McCown, B. (2000). Special symposium: *In vitro* plant recalcitrance recalcitrance of woody and herbaceous perennial plants: Dealing with genetic predeterminism. *In vitro Cellular & Developmental Biology - Plant, 36*(3), 149-154.

McCown, D., & McCown, B. (1987). North American hardwoods. In J. M. Bonga & D. J. Durzan (Eds.), *Cell and Tissue Culture in Forestry* (pp. 247–260). Dordrecht: Martinus Nijhoff.

Mendoza de Gyves, E., Royani, J., & Rugini, E. (2007). Efficient method of micropropagation and *in vitro* rooting of teak (*Tectona grandis L.*) focusing on large-scale industrial plantations. *Annals of Forest Science, 64*(1), 73-78. doi:10.1051/forest:2006090

Mitchell, R. G., & Jones, N. B. (2006). The effects of ontogenetic maturation in *Pinus patula* – Part II: Hedge cycling and field performance. *The Southern African Forestry Journal, 207*(1), 3-6. doi:10.2989/10295920609505246

Moon, H., Park, S., Kim, Y., & Kim, S. (2008). Somatic embryogenesis and plantlet production using rejuvenated tissues from serial grafting of a mature *Kalopanax septemlobus* tree. *In vitro Cellular & Developmental Biology, 44*(2), 119-127. doi:10.1007/s11627-008-9122-5

Munne-Bosch, S. (2007). Aging in perennials. *Critical Reviews in Plant Sciences, 26*(3), 123-138.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497.

Naik, S., & Chand, P. (2011). Tissue culture-mediumted biotechnological intervention in pomegranate: a review. *Plant Cell Reports, 30*(5), 707-721. doi:10.1007/s00299-010-0969-7

Naing, A. H., Kim, C. K., Yun, B. J., Jin, J. Y., & Lim, K. B. (2013). Primary and secondary somatic embryogenesis in Chrysanthemum cv. Euro. *Plant Cell, Tissue and Organ Culture (PCTOC), 112*(3), 361-368. doi:10.1007/s11240-012-0243-5

Nas, M. N., Read, P. E., Miller, V., & Rutter, P. A. (2003). *In vitro* “rejuventaion” of woody species is temporary. *Acta Hort, 625*, 211-215. doi:10.17660/ActaHortic.2003.625.22

Perera, M., Bayliss, K., & Jones, M. (2005). New Australian record for infection of trees in *Paulownia* plantations by root-knot nematodes. *Australasian Plant Pathology, 34*(3), 419-420. doi:10.1071/ap05041

Pierik, R. (1997). Factors controlling adventitious root formation on stem explants of rose (*Rosa hybrida* "Motrea") *in vitro*. *Biology of Root Formation and Development* (pp. 297-307). New York: Plenum Press.

Poethig, R. S. (1990). Phase change and the regulation of shoot morphogenesis in plants. *Science, 250*(4983), 923-928.

Primer-E. (2009). Primer 6 & Permanova + (Version 6.1.12/1.0.2): Primer-E LTD.

Riemenschneider, D., & Bauer, E. (1997). Quantitative genetic analysis of adventitious root forming ability in *Populus trichocarpa Biology of Root Formation and Development* (pp. 193-201). Rhinelander, Wisconsin: Plenum Press.

Robinson, L. W., & Wareing, P. F. (1969). Experiments on the juvenile-adult phase change in some woody species. *New Phytologist, 68*(1), 67-78.

Rosier, C. L., Frampton, J., Goldfarb, B., Blazich, F. A., & Wise, F. C. (2006). Improving the rooting capacity of stem cuttings of virginia pine by severe stumping of parent trees. *Southern Journal of Applied Forestry, 30*(4), 172-181.

Rumyantseva, N. I., Sal'nikov, V. V., & Lebedeva, V. V. (2005). Structural changes of cell surface in callus of *Fagopyrum esculentum* Moench. during induction of morphogenesis. *Russian Journal of Plant Physiology, 52*(3), 381-387. doi:10.1007/s11183-005-0057-y

Schinor, E. H., Cristofani-Yaly, M., Bastianel, M., & Machado, M. A. (2013). Sunki Mandarin vs Poncirus trifoliata Hybrids as Rootstocks for Pera Sweet Orange. *Journal of Agricultural Science, 5*(6), 190-200.

Sha Valli Khan, P. S., Kozai, T., Nguyen, Q. T., Kubota, C., & Dhawan, V. (2003). Growth and water relations of *Paulownia fortunei* under photomixotrophic and photoautotrophic conditions. *Biologia Plantarum, 46*(2), 161-166.

Shafieizargar, A., Awang, Y., Juraimi, A. S., & Othman, R. (2012). Yield and fruit quality of queen orange *Citrus sinensis* (L) Osb. grafted on different rootstocks in Iran. *Australian Journal of Crop Science, 6*(5), 777-783.

Singh, S., Bhandari, A., & Ansari, S. (2006). Stockplant management for optimized rhizogenesis in *Tectona grandis* stem cuttings. *New Forests, 31*(1), 91-96.

Smit, A., L., Bengough, G., Engels, M., van Noordwijk, M., Pellerin, S., & van de Geijn, S. (2000). *Root Methods: A Handbook*. New York: Springer.

Smith, R. S., Guyomarc'h, S., Mandel, T., Reinhardt, D., Kuhlemeier, C., & Prusinkiewicz, P. (2006). A plausible model of phyllotaxis. *Proceedings of the National Academy of Sciences of the United States of America, 103*(5), 1301-1306. doi:10.1073/pnas.0510457103

Stobbe, H., Schmitt, U., Eckstein, D., & Dujesiefken, D. (2002). Developmental stages and fine structure of surface callus formed after debarking of living lime trees (*Tilia sp.*). *Annals of Botany, 89*(6), 773-782. doi:10.1093/aob/mcf137

Taha, L. S., Ibrahim, M. M. S., & Farahat, M. M. (2008). A micropropagation protocol of *Paulownia kowakamii* through *in vitro* culture technique. *Australian Journal of Basic and Applied Sciences, 2*(3), 594-600.

Tang, Z.-Q., Chen, D.-L., Song, Z.-J., He, Y.-C., & Cai, D.-T. (2010). *In vitro* induction and identification of tetraploid plants of *Paulownia tomentosa*. *Plant Cell, Tissue and Organ Culture, 102*(2), 213-220. doi:10.1007/s11240-010-9724-6

Thomson, G. E., & Deering, T. D. (2011). Effect of cytokinin type and concentration on *in vitro* shoot proliferation of hazelnut (*Corylus avellana L.*). *New Zealand Journal of Crop and Horticultural Science, 39*(3), 209-213. doi:10.1080/01140671.2011.559253

Titon, M., Xavier, A., & Otoni, W. C. (2006). Clonal propagation of *Eucalyptus grandis* using the mini-cutting and micro-cutting techniques. *Scientia Forestalis, 71*, 109-117.

Vettori, L., Russo, A., Felici, C., Fiaschi, G., Morini, S., & Toffanin, A. (2010). Improving micropropagation: effect of *Azospirillum brasilense* Sp245 on acclimatization of rootstocks of fruit tree. *Journal of Plant Interactions, 5*(4), 249-259. doi:10.1080/17429145.2010.511280

von Aderkas, P., & Bonga, J. M. (2000). Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree Physiology, 20*(14), 921-928. doi:10.1093/treephys/20.14.921

von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., & Filonova, L. (2002). Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture, 69*(3), 233-249. doi:10.1023/a:1015673200621

Wan, X., Landhausser, S. M., Lieffers, V. J., & Zwiazek, J. J. (2006). Signals controlling root suckering and adventitious shoot formation in aspen (*Populus tremuloides*). *Tree Physiology, 26*(5), 681-687. doi:10.1093/treephys/26.5.681

Wang, J.-W., Park, M. Y., Wang, L.-J., Koo, Y., Chen, X.-Y., Weigel, D., & Poethig, R. S. (2011). MiRNA Control of Vegetative Phase Change in Trees. *PLoS Genet, 7*(2), e1002012. doi:10.1371/journal.pgen.1002012

Wendling, I., Trueman, S., & Xavier, A. (2014a). Maturation and related aspects in clonal forestry—Part I: concepts, regulation and consequences of phase change. *New Forests, 45*(4), 449-471. doi:http://dx.doi.org/10.1007/s11056-014-9421-0

Wendling, I., Trueman, S., & Xavier, A. (2014b). Maturation and related aspects in clonal forestry—Part II: reinvigoration, rejuvenation and juvenility maintenance. *New Forests, 45*(4), 473-486. doi:10.1007/s11056-014-9415-y

Xu, D., Zhang, X.-Q., & Shi, Z. (2001). Mitigation potential for carbon sequestration through forestry activities in Southern and Eastern China. *Mitigation and Adaptation Strategies for Global Change, 6*(3), 213-232.

Yadav, N. K., Vaidya, B. N., Henderson, K., Lee, J. F., Stewart, W. M., Dhekney, S. A., & Joshee, N. (2013). A review of *Paulownia* biotechnology: a short rotation, fast growing multipurpose bioenergy tree. *American Journal of Plant Sciences, 4*(11), 2070.

Yang, L., Conway, S. R., & Poethig, R. S. (2011). Vegetative phase change is mediumted by a leaf-derived signal that represses the transcription of miR156. *Development, 138*(2), 245-249. doi:10.1242/dev.058578

Zaczek, J. J., Steiner, K. C., Heuser, C. W., Jr., & Tzilkowski, W. M. (2006). Effects of serial grafting, ontogeny, and genotype on rooting of *Quercus rubra* cuttings. *Canadian Journal of Forest Research, 36*(1), 123-129.

Zhu, Z.-H., Chao, C.-J., Lu, X.-Y., & Xiong, Y. G. (1986). *Paulownia in China: cultivation and utilization*: International Development Research Centre.

APPENDIX

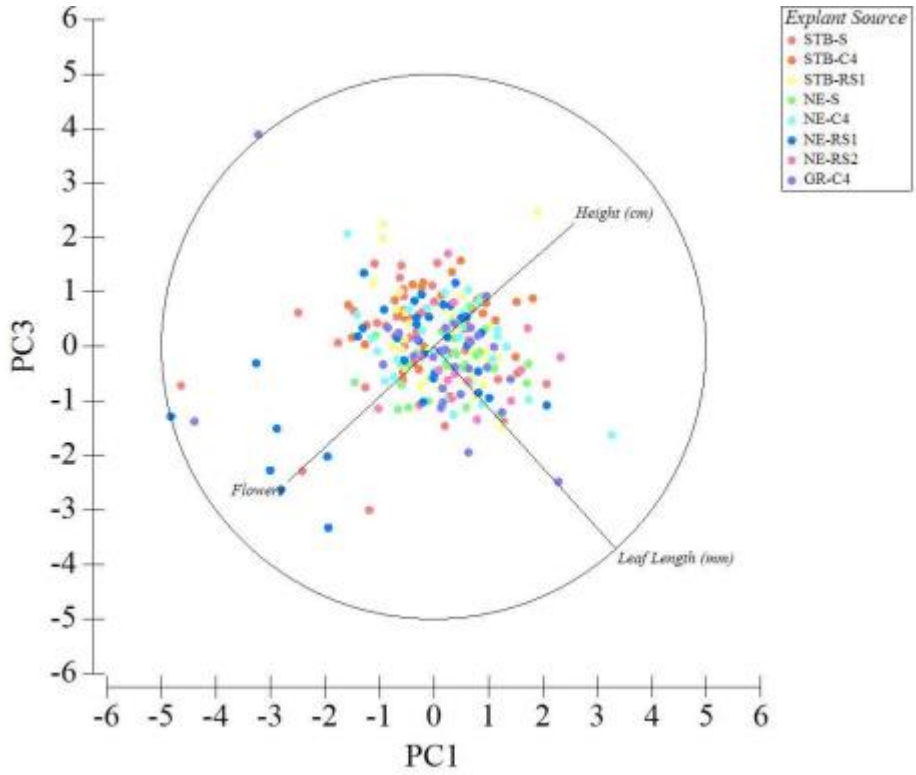

Figure 4.12 Plot of axes 1 vs 3 of principal components analysis of height, leaf and stem biomass at 24 weeks.

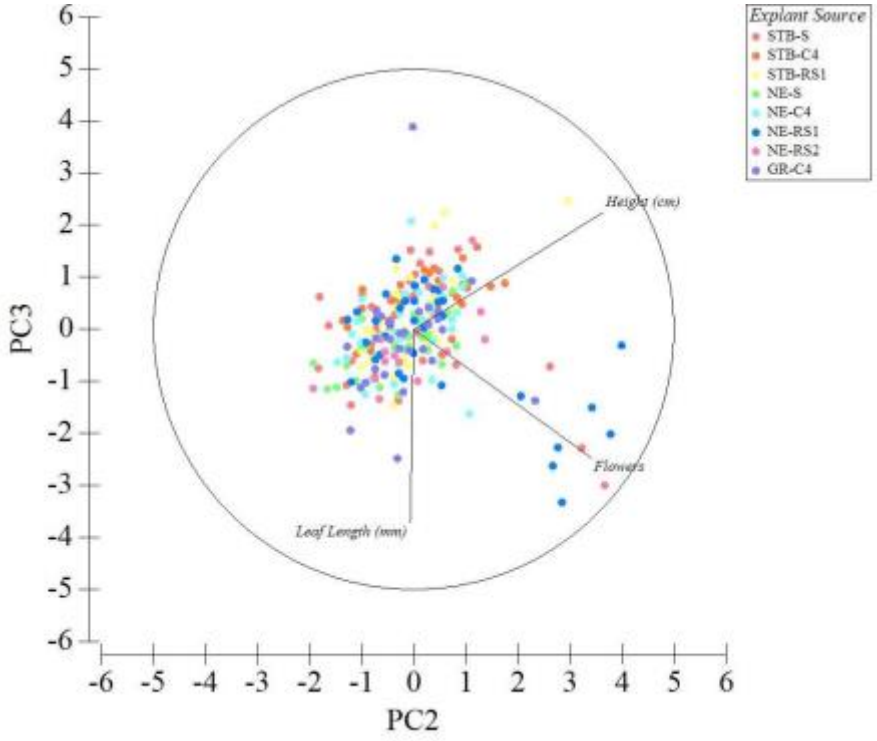

Figure 4.13 Plot of axes 2 vs 3 of principal components analysis of height, leaf and stem biomass at 24 weeks.

Table 4.4: R-statistic values for pairwise comparisons of explant sources at week 8 using analysis of similarity (ANOSIM) to test for differences in leaf length, height and presence of flowers. Values range from 0 to 1, where 0 indicates no separation of groups and 1 corresponds to complete discrimination between groups. Pairwise comparisons are significant if P<0.1%.

Explant source	R stat	Significance
STB-S, STB-C4	0.022	9
STB-S, STB-RS1	0.054	1
STB-S, NE-S	0.048	1
STB-S, NE-C4	0.041	3.7
STB-S, NE-RS1	0.002	36.4
STB-S, NE-RS2	0.067	17.6
STB-S, GR-C4	0.072	1.2
STB-C4, STB-RS1	0.035	5.3
STB-C4, NE-S	0.102	0.2
STB-C4, NE-C4	0.02	14.8
STB-C4, NE-RS1	0.029	5.6
STB-C4, NE-RS2	0.161	0.9
STB-C4, GR-C4	0.097	0.4
STB-RS1, NE-S	0.012	22.3
STB-RS1, NE-C4	0.003	37.5
STB-RS1, NE-RS1	0.014	14.9
STB-RS1, NE-RS2	0.098	9.3
STB-RS1, GR-C4	0.012	27.3
NE-S, NE-C4	-0.004	45.8
NE-S, NE-RS1	0.035	7.1
NE-S, NE-RS2	0.053	18.4
NE-S, GR-C4	-0.014	73.9
NE-C4, NE-RS1	0.011	22.8
NE-C4, NE-RS2	0.02	33.7
NE-C4, GR-C4	-0.003	45.6
NE-RS1, NE-RS2	0.011	41.4
NE-RS1, GR-C4	0.007	29.5
NE-RS2, GR-C4	-0.002	43.5

Table 4.5: R-statistic values for pairwise comparisons of explant sources at week 12 using analysis of similarity (ANOSIM) to test for differences in height, stem biomass and leaf length. Values range from 0 to 1, where 0 indicates no separation of groups and 1 corresponds to complete discrimination between groups. Pairwise comparisons are significant if P<0.1%.

Explant source	R stat	Significance
STB-S, STB-C4	0.053	10.5
STB-S, STB-RS1	-0.029	68
STB-S, NE-S	0.05	10.1
STB-S, NE-C4	-0.021	58.9
STB-S, NE-R1	-0.01	44.2
STB-S, NE-RS2	-0.014	48.2
STB-S, GR-C4	-0.007	42.7
STB-C4, STB-RS1	0.007	32.6
STB-C4, NE-S	-0.024	61.7
STB-C4, NE-C4	0.009	30.2
STB-C4, NE-R1	0.141	2.3
STB-C4, NE-RS2	0.047	25.9
STB-C4, GR-C4	0.047	12.8
STB-RS1, NE-S	-0.015	54.3
STB-RS1, NE-C4	-0.043	85.6
STB-RS1, NE-R1	0.019	25
STB-RS1, NE-RS2	-0.078	78.6
STB-RS1, GR-C4	-0.023	59.9
NE-S, NE-C4	-0.022	61.1
NE-S, NE-R1	0.128	2.9
NE-S, NE-RS2	-0.086	85
NE-S, GR-C4	0.023	21.7
NE-C4, NE-R1	0.036	17.9
NE-C4, NE-RS2	-0.112	90.2
NE-C4, GR-C4	-0.039	84.8
NE-R1, NE-RS2	0.129	10.1
NE-R1, GR-C4	0.039	16.2
NE-RS2, GR-C4	-0.063	68

Table 4.6: R-statistic values for pairwise comparisons of explant sources at week 24 using analysis of similarity (ANOSIM) to test for differences in height, stem biomass and leaf biomass. Values range from 0 to 1, where 0 indicates no separation of groups and 1 corresponds to complete discrimination between groups. Pairwise comparisons are significant if P<0.1%

Explant source	R stat	Significance
STB-S, STB-C4	-0.041	87.1
STB-S, STB-RS1	-0.028	71.3
STB-S, NE-S	-0.031	68.3
STB-S, NE-C4	0.065	8.9
STB-S, NE-RS1	-0.006	44
STB-S, NE-RS2	0.072	20.3
STB-S, GR-C4	-0.038	84
STB-C4, STB-RS1	-0.03	69.6
STB-C4, NE-S	-0.025	58.7
STB-C4, NE-C4	0.028	19.8
STB-C4, NE-RS1	-0.02	57.7
STB-C4, NE-RS2	0.019	34.1
STB-C4, GR-C4	0.002	37.1
STB-RS1, NE-S	-0.05	87.8
STB-RS1, NE-C4	-0.016	53.4
STB-RS1, NE-RS1	-0.052	91.5
STB-RS1, NE-RS2	0.053	20.6
STB-RS1, GR-C4	-0.024	66.2
NE-S, NE-C4	-0.017	51.5
NE-S, NE-RS1	-0.047	82.4
NE-S, NE-RS2	0.138	6.5
NE-S, GR-C4	-0.033	72.5
NE-C4, NE-RS1	-0.051	89.5
NE-C4, NE-RS2	0.009	37.3
NE-C4, GR-C4	0.04	15.5
NE-RS1, NE-RS2	0.06	20.4
NE-RS1, GR-C4	-0.015	51.9
NE-RS2, GR-C4	0.071	21.5