# The Examination of Developmental Potential in Forest Species through *in vitro* Grafting of Oak (*Quercus robur and Quercus petraea* )

Thesis presented for the degree of

Master of Science
by
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So long and thanks for all the fish!!!!

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: Eamonn McGowan ID Number: 93701306

Date: 25/9/1997

## ABBREVIATIONS

| | |
|---|---|
| % | percentage |
| µ | micro ($10^{-6}$) |
| µl | microlitre ($10^{-6}$ litres) |
| ABA | Abscisic Acid |
| AP | Adolescent Petraea |
| BA | Benzyladenine |
| BAP | 6 - Benzyaminopurine |
| CE | Capillary Electrophoresis |
| CK | Cytokinin |
| cm | centimetres ($10^{-2}$ metre) |
| $CO_2$ | carbon dioxide |
| DFR | Dihydroflavonol reductase |
| g | gram weight or units of gravitational force |
| GA | Gibberellic acid |
| $GA_3$ | Gibberellic acid 3 |
| $GA_{4+7}$ | Gibberellic acid 4 + 7 |
| HPLC | High Performance Liquid Chromatography |
| i.d. | Internal diameter |
| IAA | Indole Acetic Acid |
| JP | Juvenile Petraea |
| JR | Juvenile Robur |
| kPa | Kilo Pascals |
| l | Litre |
| M | molar |
| mg | milligram |
| mins | minutes |
| mm | millimetre |
| mM | millimolar |
| M & S | Murashige and Skoog |
| mol/m/s | mol/metre/second |
| MP | Mature Petraea |
| MR | Mature Robur |
| $^{o}C$ | degrees centrigade |
| pH | $\log_{10}$ of the Hydrogen ion concentration |
| PP | Phoenix Park |
| RM | Rooting medium |
| rpm | revolutions per minute |
| s | seconds |
| SDS | Sodium Dodecyl Sulphate |
| v/v | volume per volume |
| W | Watt |
| WPM | Woody Plant Medium (Lloyd and McCown, 1980) |
| Z | Zeatin |
| ZR | Zeatin riboside |

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## ABSTRACT

**The Examination of Developmental Potential in Forest Species through *in vitro* Grafting of Oak (*Quercus robur and Quercus petraea* )**

**by**

**Eamonn Mc Gowran B.Sc.**

As plants get older they go through a gradual developmental ageing process, from a juvenile phase characterised by rapid growth, good rooting potential, and orthotropic growth, to a mature phase characterised by slow growth, poor rooting potential and plagiotropic growth. This is of considerable importance from the viewpoint of propagation of 'elite' trees , since it is usually impossible to identify high quality plants at the seedling stage and difficulties in the vegetative propagation of adult woody plants arise due the physiological changes associated with maturation. The process of such maturation is very complex and poorly understood. The development of tissue culture techniques, has allowed for the mechanisms underlying maturation and rejuvenation of woody species to be better understood. The technique of cascade grafting of mature tissue to juvenile tissue has yielded 'rejuvenated' in several woody plant species. However the techniques of 'rejuvenation' rely on the availability of good quantitative markers for juvenile and mature plants. This study was concerned with two aspects of rejuvenation of mature oak material. The establishment of good quantitative and qualitative markers for *in vitro* growing juvenile and mature *Quercus robur* and *Quercus petraea* plants to enable the progress of rejuvenation to be assesed and the development of a system for *in vitro* cascade grafting of *Quercus* material.

Shoot cultures of *Quercus* species of juvenile, adolescent and mature origin were examined for a range of morphological and physiological markers of juvenility and maturity *in vitro*. Morphological Criteria examined were angle of the shoot to the horizontal, stem length, stem diameter (tip, mid, base), leaf number, scale leaf number and shoot number. Image analysis was also carried out to determine leaf area, size, and breadth and length of leaves. Mature *Q. robur* clones showed a larger mid-stem diameter than juvenile clones, while mature *Q. petraea* clones were characterised by larger stem diameters at the apex, greater number of leaves on the shoot, shorter shoots and plagiotropic growth. Discriminant analysis on data for *Q. robur* and Q. *petraea* allows us to propose the following formulae for discrimination of juvenile and mature shoots where a negative value for Discriminant Score (D) indicates juvenility.

*Q. petraea*

Discriminant Score D = - 1.308 - 0.0351*Angle($^{o}$) + 2.41*Tip diameter(mm) + 1.435*Mid - Stem diameter(mm)

*Q. robur*

Discriminant Score D = - 3.546 + 2.418*Tip diameter(mm) + 2.202*Mid-Stem Diameter(mm)

*Q. robur* clones derived from stump sprouts and desingnated as juvenile scored negatively indicative suggesting a juvenile status for these clones. Clones sourced from a hedged grafted *Q. robur* tree of mature origin scored positively indicating a mature status. Clones initiated from a 20-25 year old *Q. petraea* tree displayed morphology *in vitro* consistent with mature status and scored positive on the discriminant score. Image analysis of leaf morphology suggests that for Q. *robur* clones, surface area, perimeter, lamina length and width of leaves are greater in mature clones whilst the opposite is the case for *Q. petraea*.

Growth rate was highly variable both within a clone and with time of culture and did not provide a suitable marker for juvenility/maturity for *Quercus* species. High intra clonal variability in the ability to form roots and high levels of shoot tip necrosis makes rooting an unsuitable marker for juvenility/maturity for *Quercus* species.

Biochemical studies was problematic and yielded no definitve markers of juveniliy/maturity in *Quercus*.

*Quercus* material of juvenile origin was successfully grafted under *in vitro* conditions using a split agar petri dish. Apical media consisting of and basal media containing provided best support medium for development of shoots from scion. Heterografting of mature *Quercus* to juvenile *Quercus* was possible but cascade grafting proofed diificult due to loss of material. The progress of rejuvenation could not be followed with developed discriminant formula due to the lack of material. This study has further advanced the limited understaniing of maturation and rejuvenation. The use morphological markers that indicate differences between juvenility and maturity, has enabled the development of a formula that gives a discriminant score which provides a means for identifying the acquisition of juvenile traits in Oak clones growing *in vitro* and the possiblity of defining the time frames(s) of the main maturation process within a period of approximately 20 years.

# Chapter 1

## Introduction

## 1.0 Introduction

Of all the natural resources on this planet, forestry has been one of the most utilised and exploited by man. Rich in diversity and extent, forests have for centuries provided a valuable source of wood for construction materials, fuel and paper, fruits, oils and medicines (Hammatt, 1992). More recently it has become apparent that forests, particularly those of the vast rainforest regions, play a vital role in maintaining the global hydrological cycle and in controlling $CO_2$, its importance in the greenhouse effect by now well documented (Myers, 1990; Proops *et al.*, 1993; Read, 1994). It has been calculated that 50 % of the oxygen added to the atmosphere each year comes from the tropical rain forests of the Amazon. Around a third of the worlds' land mass (29-34%) is covered by forest while a further small area is covered by commercial fruit orchards. Broad-leaved tropical rainforests comprise a third of this forest resource, and coniferous boreal forests account for a further 20%. Another 20% of the forests of the world consist of open forest with the final 20 % being scrub, declining forest and planted agricultural land.(Forestry Industry Committee of Great Britain, 1987). Due to demands from wood pulping and construction up to 3% of the tropical rainforests are removed annually whilst an even greater area of tree cover is removed in the third world for fuel and agriculture (Hammatt, 1992).

In Ireland, about 575,000 hectares or 8% of the land is covered in forest. This figure has increased from a 1% coverage at the turn of the 20th century. Nevertheless, Ireland remains the least forested area within the European Union which has an average of 24% land under forest. Whilst a mixture of both broad-leaved and coniferous tree species are planted, the latter having a higher timber production predominate. The four main conifer species currently planted are Sitka spruce, Lodgepole pine , Douglas fir, and Norway spruce with 84% of the productive forest area under conifers (Department of Agriculture, Food and Forestry, 1996). Broad-leaved tree species, particularly native ones such as Ash, Birch, Cherry,Oak, Rowan and Sycamore are being encouraged on suitable sites with a view to quality hardwood timber production as well as improvement of wildlife and amenity areas (ENFO, 1992). Such is the success of grants that levels

of broadleaf planting are rising steadily and constitutes around 20% of the private sector's planting.

Economically the industry associated with forestry in Ireland is highly important. The contribution to the economy of the sawnwood and panel board sub sectors alone have been estimated at IR£ 87m (1993). Total employment in the forestry and wood products sector is about 16,000 people. Of these more than 7000 are employed directly in the management of public forests. The bulk of the remainder are employed in downstream industry. It is estimated that every additional 1,000 hectares of planting generates 100 jobs on average and it is Irish government policy to reach an annual planting rate of 25,000 hectares and attain a 17% coverage by the year 2030 (Department of Agriculture, Food and Forestry, 1996). This is actively being encouraged by the provision of planting grants ( Forestry Operational Programme, 1991). Despite increased production of timber Ireland is a net importer of wood and wood products. The estimated minimum domestic production required is approximately 10 million cubic metres per annum. Ireland's current annual timber production level is 2.2 million cubic metres and will reach only 6.5 million cubic metres per annum by the year 2035. The EC as a whole is only 50% self sufficient in wood and consumption is increasing. By the year 2015 Forbairt predicts that timber exports could rise 15 fold to around £1 billion (Sheridan, 1995). In the year 1995 to 1996 it is estimated that over 20,000 hectares of forest was planted. This required over 60 million plants. To reach the predicted export levels for the year 2015 would require a volume of 4.5 million cubic metres to be harvested. It is also calculated that by the year 2020, a total of 11,000 full time jobs could be created in the forestry sector.

On a global scale, as the world population increases, immense pressures will be placed on the worlds supplies of trees. Forests are being harvested at a faster rate than they are regenerated either naturally or artificially hence a shortage of wood and wood products has been forecasted for the end of this century (Keays, 1974). Disease, pests and fires are placing a further strain on our ever decreasing supply of forestry. Thus there is an immediate demand on the need for research to increase the productivity of trees, produce larger numbers of improved and fast growing trees (Thorpe and Biondi, 1984). and improve existing tree breeding programs.

Fundamental to this requirement will be the development of techniques enabling the cloning of tress with elite properties of interest. Such stock will likely be sourced from the wild or existing forest plantations. The techniques of tissue culture have become exceptionally important in achieving this aim (Gupta *et al.*, 1993). Although tissue culture culture technology may not supplant traditional tree breeding technologies (Karnosky, 1981), tree improvement researchers are now able to use tissue culture techniques to evaluate genotypes in experiments of growth rates, cold hardiness, disease resistance and tolerance to drought (Manders *et al.*, 1992). Of a greater interest to the tree breeder is the ability to clone mature 'Elite' trees with proven desired qualities through tissue culture techniques. Such trees are superior individuals displaying either industrially significant qualities such as enhanced growth rates and superior wood quality, or improved ornamental characteristics, such as branching and good form.

Elite trees can be propagated either sexually by seed or asexually by vegetative clonal propagation strategies. Juvenile trees are in general readily cloned by conventional techniques but the ease of propagation of many trees tends to diminish as they approach a size sufficient to allow reliable evaluation of their crop potential (Ballester *et al.*, 1990). Elite trees grown from seed must prove themselves as elite, which may take 10 years or longer. By this time they have matured and in most cases are recalcitrant to vegetative propagation. Hence methods of rejuvenation or reversal of maturation are required.

The transition from the adult often occurs gradually and some parts of an otherwise adult tree may retain juvenile or transient characteristics for many years. Such parts include epicormic shoots, root suckers and stump sprouts and offer material that is generally easier to establish *in vitro* than tissues isolated from the upper branches of the same tree ( Jones, 1991). However not all adult trees feature material with juvenile properties and such adult tissue must be rejuvenated in some way to allow *in vitro* establishment. In recent years several rejuvenation methods have been reported including severe pruning, hormonal treatment, serial grafting and meristem culture.

Both rejuvenation of mature material and the process of maturation of juvenile material are complex phenomena, probably similar to those regulating cellular differentiation and the events that underline them are poorly understood (Franclet *et al.*, 1987). With the development of tissue culture techniques, the mechanisms of maturation and rejuvenation of woody species will be better understood and utilised to allow the clonal propagation of 'truly elite' trees.

## 1.1 Description and Developmental Basis of Phase Change

In the development of all woody plants from seed there is a so called juvenile phase lasting up to thirty to forty years in certain forest trees, during which flowering does not occur and cannot be induced by normal flowering initiating treatments. In time, the ability to flower is achieved and maintained under natural conditions; at this stage , the tree is considered to have attained the adult or sexually mature condition. The length of the juvenile period can be influenced by environmental and genetic factors. The transition from the juvenile to the mature phase has been referred to as phase change by Brink (1962), ontogenetic ageing by Fortanier and Jonkers (1976) and meristem ageing by Oleson (1978). Associated with the transition from the juvenile to the mature state are progressive changes in a range morphological and developmental attributes such as leaf characteristics, growth patterns, and branching. Changes in such characteristics during development vary from species to species. Phase change is of considerable theoretical importance relative to morphogenetic control, differentiation and determination in plant development. It has a practical significance given that breeding efficiency of woody perennials coupled with the selection of improved cultivars is inversely related to the juvenile period. The ease of cuttage propagation for all woody perennials is strongly affected by ontogenetic age and the quantity and quality of productivity of a forest tree species is related to its degree of maturity (Hackett and Murray, 1993).

### 1.1.2 Ageing in tree species

In woody plants, ageing is a complex phenomenon which has far reaching consequences for vegetative and generative reproduction. Difficulties in the vegetative propagation of adult woody perennials and of adult woody plants arise due to the physiological changes associated with maturation (Meier-Dinkel and Kleinschmit, 1990). Fortanier and Jonkers (1976) classified the ageing or phase change process into three aspects: a chronological, an ontogenetical and a physiological one. Chronological ageing is defined as "getting older" and refers to the period of time since the germination of a seedling. Physiological or somatic

ageing refers to a loss of vitality during the life of the tree ending with the death and is mainly caused by an increase in size and or structural complexity of the tree (Borchert, 1976). Ontogenetical ageing which is localised in the meristem, at the level of the individual cell or of the entire meristem (Hackett, 1985) is defined as the genetically programmed process of phase change resulting in different phases of development: the embryonic phase, the juvenile or seedling phase, the transition phase and the mature phase.

#### 1.1.2.1 Embryonic phase

This phase begins with the formation of a zygote, and lasts until the formation of a mature, dormant embryo within the seed. Embryonic tissue has a high regeneration capacity and is therefore often used as an explant source for *in vitro* cultures. Usually zygotic embryos are used for the induction of adventitious bud formation, especially in conifers and has been used for example in *Picea abies*, *Pinus radiata* , *Pinus pinaster*, *Pseudotsuga senziesii*. Embryonic tissue also acts as source of somatic embryos (Meier-Dinkel and Kleinschmit, 1990).

#### 1.1.2.2 Juvenile phase

The juvenile phase begins with the generation of the seedling and is followed by the transition phase. According to Doorenbos (1965) the juvenile phase of woody plants is characterised apart from it's morphological properties by a greater readiness to form adventitious roots and an inability to flower. Borchert (1976) felt that the existence of one true uniform juvenile state for all woody species "must be seriously doubted". He argued that if such a state existed then all juvenile characters should change at the same rate with an increase in the physiological age of a tree. Observations have shown that traits change at different rates. No one model can be applied to all trees but certain correlation's have been deduced from frequent observations: Shoots with juvenile foliage often possess high rooting capacity; the transition from juvenile to adult foliage coincides with a decline in shoot vigor; flowers are formed on plants with adult foliage and low vigor; plants

with juvenile foliage flower and root with great difficulty (Borchert, 1976). The average duration of juvenility varies from species to species (Table 1.1.2.2.1). Birch have a comparatively short juvenile phase (5-6 years) whereas oak and beech stay in this phase for some decades (Clark, 1983). However these dates only refer to periods until flowering first occurs. In single individuals the length of the phase is influenced by environmental, genotypical and nutritional factors (Doorenbos, 1965). The length of the juvenile phase is related to the ultimate size of the plant and changes in morphology and physiological attributes are the direct consequence of an increasing complexity and size of the tree (Borchert, 1976). In general, shrubs have a shorter juvenile period than trees. Sexual reproduction structures are extremely rare during this phase although some pine seedlings have displayed isolated instances of stroboli (Greenwood, 1987).

### 1.1.2.3 The Transition phase

This phase is typically characterised by a gradual change in morphological, anatomical, physiological, biochemical and growth characteristics from the juvenile to the mature state. These different characters of a plant can chronologically change quite differently and the changes in such characteristics vary from species to species. No distinct change in any characteristic is apparent at the time when ability to flower is attained (Hackett, 1985).

Attainment and maintenance of the ability or potential to flower is the only consistent criterion available to assess the termination of juvenility and the beginning of the mature phase. However Franclet (1983) felt this may be coincidental as flowering *Eucalyptus* seedlings which can be readily propagated may be found in the nursery. Although flowering occurs only after 25 - 40 years in some forest trees like *Quercus, Fagus, Abiea,* or *Picea,* this does not mean that the trees are completely juvenile before the first flower onset. Other characteristics especially those affecting the vegetative propagation potential do change earlier. The ability of cuttings to form adventitious roots decreases early in the development of seedlings. At 2-3 years oak cuttings give an 80% rooting rate, by 8 years this rate varied between 0 - 95% and decreased with increasing age.

| Species | Length of Juvenile period |
|---|---|
| *Rosa* (hybrid tea) | 20 - 30 days |
| *Vitis* spp. | 1 year |
| *Prunus* spp | 2-8 years |
| *Malus* spp | 4-8 years |
| *Citrus* spp | 5-8 years |
| *Pinus sylvestris* | 5-10 years |
| *Hedera helix* | 5-10 years |
| *Betula pubescens* | 5-10 years |
| *Pyrus* | 6-10 years |
| *Sequoia sempervirens* | 5-15 years |
| *Pinus monticola* | 7-20 years |
| *Larix decidua* | 10-15 years |
| *Fraxinus excelsior* | 15-20 years |
| *Acer. pseudoplatatanus* | 15-20 years |
| *Thuja plicata* | 15-25 years |
| *Pseudotsuga menziesii* | 20 years |
| *Pinus aristata* | 20 years |
| *Sequoiadendron giganteum* | 20 years |
| *Picea abies* | 20-25 years |
| *Tsuga hetrerophylla* | 20-30 years |
| *Picea sitchensis* | 20-35 years |
| *Quercus robur* | 25-30 years |
| *Abies amablis* | 30 years |
| *Fagus sylvatica* | 30-40 years |

Adapted from: Clark (1983)

**Table 1.1.2.2.1 Length of Juvenile Period in Some Woody Plant Species**

In some studies this rooting potential has been thrown into doubt. In studies of Douglas Fir Roberts (1979) wrote "it is well established that the rooting potential of most conifer cuttings decreases with increasing age of the tree from which the cuttings were taking. It has been assumed that this was due to the onset of flowering, vegetative maturity, or loss of juvenility. A ten year study of Douglas fir shows that this may be the wrong conclusion . Cuttings from fifteen to eighteen year old trees which are now in cone production are rooting as well as at any time in their history". Vegetative and growth characteristics also change gradually during this phase (Greenwood, 1987). In Loblolly pine the capacity for height and diameter growth, as well as the number of branches produced per unit of stem length, decline over at least a 12 year period (Greenwood, 1984). In contrast, needle length and diameter as well as reproductive competence increase during this period. Many morphological traits change gradually with time including branching habit, foliar morphology, pigmentation and phyllotaxy in addition to reproductive competence and growth rate (Greenwood, 1984; Rogler and Hackett, 1975).

#### 1.1.2.4 Mature state

Maturation can be described as the ongoing process of phase change which results in relatively permanent developmental changes. Maturity is taken to be reached when the tree obtains and maintains its ability to flower in natural conditions i.e. its sexually mature state or reproductive state. The mature growth phase is relatively stable. Reversion to the juvenile condition does not generally occur as a result of asexual propagation such as cuttage or graftage involving a single bud and a small piece of stem, although flowering may be delayed to varying degrees by these techniques, depending on the species. Reversion to a juvenile form occurs naturally following sexual reproduction. In some species such as apple, apomictic seed formation gives rise to juvenile seedlings which have the same genotype as the mother tree. Generally on attainment of the mature state the capacity for height and diameter growth reaches a minimum and no further changes in foliar or branching capacity are expected (Greenwood, 1987). Table 1.1.2.4.1

| Characteristics | Juvenile | Mature |
|---|---|---|
| Nucleus | Small, surrounded by a thin layer | Reticulated and elongated surrounded by endoplasmic reticulum membrane |
| *Chromatin* | *Decondensed* | *Condensed methylated DNA*<br>*Polyploid euchromatin* |
| Ribosomes | Free | Associated to membranes |
| *Apical meristems* | *Small dome, few large cells* | *High dome, numerous small cells*<br>*Increased RNA* |
| Leaves | Typical form (simple or complex)<br>Large epidermic cells<br>Winter retention<br>Unequal opposed stomate distribution | Typical form (complex or simple)<br>Dense venation<br>Thick<br>Amphistomatism<br>Frost resistant |
| *Branches* | *Acute angle*<br>*Thick and long* | *Obtuse angle*<br>*Thin* |
| Thorns | Frequent | Rare or none |
| *Bark* | *Thick* | *Thin* |
| Cuttings | Easy rhizogenesis<br>Easy recovery of vigorous growth<br>Quick recovery of orthotropy when cuttings originally plagiotropic<br>Quick formation of tap roots | Slow rooting<br>Low and durable rate of growth<br>Low recovery of orthotropic habit<br>Slow recovery of tap roots |
| *Biochemical* | *None or weakness of ABA and other inhibitor content*<br>*High content in endogenous auxins*<br>*High total peroxydasic activity at the basal part of excised cuttings*<br>*High K/Ca ratio* | *High basal content in ABA and other inhibitors*<br>*Low content in endogenous auxins*<br>*Low total peroxydasic activity at the basal part* |

Adapted from Franclet (1993)

**Table 1.1.2.4.1 Characteristics associated with juvenile and mature trees**

gives an overview of characteristics associated with different phases of development.

### 1.1.3 Theories of mature and juvenile zones in trees

Franclet (1983) states that all parts of a tree do not mature equally, in fact there are probably seasonal cycles where meristems are juvenile or mature. The existence of juvenile zones in a tree has long been recognized. Passacker (1947) showed that the part of the tree near the root remains juvenile while the other parts are more mature. It can be demonstrated that the upper and peripheral parts of a plant first obtain mature characteristics, while the basal and interior parts retain juvenile characters (Figure 1.1.3.1). In seedling birch, where trees are induced to flower very early under conditions of maximal growth in conjunction with stem girdling, the first- formed branches near the base do not flower whereas those less than 100 cm up the stem flower quite profusely. Similarly adventitious rooting and bud initiation potential seem to be related to ontogeny, but in an inverse fashion. In Eucalyptus seedlings, it has been shown that the cotyledonary node has a very high rooting potential but by the fourth node in *Eucalyptus ficifolia* F.muell. and the fifteenth node in *E. grandis* rooting capacity is nearly zero. It has also been observed that cuttings taken from shoots in the basal region have a higher capacity to form adventitious roots than those taken from shoots in the upper part of the plant. The trunk and basal portion of main branches of a seedling tree of citrus or *Geditsia triacanthos* L. retains the ability to form thorns whereas the upper and peripheral region of the tree is nearly thornless. Leaf retention in *Fagus sylvatica* displays a similar trend. These findings suggest that juvenile characteristics such as rooting potential may be preserved at the base of the plants in ontogenetically young tissue while maturation occurs in the periphery of the plant in ontogenetically older but chronologically younger tissues (Hackett, 1985). Franclet (1983) adapted the theories of Krenke and proposed a more complicated view of juvenile and mature zones within trees. He argued that juvenile territories may be found in the crown of old trees (Figure 1.1.3.2) and the duration of the juvenile stage of apical meristems during each flush or cyclical growth, becomes shorter when

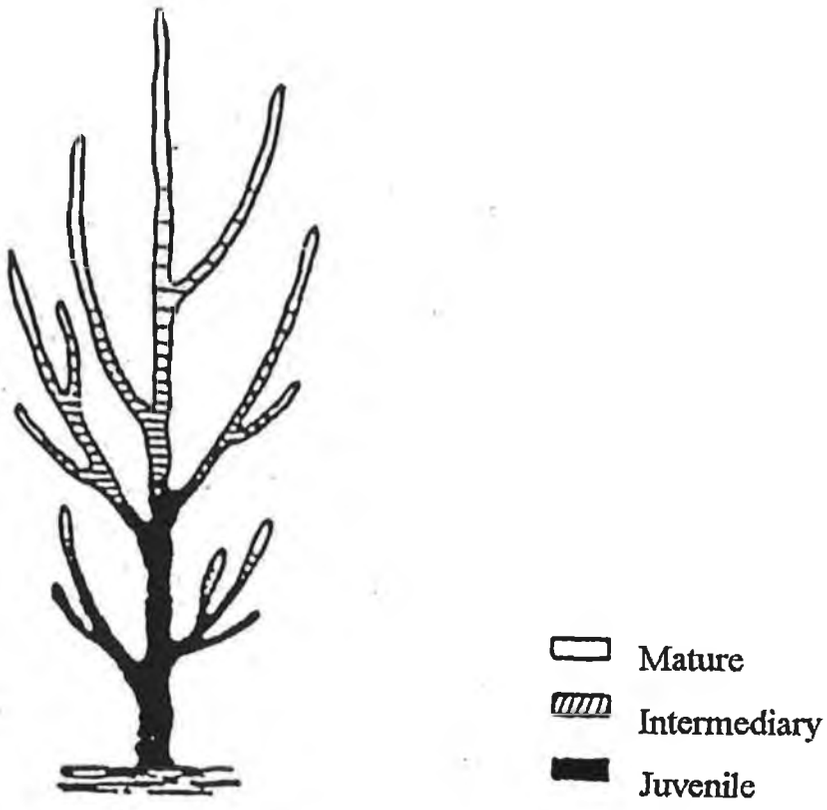



**Figure 1.1.3.1 Zonal variations in location of different maturation phases on a mature seedling of a woody plant (After Passacker, 1947)**

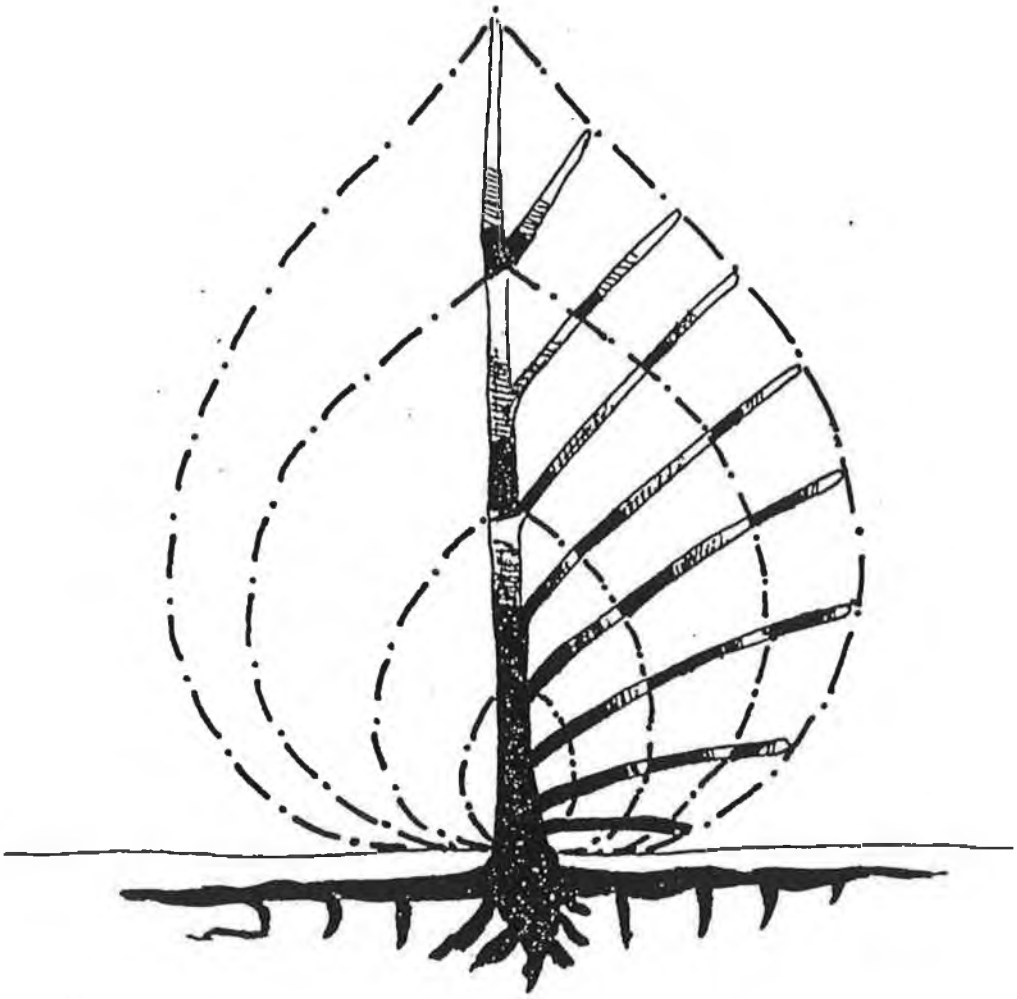


**Figure 1.1.3.2 Juvenile, intermediary and mature zones on a tree (Adapted from Franclet, 1983)**

taking place further from the root. In the mature stage and in some species, the juvenile zones may disappear almost completely.

### 1.1.4 Switches controlling attainment of maturity

The fundamental question to be faced in addressing phase change is whether maturation is controlled by single switches or a composite of processes. Studies of time course changes of six vegetative characteristics and flowering in *Larix laricina* revealed an interesting phenomenon. Greenwood *et al.* (1989) grafted scions from donor trees ranging in age between 1-74 years onto 2 year old seedling rootstocks and noted two distinct time course patterns for vegetative characters. Orthotropic and secondary branching of grafted scions decreased with scion donor age up to 5 years and showed little change thereafter. In contrast height, stem diameter, chlorophyll content and rooting ability of grafted scions all displayed a linear function of the $\log_{10}$ of donor tree age. Greenwood *et al.* (1989) concluded that although there was evidence of two distinct time lines, the question remaining was whether these vegetative maturation changes varied independently of one another. It was further noted that flowering scions from juvenile donor trees produced more stroboli per tree than did scions from mature trees. The conclusion drawn from this result was that the achievement of a minimum size is more important than the maturation state for flowering in *Larix*. This further suggests that the mechanism for the acquisition for flowering potential is not closely related to mechanisms for changes in the vegetative maturation characteristics studied. Hackett (1993) suggests that alternatives to the single switch mechanism should be considered. There may be several switches in parallel one for each character or separate switches for sets of characters. In contrast, it may be possible that there are switches in series such that a change in one character or more characteristics causes changes elsewhere in the plant. The process could be more complex as there might be two stage, multi stage or cascade switches. The latter possibilities have been backed by observations in rejuvenation of mature *Hedera helix* induced by Gibberellic acid 3 ($GA_3$) applications (Rogler and Hackett, 1975a). Such treated plants show varying responses to differing levels of $GA_3$. At low doses stable changes in aerial rootlet

and athocyanin formation can be induced and flowering ability repressed whereas changes in leaf shape and phyllotaxy do not occur. This effect increases such that a saturating dose results in the attainment of juvenile characteristics. The differential sensitivity of individual characteristics indicates that $GA_3$ may act through several mechanisms in this very complex process of phase change. To fully understand the events controlling the attainment of maturity in any species, will involve greater studies of the biochemical, physiological and genetic control of phase change. Genetic studies particularly of plants displaying characteristics due to mutations offer the greatest understanding of the events surrounding maturation.

## 1.2 Physiological, Biochemical and Genetic basis of phase change

Morphological, histiological and biochemical criteria have been utilised as a means of defining juvenility and maturity in many different plant species. All of these parameters could allow a quantitative and qualitative approach to defining the phenomenon. Factors such as the physiological state of the plant, the concentration or type of active substances of plant metabolism are affected by environmental factors and the levels of substances differ between juvenile and adult plants and differing woody species (Haffner *et al.*, 1991). Rooting capability and orthotropic, or upright, growth of apical meristems are well established in juvenile material, while plagiotropic, or horizontal, branch habit and reduced rooting capability are features of mature material. (Franclet, 1983).

The juvenile and mature phases of woody plants usually may be characterised by morphological or physiological features such as shape or physiology of leaves, by occurrence of certain shoot types or phyllotaxis or the inability and ability to flower under natural conditions (Meier-Dinkel and Kleinschmit, 1990; Poethig, 1990). Other criteria include rooting ability and orthotropic or plagiotropic growth. Examples of woody species with clear juvenile phases distinguishable by morphological and physiological criteria are *Hedera helix*, *Vitis vinifera*, *Eucalyptus* and *Acacia* (Doorenbos 1953; Mullins *et al.*, 1979; Poethig, 1990). In the common ivy *Hedera helix*, morphological differences between the adult and the juvenile phases are particularly marked and can be easily distinguished by differences in leaf shape, phyllotaxy, stem shape and growth habit. Cuttings from the juvenile form are easily rooted as opposed to the difficult to root mature form cuttings (Hess, 1959). Rogler and Hackett (1975) utilised these distinct differences in morphology to develop a qualitative system similar to that used in plant genetics and pathology (Bringhurst *et al.*, 1961). $GA_3$ induced morphological reversion of mature ivy to the juvenile form was quantitatively characterised by the developed scoring system based on morphological differences. Doorenbos (1954), in studies of graft combinations in ivy, concluded that the reversion from adult morphology to that of a juvenile form clearly demonstrated that a juvenile stock may rejuvenate an adult scion. In the conifer species *Sequia sempervirens* both

morphological and physiological differences between juvenile and mature clones are noticeable. Differences in leaf and stem morphology have been observed as well as orthotropic and plagiotropic growth differences (Arnauld *et al.*, 1993). These differences have been exploited to argue for reversal of mature status by rejuvenation techniques (Huang *et al.*, 1992; Arnuald *et al.*, 1993).

Cuttings from tissue culture colonies of non juvenile blueberry, *Vaccinium ashei*, grown *in vivo* rooted as though juvenile. Leaf and stem measurements indicated tissue culture cuttings were close in morphology to 4-6 month old seedlings having small leaves, thin stems and short internodes (Lyrene, 1981). Mature Birch (*Betula*) is characterised by long internodes, warty stems, stout shoots, acute or rounded leaf bases, acute leaf tips and low adventitious rooting potential (Brand and Lineberger, 1992). Adventitious root initiation and restored rooting competence has been used as a marker of rejuvenation in globe artichokes, *Hedera helix* and mature avocado, *Persa americana*, shoots (Moncousin, 1982; Pliego Alfaro *et al.*, 1987; Geneve *et al.*, 1991). The ease of root regeneration of oak cuttings generally declines with the age of the mother tree and is a possible criterion for juvenility. However the variation in rooting between clones of any given age may be very high. This may be an especially serious problem in trees of age five years or older where rooting of any one clone may vary between 0% and 100% (Meier-Dinkel and Kleinschmit, 1990). This makes rooting an unreliable marker for the juvenility status of the tree.

Peroxidase activity at the basal part of excised cuttings was observed to be high in juvenile material, while low in mature material and are considered as a marker for rooting potential (Moncousin, 1982; Quoirin *et al.*, 1974). Vershoore-Martouzet (1985) noted differences in Potassium (K) to Calcium (Ca) ratios in buds of *Sequoia sempervirens* depending on their position on the plant and the K/Ca ratio decreased with ageing. On the other hand, in Douglas fir, rejuvenation produced by *in vitro* subculturing is characterised by a decrease in the Potassium/Sodium ratio (Bekkaoui, 1986). Dechamps (1986) recommended using K/Ca ratios as a marker for juvenility in Douglas fir and Eucalyptus cultivated by *in vivo* methods. Findings should indicate an increase in these ratios as the meristem matures.

Many studies have indicated a relationship between phenolic content and juvenility, rejuvenation and maturation. The number of phenolic compounds increases with maturation in the chestnut. In *H.helix* the mature phase appears to be characterised by inactivation of one or more enzymes involved in the biosynthesis of active polyphenols and flavanoids (Hackett *et al.*, 1989). Jay Allemand (1987) found that severe pruning of walnut trees severely affected phenolic content in new shoots, finding it similar to that of juvenile individuals. Rejuvenation of walnut hybrids has also been characterized by three ratios of five different polyphenols.

Phytohormones are involved in maturation. Maturation phase changes are related to large phytohormone changes in buds and the apical part of the stem. Auxin has a negative effect on plagiotropy in conifers, an indicator of maturation, whilst twice as much auxin is required to root mature cuttings of *Ficus pumilla* than in its' juvenile counterpart. It is argued that auxin levels decrease in mature parts of trees due to either a reduction of auxin transport to roots as a tree increases in size or else a decrease in auxin supply to mature meristems. During maturation auxin levels decrease less rapidly than those of cytokinins. Douglas fir maturation is characterised by a decrease in the Zeatin/Indole Acetic Acid (Z/IAA) and Zeatin Riboside/Indole Acetic Acid (ZR/IAA) ratios. Contrary to this, Abscisic Acid/Indole Acetic Acid ratios increase as *Sequoia sempervirens* matures. Gibberellins have been implicated in the morphological reversion of *H.helix* from the adult leaf type to juvenile type. The natural gibberellin like substance $GA_3$ aids rejuvenation (Rogler and Hackett, 1975). It is hypothesised that $GA_3$ appears to involve elongation and indirectly affects morphology. However the role of Gibberellic acid (GA) in maturation is still debated. These substances promote flowering in conifers whilst in some cases their application does not "offset a genetic indisposition of trees to flower" (Haffner *et al.*, 1991). Furthermore GAs' are associated with increased vigor of apple trees *in vivo* ( Looney *et al.*, 1988), vigour being a trait associated with juvenility. The role of Cytokinins (CK) in maturation is still open to discussion. CKs affect reactivity and growth of buds in many species (Franclet, 1991) and have been shown to induce apex rejuvenation in mature Douglas fir (Bakkoui, 1986). Contrary to this, a relationship between increased CK levels and lack of rooting ability in Poplars (Okoro and Grace, 1978)

is evident. Benzyl-adenine has a role in both the promotion and reversal of maturation of conifers (Greenwood, 1987). Abscisic acid (ABA) levels are higher in mature tissues than in juvenile tissue (Hackett, 1985). Rogler and Hackett (1975b) have reported that the $GA_3$/ABA ratio has more importance than the absolute value of the two substances in controlling reversion to the juvenile phase from the adult phase in *H.helix* and stabilisation of the mature form by ABA probably occurs via regulation of the GA level in the plant. Although the action of the root produced plant growth regulators (GAs' and CKs) in maturation is unsure, Auxins, Gibberellins, Cytokinins and Abscisic Acid are related to the maturation phenomenon. The ratios between these four phytohormones seem to better determine the induction and stabilisation of the phase changes than their respective absolute values (Haffner *et al.*, 1991).

At a nucleic level, variations in composition of nucleic acids between juvenile and mature material are evident. Differences have been found in total, soluble and ribosomal Ribonucleic Acid (RNA) between the two phases (Zimmerman *et al.*, 1985). Waering and Frydman (1976) reported quantitative RNA variations during maturation of *H.helix*. In *Ficus pumilla*, total RNA levels are higher in juvenile individuals. Evident in this species is an increase in RNA levels and cambial activity at maximum rooting. It has been proposed that only few genes are active in the mature phase (Zimmerman *et al.*, 1985). Consequently the RNA transcribed represents a small proportion of total DNA suggesting that the molecular basis of phase change depends on an alteration of the transcription rate of certain DNA sequences (Zimmerman *et al.*, 1985). Such alteration could be associated with methylation of cystosine in DNA, since older trees of *Picea abies* showed a greater cystosine methylation than the youngest ones (Haffner *et al.*, 1991).

Lawson and Poethig (1995) argue that it is likely that phase change and the identity of a plant structure is regulated by homeo-box genes. These genes may interact to downregulate the different phase specific traits. Chronological ageing or time dependent phase change in plants is another factor in plant growth. Some preliminary work on *Arabidopsis* has identified a gene called *paused (psd)*. Leaf primordia from *psd 1* mutants were produced later during embryogenesis than in

normal plants. When these first true leaf primordia did form, they morphologically resembled leaves formed at the same chronological time in normal plants. That is the first true leaf primordia of *psd* mutants were morphologically older than those of normal plants, but were morphologically similar if compared on a chronological basis rather than on a spatial position on the plants (Lawson and Poethig, 1995). Consistent with the idea of genes regulated by a clock mechanism, precocious leaf development during embryogenesis in *Arabidopsis* was attributed to mutations in *extracotyledon 1*, *extracotyledon 2* and *amp 1* genes (Lawson and Poethig, 1995). Furthermore, phase specific genes have been identified. *Teopod 1*, *2* and *3* (*Tp 1*, *Tp 2* and *Tp 3*) mutants of *Arabidopsis* prolong the expression of early vegetative traits but only slightly delay the onset of late vegetative traits. It is possible that *Teopod* genes are regulated by Giberellic Acid levels and by the other genes in a downstream regulation fashion. *Glossy 15* (*gl15*) mutants in *Arabidopsis* cause precocious expression of adult traits and may regulate *Teopod* genes. It is possible that the *gl15* encodes for an epidermal growth factor that maintains juvenile traits. (Lawson and Poethig, 1995).

In *Hedera helix*, juvenile phase ivy produces anthocyanin while adult phase ivy does not. Murray and Hackett (1991) have demonstrated that the lack of anthocyanin accumulation in the collenchyma cells of stems and mesophyll cells of leaves of mature phase plants is due to a lack of expression of activity of dihydroflavonol reductase (DFR). This enzyme catalyses a late step in the production of anthocyanin. They have shown that the lack of the DFR is due to the lack of the transcription of the gene for DFR. Whether the phase specific expression of the DFR gene depend on *cis*-acting factors such as DNA cysteine methylation, or is regulated higher in a signal transduction pathway remains to be determined (Lawson and Poethig, 1995).

Waering and Frydmman (1976) observed differences in proteins during maturation in *H.helix* however such differences were not great enough to distinguish between juvenile and mature clones of walnut. More recently Hackett *et al.* (1989) showed that the juvenile phases of *H.helix* can be characterized by two polypeptides. Bon and Monteiuus reported that rejuvenation of *Sequioadendron giganteum* by micrografting was accompanied by a decrease in meristematic

proteins. Bon (1988) identified a membrane associated protein, labeled J16, with an approximate molecular weight of 16 KD specific to juvenile individuals and rejuvenated individuals of *S. giganteum*. Careful consideration must be paid to the fact that the control mechanisms for maturation are probably the result of the products or regulatory activities of many different genes. In trying to understand the events surrounding maturation and phase change there is a temptation to obtain a single biochemical marker of juvenility or maturity (Bon, 1988; Lawson and Poethig, 1995).

### 1.3 Procedures to recover juvenile material from mature trees

Rejuvenation is reverse maturation, the reversion from a mature state to a juvenile phase thereby obtaining the vigorous growth and adventitious rooting ability of the juvenile phase. Various methods have been devised over the last few decades. Procedures to obtain juvenile material from mature plants is of considerable importance for the micropropagation of woody plants considering that *in vitro* establishment of shoots and rooted explants is highly influenced by the maturation state of the tissue used as a primary explant. Juvenile material may be obtained or isolated from mature plants by use of juvenile parts of mature plants or rejuvenation of mature parts of plants (Hackett and Murray, 1993).

#### 1.3.1 Use of juvenile parts of mature plants

Methods in this category rely heavily on observation and experiments that indicate that ontogenetically juvenile material is found at the base of plants particularly the base of trunks and in the lower branches. It is standard horticultural practice to select and propagate shoots from the mature plant of seedling origin that displays all or part of its juvenile characteristics. There are several sources for such material including lower parts of the crown branches, epicormic shoots on the trunk and the base of primary branches and epicormic shoots obtained as a result of severe pruning. Cuttings taken from the lower region of crown branches have been shown to have higher rooting than those from the upper regions of the crown branches of the same tree in *Picea abies L.* Karst. Similar findings persisted in several other investigations of species *Pseudotsuga menziesii* Mirb. Franco, *Pinus radiata*, *Pinus strobus*, and *Pinus virginiana* Mill. (Black, 1972). Epicormic or latent buds (Hackett, 1985) on the base of trunks and primary branches are a source of shoots that often have juvenile morphological characteristics and high rooting potential. Cuttings from such material has resulted in the successful propagation of several tree species (*Glesditsia triacanthos* L. *Eucalyptus rostrata*, *E. polyanthemos Carya illinoinensis* and *Olea euroaea* L.). Severe pruning or hedging of trees can result in the production of epicormic shoots (Arnaud *et al.*, 1993). Boulay (1978), using this

method, successfully produced significant quantities of plants propagated *in vitro*, from basal shoots of the tree *Sequoia sempervirens*. At AFOCEL a cloning program for Norway spruce *Picea abies* was developed through severe hedging (Franclet, 1983). The application of the cytokinin benzylaminopurine (BAP) has also resulted in epicormic shoots being forced into growth (Hackett, 1985).

### 1.3.2 Rejuvenation of mature parts of plants

The rejuvenation of mature parts of a tree is based on the conclusion that although the mature related characteristics are relatively stable, they are reversible under certain conditions (Rogler and Hackett, 1975). Some characters do appear to be easily manipulable and certain treatments may induce one character but not all. Most investigations indicate that shoots arising adventitiously are to some degree juvenile in appearance, exhibit delayed flowering and a greater potential to root in comparison to shoots from more mature parts of the plant. Some observations are for shoots arising from roots and this poses a problem in that the maturity of roots is uncertain. Barlass and Skene (1980) found plantlets originating adventitiously from leaf primordia of mature grape shoot apices were juvenile in morphology and flowering ability. Garner and Hatcher (1962) observed that adventitious shoots from *Ilex aquifolium* had juvenile foliage and that adventitious shoots arising from sphaeroblasts on stems of clonal apple have a high rooting potential and low flowering ability. The use of exogenous hormones opened up another avenue in rejuvenation of plants. The first attempts were with *Hedera helix*. Robbins (1956) argued that a possible "rejuvenating substance" suggested by Doorenbos (1955) was gibberellic acid. Rogler and Hackett (1975) investigated the effects of applying varying doses of $GA_3$, $GA_{4+7}$ and $GA_1$ on *Hedera helix* and found varying degrees of rejuvenation, the stability of the induced characteristic through $GA_3$ being dependent on the dose applied. The application of $GA_3$ to other species (mature pear, *Citrus*, *Prunus*) resulted in inhibition of flowering and an induction of juvenile morphological characters (Hackett 1985).

Although the effects on rooting potential were not reported,

Hatcher (1959) demonstrated that severe pruning and hedging of rootstock clones delayed flowering and increased vegetative vigor and rooting. Black (1972) used severe pruning of mature *Pseudotsuga menziesii* to enhance vigor and rooting of cuttings from this species. Similarly Franclet (1979) applied severe pruning to several conifer species to obtain cuttings with higher rooting potential from the mature crowns of trees of seed origin. In *Pseudotsuga menziesii* shoots had juvenile growth characteristics after the first year and increased rooting potential after the second. Rooted *Picea Abies* cuttings obtained as above grew orthotropically, however Franclet noted that in this case the rejuvenation of foliage characteristics was incomplete when compared to those of 2 to 3 year old seedlings. From the above work, Hackett (1985) proposed that it was unlikely that severe pruning yielded a fully rejuvenated plant. Franclet (1983) claimed that "repeated cutting favours rejuvenation which is practical for the true cloning of old trees. Rejuvenation is a slow process and is probably incomplete". Franclet (1983) felt that the term invigoration might best apply and may play an important role for further ontogenetic rejuvenation.

Although mature meristems are quite stable *in vitro* and *in vivo*, *in vitro* culture has been shown to affect their phase related characteristics. Both the length of culture and the number of subcultures involved seem to play a role in these changes. Studies carried out involving culture media and the variation of culture conditions suggest an important role with respect to rejuvenation *in vitro*.

The *in vitro* culturing of *Pinus pinaste* onto medium containing Benzyladenine (BA) resulted in the development of new buds from previously elongated buds growing on developing needle fascicles (David *et al.*, 1978). This indicated a rejuvenation of primary meristems. The less elongated the buds, the more juvenile the foliage characteristics on the newly derived buds. High concentrations of BA and low sucrose concentrations facilitated juvenility. In studies of the conifer *Sequoia sempervirens*, Boulay (1979) showed that the inclusion of activated charcoal in culture medium resulted in an explant, originally displaying plagiotropic shoots, undergoing an increase in the number of orthotropic shoots, the increase being related to number of subcultures. Root potential also increased with subculturing and Boulay suggested that culture conditions were

aiding ontogenetic rejuvenation. Further studies showed that by alternating multiplication media, one month on cytokinin media, one on cytokinin free media including activated charcoal, clones that were free from hyperhydration could be produced. That this method may have a general applicability is demonstrated by the fact that clones of the famous ARC 154 (worlds tallest tree) have been produced with the method established by Boulay (Franclet 1991). Mature shoot tips of an ancient grape clone 'Cabernet Sauvignon' were serially *in vitro* subcultured and reverted to a more juvenile form (Mullins *et al.*, 1979). Juvenility was indicated by a lack of tendrils and spiral phyllotaxy.

Thornless blackberries cultured from shoot tips produced monophyllous leaves (Broome and Zimmernam, 1978) and production of monophyllous leaves in culture was maintained by repeated subculturing (Zimmerman, 1981). Apple cultivars cultured from shoot tips also show some reversion in leaf shape in tissue culture (Sriskandarajh *et al.*, 1982), the reversion manifesting itself as lobed leaves, irregular serrations and thinner leaf blades. This morphological reversion to a juvenile form perhaps suggests a reason for the increase in rooting potential also observed after a certain number of subcultures. Lyrene (1981) also noted an increase in rooting potential and changes in morphological characteristics.

## 1.4 Grafting as a means of rejuvenating mature material

Grafting is an ancient horticultural practice where it has been used as a method of vegetative propagation, production of virus free plants and the maintenance of tree species. It was known to the Chinese as early as 1560 B.C. and to Aristotle, Theophrastus and others of the Hellinistic and Roman times (Stoddard and Mc Cully, 1979). Grafting has been used to obtain special forms of growth, to improve the growth of certain clones of plants, to improve fruit quality, enhance disease resistance, investigate graft incompatibility, improvement of grafting techniques, improve winter hardiness and to facilitate the adaptation of plants to different environments (Edriss and Burger, 1984; Huang *et al.*, 1992; Murashige *et al.*, 1972; Navarro *et al.*, 1975; Parkinson, 1983). More recently grafting has been investigated as a means to rejuvenate and micropropagate adult material (Pullman and Timmis, 1992; Meier and Reuther, 1994; Ewald and Kretzchmar, 1996).

### 1.4.1. Events surrounding graft union formation

Though most of the extensive literature on grafting deals with practical aspects of grafting technique, several authors have described the histology, anatomy and events surrounding graft union (Gebhardt and Goldbach, 1988; Shimomura and Fuzihara, 1977; Stoddard and McCully, 1979; Yeoman *et al.*, 1978). All of these authors report the same sequence of detailed events. Briefly, cells killed by the initial cut form a brown layer and the living cells beneath become meristematic and form callus. This brown layer eventually disappears. Cohesion of stock and scion due to callus cell adhesion follows. In autografts of pea root and in tomato autografts initial green staining with toluidine blue is replaced by metachromic pink to purple staining after several days (Stoddard and McCully, 1979). This suggests that the initial material from cut cells contains lignins and polyphenols while the later secretions are of different materials probably pectin and cell wall precursors. Proliferation of callus, new vascular tissue and new cambium result in the vascular connection between stock and scion and then formation of successful graft union. Studies in autografts of *Sedum telephoides* (Moore, 1984)

indicated that initial cohesion of stock and scion contributed 13% of graft tensile strength, fusion of callus cells contributed 44% of tensile strength and vascular redifferentiation contributed 43%. The callus tissue that forms after a graft has been prepared, acts as a bridge across which water and nutrients can bypass damaged vascular tissue. Eventually the cambia of the graft components unite and produce new xylem and phloem, leading to a restoration of vascular flow pathways. The union of the vascular elements of stock and scion following grafting is thought to be the critical event in successful union (Gebhardt and Goldbach, 1988). Arguments in favour or against recognition systems in graft union formation have been discussed elsewhere by other authors (Yeoman *et al.*, 1978; Yeoman, 1984; Moore and Walker, 1981; Moore; 1986).

### 1.4.2 Rejuvenation through grafting

The first studies into rejuvenation were undertaken by Doorenbos (1954) and involved grafting mature and juvenile *Hedera helix* plants. Carrying out experiments in the greenhouse, Doorenbos made several graft combinations such as mature scions onto juvenile rootstocks, juvenile scions onto mature rootstocks and mature scions onto mature stocks. He also investigated the presence of leaf material on scion and stock. It was found that rejuvenation effects were observed when a leafless mature scion was grafted to a leafy stock. Doorenbos argued that complete rejuvenation was evident in a graft comprising of a juvenile leafy scion to a mature leafless stock.

In a comprehensive review of rejuvenation in woody species, Hackett (1985) claimed "the mature state is normally quite stable during vegetative propagation techniques, including grafting on seedling rootstocks. Generally such grafting causes an increase in vegetative vigour and some delay in flowering, but similar changes are also expected when such a scion is grafted onto a mature clonal rootstock". Although vegetative propagation yields material that can be rooted and/or cultured *in vitro*, oaks are still quite difficult to root compared with other forest trees. Serial grafting may open the way to rejuvenation of oak and to the mass propagation of oak species. The technique has been successfully applied to oak

species in trials on *Q. acutissima* clones (Moon and Yi, 1993). A number of reports have shown some rejuvenation, due to grafting adult scions to juvenile rootstocks, (Dumas *et al.*, 1989; Franclet, 1979; Monteuuiss, 1986; Pieringer and Hanks, 1965; Stoutemyer and Britt, 1961). Franclet (1979) investigated rejuvenation *in vivo* through serial grafting of adult scions onto seedlings and suggested maximum miniaturization of the ramet to enhance transmission of hormonal 'juvenility inducing substances'. *In vivo* grafting of scions from a 30 year old chestnut tree onto rootstocks grown from seeds of the same tree resulted in an enhanced *in vitro* establishment of cultures (Ballester *et al.*, 1990). Ballester *et al.* (1990) subjected grafted material to varying spray treatments with 6- Benzylaminopurine (BAP) and found that explants from ungrafted branches failed to grow *in vitro*, whereas establishment *in vitro* was achieved for 22% of explants from grafts given no prior BAP treatment, 43% of those grafts treated with BAP 2 hours immediately before culture and 100% for those grafts that had been sprayed with BAP during the last two weeks prior to culture. After 1 year of culture, explants from the latter treatments displayed the best multiplication rates. Ballester *et al.* (1990) felt that a combination of grafting and BAP spraying *in vivo* offered an efficient alternative to the laborious, time consuming cascade grafting technique.

Grafting has also been shown to have a rejuvenation effect on Eucalyptus species (Franclet, 1983). In 1956 while grafting pieces of sprouts from a possible hybrid of *E. tereticornis* X *camaldulensisto* plants in the first seed orchard, Franclet noticed that the grafts were producing juvenile like foliage. In further studies with Eucalyptus, twigs from the crown of an eighty three year old *E. camaldulensis* were grafted to seedlings initiated from seeds of the donor tree. A series of treatments were applied to the grafted twigs, in treatment one only one graft per year was carried out. Treatment two consisted of two grafts per year with the final treatment being serial grafts carried out at two to three month intervals. Only the third treatment rejuvenated the clone. Soon after the third grafting, clones with recovered juvenile characteristics could be propagated. *In vitro* grafting of adult avocado (*Persea americana* Mill.), as scions, to *in vitro* grown seedling rootstocks resulted in the acquisition of juvenile characteristics to adult material (Pliego-Alfaro and Murashige, 1987). About 50% of shoots that emerged from adult scions showed

increased rooting competence. The shoots resulting from grafts of adult buds onto juvenile rootstocks rooted with a frequency of around 30%. However regrafting of shoots onto fresh rootstocks did not increase the incidence of rooting. Grafted adults produced less callus, their leaves grew more rapidly, the stems elongated more rapidly than those of ungrafted adults and displayed growth characteristics resembling those of juvenile shoots. Pliego-Alfaro and Murashige (1987) argued that this restored competence reflected a developmental phase reversal from adult to juvenile rather than a simple transfer of rooting cofactors from rootstock to scion. Pullman and Timms (1992), in studies of *in vitro* grafting of adult buds of Douglas Fir to juvenile seedling rootstocks, reported a significant acquisition of juvenile-like characteristics. However studies showed that the juvenile characteristics displayed on *in vitro* rejuvenated buds were not fully equivalent to those of cotyledon derived shoots. Nevertheless, they felt that the technique serves as a suitable point for further rejuvenation through embryogenesis. In studies on the grafting of micropropagated apple tree scions to micropropagated rootstocks, Jones and Hadlow (1989) noted that the micropropagated trees displayed juvenile like characteristics such as more vigorous shoot growth and delayed cropping when compared to trees produced through conventional methods. All grafting was carried out in the greenhouse by saddle grafting and tying the graft union with cling film for the first month. Jones proposed that the attainment of the juvenile like characters was a rejuvenation of tissue through micropropagation however one must note the influence of the grafting regime.

Repeated grafting of shoot tips onto juvenile rootstocks *in vitro* resulted in a rejuvenation of *Sequoia sempervirens* (Huang *et al.*, 1992). Morphogenic competence and related biochemistry were restored progressively and restored traits were retained for 3 years. The grafting scheme followed by Huang *et al.*, is outlined in Figure 1.4.2.1 Grafting was achieved by simply inserting an *in vitro* cultured scion wedge into a longitudinal incision of an *in vitro* cultured rootstock. Grafted stems were transferred to nutrient medium. Over 90% of grafts were successful. After 8 weeks in culture, regrafts were carried out and key juvenile traits of increased incidence of rooting and number of roots, elongation and branching of

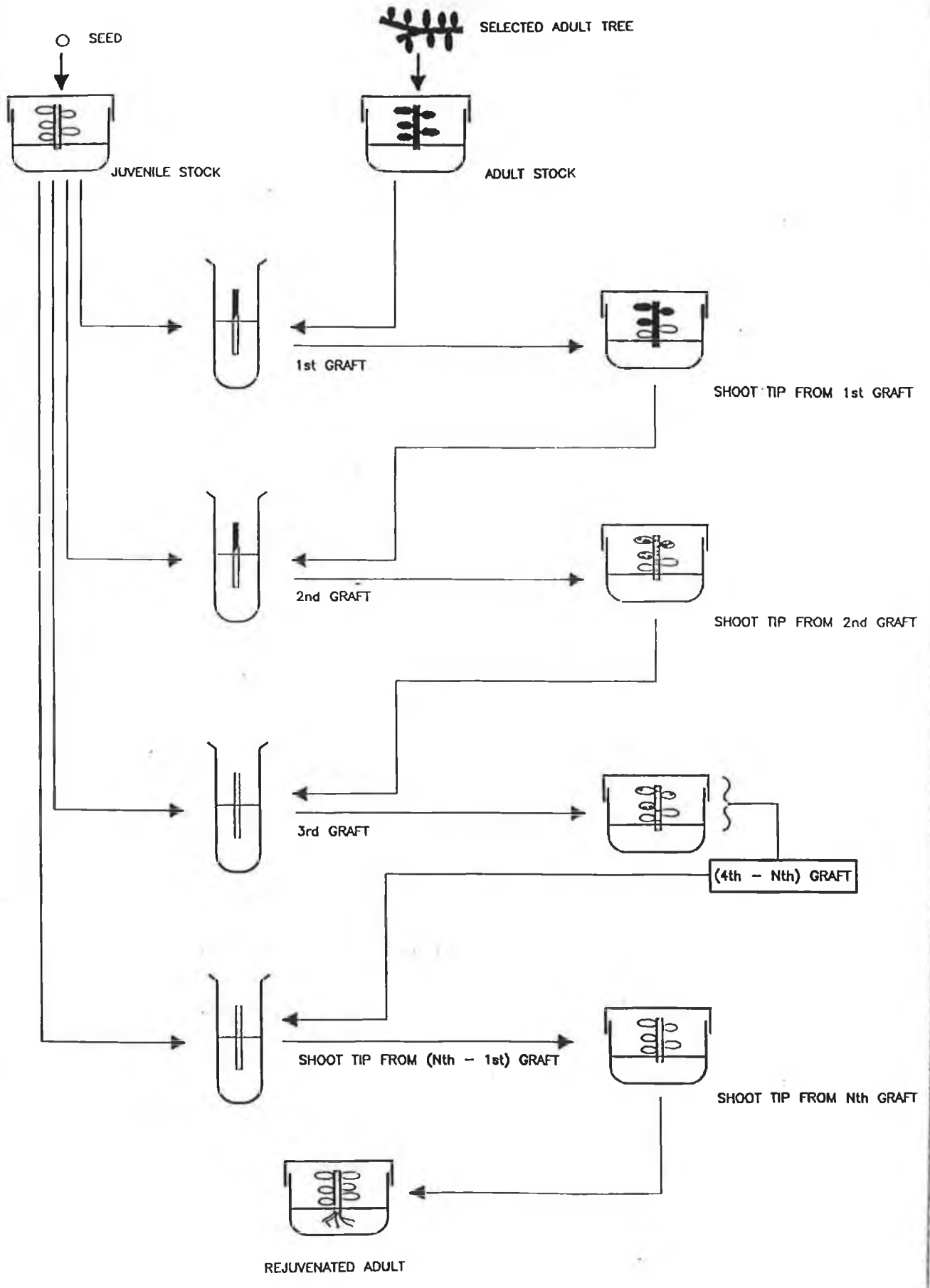



**Figure 1.4.2.1 Scheme for Cascade grafting (Adapted from Huang *et al.*, 1992)**

stems, vigor of roots and stems, restoration of juvenile leaves and competence for adventitious shoot differentiation plus diminishment of callus growth and reduction in mortality of explants were observed after four successive grafts. Coupled with such morphological observations was the absence of some distinctive proteins and the appearance of others in the leaves of rejuvenated *S. sempervirens* when compared with those of adult material. Arnauld *et al.* (1993) similarly reported morphological characteristics indicating an apparent rejuvenation of *Sequoia sempervirens* through micro-grafting. Apices from a mature *in vitro* maintained clone were grafted onto an oblique section of a rootstock at 1 cm distance from the roots. Grafted apices which grew acquired a growth rate and morphology similar to juvenile clones. Micro-grafting also increased the number of shoots which rooted, the speed of rooting and the quality of root system. After transplanting to *ex vitro* the rooted stems displayed an orthotropic growth habit. However this reverted to plagiotropic growth after several weeks. After 2 years a small number of the original grafted apices transplanted *ex vitro* were displaying orthotropic growth.

Grafting *in vitro* offers many advantages to the more conventional methods of grafting *in vivo*: *In vitro* grafting can be achieved with substantially smaller and more juvenile rootstocks; Regrafting can be carried out at shorter intervals and significant levels of regrafting can be achieved in these short intervals (Huang *et al.*, 1992); *In vitro* grafting also brings the influence of the rootstock closer to the scion and in the case of micro-grafting of buds to scions, specific organs can be selected for grafting; *In vitro* grafts can be carried out under sterile conditions and the environmental conditions greatly controlled; Hormonal treatment may be varied and substances of interest maintained in or near the graft union at high and defined concentrations; *In vitro* grafting allows a better observation of the grafting procedure and surrounding events (Parkinson *et al.*, 1990).

Problems in grafting scions to stocks manifest in many ways. Initial problems of graft assembly and the proper attachment of scion to stock, particularly *in vitro*, present the researcher with the first true test. Other problems include the protection of the union following initial assembly and during healing . The removal of grafting devices that aid assembly also have had a significant effect on the graft success. (Obeidy and Smith, 1991). *In vitro* graft unions of apple have been

connected using a filter paper bridge to hold stock and scion together and to aid and facilitate callus formation (Huang and Millikan, 1980). The application of an elastic strip for the protection of graft zones in peach microcultures was utilized by Jonard *et al.* (1983). Parkinson and Yeoman (1982) grafted two halves of explanted internodes of *Lycopersican esculentum*, *Datura stramonium* and *Nicandra physaloides* successfully using silicon tubing to act as a support for the graft union. The grafting technique was further enhanced by utilising a split agar Petri dish that allowed for the manipulation of growth media and hormone application to the scion and stock. Gelbhardt and Goldbach (1988) investigated the use of translucent silicone tubing as a support and as a means to maintain contact between scion and stock in *Prunus* microcuttings as did Richardson *et al.* (1996) when examining graft union formation in *in vitro* micrografts of tissue cultured apple (*Malus domestica.* Borkh). Jonard *et al.* (1990) investigated graft incompatibilities in apricot and lemon trees using *in vitro* techniques. Using split agar and varying hormonal conditions, internode association was examined. In this case no support mechanism at the interface was utilised. The provision of a suitable support that will maintain graft integrity and that can be removed easily without damage occurring to the graft union has a major role in ensuring grafting is successful (Obeidy and Smith, 1991).

### 1.4.3. Transmission of rejuvenation factor across Graft Union

Doorenbos (1954) in obtaining a rejuvenation of apical meristems through *in vivo* grafting demonstrated that a juvenile stock may influence an adult scion, causing the latter to lose its' capacity to flower. In 1958, Muzik and Cruzada, in repeated grafting of mature *Hevea brasilensis* to juvenile plants, observed similar findings. This led the researchers to propose that the juvenile plant may transmit a substance to the adult which induces the adult to assume juvenile characteristics. Further studies undertaken indicated that the 'factor' is not readily soluble in ordinary solvents but may be slowly absorbed and accumulated by the scion in sufficient amounts to induce a change in rooting ability. Geneve *et al.* (1991), in studies of reciprocally grafted leaf cuttings of *H.helix*, argued that the positive effect of a juvenile lamina in inducing rooting in a mature petiole "provided strong

evidence for a transmissible factor produced in the juvenile lamina that promoted initiation of rooting in mature tissue". No evidence for the presence of an inhibitor of root initiation being supplied from mature lamina to juvenile petioles was forthcoming. Similarly, Mullins (1985) reported no evidence for a transmissible rooting inhibitor in an *in vitro* grafting study with non- rooting (mature) and rooting (rejuvenated) microcuttings of Jonathan apple. Parkinson (1983) demonstrated the transport of colloidal iron oxide particles across the graft union in *N.physaloides* and *L.esculentum* homografts showing that full vascular continuity is re-established across grafts and that this is contiguous with the pre-existing vascular tissues. Warren Wilson (1982) proposed that the presence of large, vigorous scions with leaves and flowers would indicate that vascular connections across graft unions were capable of transporting water, minerals and hormones. The graft associated rejuvenation of *S. sempervirens* (Huang *et al.*, 1992) is displayed as progressive increases in incidence of rooting and juvenile characteristics. Of more importance is the absence of distinctive proteins and the appearance of others in rejuvenated leaves of *S. sempervirens* when compared with proteins of adult leaves by SDS PAGE. The protein observations coupled with that of Bon (1988), a 16kDa shoot apex protein in juvenile *S. giganteum*, and Bon and Monteuuis (1991) suggest the transmissibility of a signal through cells of the graft union. Huang *et al.* (1992) suggests that the signal is probably a small sized molecule(s). Given that prolonged retention of restored traits (over 3 years) *in vitro* occurred in the Hung *et al* experiments, it is also possible that the molecules are self replicating, perhaps nucleic acids and if so capable of resisting enzymatic destruction.

**Aims**

Rejuvenation of mature material has been achieved for several woody plants. Several techniques have been employed including the repeated sequential grafting of adult scions onto juvenile rootstocks. It may be possible to 'rejuvenate' mature *Quercus* material through *in vitro* grafting. Plant material so treated may respond like juvenile tissues, and hence be more receptive to vegetative propagation both *in vitro* and in the greenhouse However with *Quercus* several obstacles remain to be overcome. In order to follow the progress of rejuvenation we need easily measured qualitative and quantitative criteria of juvenility and maturity and these are currently lacking in oak . The procedures must also be non-destructive of the shoots. Secondly a technique for successful *in vitro* grafting of mature *Quercus* material to juvenile *Quercus* material needs to be established and investigated.

The aims of this thesis are as follows :-

(1) To establish good quantitative and qualitative markers for *in vitro* growing juvenile and mature *Quercus robur* and *Quercus petraea* plants. To provide such markers *in vitro* growing shoot cultures of *Quercus* species of juvenile, adolescent and mature origin will be examined for a range of morphological, physiological and biochemical markers of juvenility and maturity.

(2) To devise a system for *in vitro* grafting of *Quercus* material and to establish the conditions in which autografts, homografts and hetrografts of *Quercus* material will graft successfully

(3) To evaluate rejuvenation of mature *Quercus* material through sequential grafting by applying established markers of juvenility and maturity.

# Chapter 2

# MATERIALS AND METHODS

## 2.1 Source of Plant Material

Unless otherwise stated all *Q. robur* and *Q. petraea* clones were donated from the laboratories of Teagasc, Kinsealy Research Centre, Co.Dublin, Ireland.
Table 2.1.1 outlines sources of all *Q. robur* and *Q. petraea* clones.

### 2.1.1 Juvenile Clones

Clones JR2, JR3, JR4 were initiated from 7 months seedlings of *Q. robur* (NL3 superior road stand (Veenendaal -de Klomp, Netherlands). Clones JR1 and JR5 were derived from stump sprouts taken from the base of a 100 year old *Q. robur* (NL3 superior road stand, Veenendaal -de Klomp, Netherlands) (Evers, 1992) and these were designated as juvenile because of their origin.

*Quercus petreaea* clone JP1 was derived from embryos germinated *in vitro*. Clone JP2 was donated by Dr. Eva Waindinger, Forschungszentrum Seibersdorf, Austria, and was initiated from a *Q. petraea* seedling. Juvenile clone JP3 was initiated in the laboratories of Dublin City University from shoot sections of a 7 month old *Quercus petraea* seedling (Natural oak forest, Donegal) from Dr. Sean Mac an tSaoir, Department of Applied Plant Science, The Queens University of Belfast, Newforge Lane, Belfast, U.K.

### 2.1.2 Mature and Adolescent Clones

Branch tips from the lower branches of one *Q. robur* and three *Q. petraea* oak trees c.a. 150 year old (Kilmacurragh, Co. Wicklow, Ireland) that were known to be capable of flowering were collected and grafted to 2 year old seedlings. New shoots from grafted plants were used to initiate cultures of clones: MR1 (*Q. robur)*; MP1, MP2 and MP3 (*Q. Petraea*). A mature clone (MR2) of *Q. robur* 'Fastigiata' was initiated from adult budwood, of an unknown age, of severely pruned hedge plants (East Malling, Kent, England) which had been grafted onto 2 year old seedling rootstocks before culture initiation. (Marks and Simpson 1993).

| Clone identity | Origin | Species |
|---|---|---|
| JR1 | Stump sprouts, base of 100 year old tree , Netherlands | *Q. robur* |
| JR2 | Seedling, 7 months, Netherderlands | *Q. robur* |
| JR3 | Seedling, 7 months, Netherderlands | *Q. robur* |
| JR4 | Seedling, 7 months, Netherderlands | *Q. robur* |
| JR5 | Stump sprouts, 100 year old tree , Netherlands | *Q. robur* |
| JP1 | Seedling, Kilmacurragh, Ireland | *Q. petraea* |
| JP2 | Seedling, Austria | *Q. petraea* |
| JP3 | Seedling, Donegal, Ireland | *Q. petraea* |
| AP1 | Branch tips, c.a . 20 years, Kilmacurragh, Ireland | *Q. petraea* |
| MR1 | Branch tip of mature oak, c.a .150 years Kilmacurragh, Ireland | *Q. robur* |
| MR2 | Adult budwood of pruned hedged plants, East Malling, Kent. | *Q. robur* |
| MP1 | Branch tip of mature oak, c.a .150 years Kilmacurragh, Ireland | *Q. petraea* |
| MP2 | Branch tip of mature oak, c.a .150 years Kilmacurragh, Ireland | *Q. petraea* |
| MP3 | Branch tip of mature oak, c.a .150 years Kilmacurragh, Ireland | *Q. petraea* |
| PP 1 - 32 | Epicormic shoots, branch sections, 100 year old tree, Phoenix park, Ireland | *Q. robur* |

**Table 2.1.1 Source of *Q. robur* and *Q. petraea* clones**

### 2.1.3 Phoenix park clones

Clones PP1 to PP 32 were initiated in the laboratories of Dublin City University from epicormic buds of a branch section sourced from a stand of 100 year old *Quercus robur* trees situated in the Phoenix Park, Dublin, Ireland.

#### 2.1.3.1 Initiation of Phoenix Park Epicormic Shoots

The lowermost branch approximately 2.5m from the base exhibiting strong orthotropic growth and approximately 12m in length was removed with a chainsaw. The branch was then cut into 32 segments, each of approximately 0.3m in length. The branch segments were labeled as section 1 - 32, section 1 being that closest to the original tree trunk. The following day sections were transferred to the greenhouses with insect control under natural light and temperature conditions at Teagasc Research Centre Kinsealy, Co.Dublin, Ireland. The sections were vigorously scrubbed with a wire brush to remove external dust and moss. Each section was soaked twice with a disinfection solution comprising 250 mls of commercial Savlon (ICI ltd, England) in 8 litres of water and rinsed with a jet of water. Sections were then placed into 8 litre buckets filled with tap water. Sections 3, 7, 11, 15, 19, 23, 27, 31 were placed into a thermostatically controlled greenhouse with insect control under a 16 hr photoperiod at a constant temperature of $24^{o}C \pm 2^{o}C$. The remaining sections were maintained in a greenhouse with insect control under natural light and temperature conditions. The water in each bucket was replaced with fresh water every 1 - 2 weeks to alleviate retardation of budding caused by phenolics leaking from branch sections

### 2.2 Sterilisation Procedure

Unless otherwise stated all meterials and chemicals were sterilised by autoclaving (SS-325 Portable Autoclave , Tomy Seiko Co. Ltd.,Tokyo, Japan). To conserve sterility, utensils were first wrapped in at least two layers of aluminium foil or

placed into 250 ml conical flasks and sealed at the neck with aluminium foil. Media and utensils were then autoclaved for 20 minutes at 103.4 kPa.

### 2.2.1 Sterilisation of buds

Shoot sections approximately 3 - 4 cm with closed buds were excised using a scalpel and forceps. Leaf material was removed and stem sections placed in Beatson jars and sterilised as follows:

After a 30 second rinse in 85% ethanol, they were given a 5 minute treatment in 0.1% solution of Mercuric chloride ($HgCl_2$) containing two drops of Tween 20®. Shoot sections were then rinsed 3 times in sterile distilled water (3 minutes per rinse) followed by a 20 minute treatment in Calcium Hypochlorite ($Ca(OCl)_2$) (7 % solution) with a second series of 3 rinses with sterile distilled water (3 minutes per rinse).

### 2.2.2 Conservation of Sterility

Unless otherwise stated all manipulation, micropropagation and sterilisation of plant material was carried out under laminar air flow conditions in a laminar air flow cabinet (Gelman Sciences, Clean Bench Model HLF-120).

Throughout all procedures Latex examination gloves were worn. These were surface sterilised by swabbing with Absolute Ethanol prior to use, during manipulations, and upon contact with non sterile material. Standard sterile techniques were maintained throughout. Before and after use, surfaces of the laminar flow cabinet were swabbed with absolute ethanol. Scalpels and forceps were resterilised after each manipulation by placing into a K-MIDT Bead Steriliser (K-Midt Ltd., Freiburg, Switzerland), for 7-10 seconds, set to 220$^{o}$C.

## 2.3 Culture Medium

Unless otherwise stated, all plant material was cultured on a Woody Plant Media (WPM3) based on the woody plant media of Lloyd and McCown (1980) (Table 2.3.1.). The pH of the media was adjusted to pH 5.7 using 1M KOH prior to autoclaving. All media components were obtained from Sigma Chemicals, Poole, Dorset, England. Media was dispensed into 6oz Borosilicate glass Beatson jars (32 mls of medium per jar) and autoclaved.

## 2.4 Growth conditions

Unless otherwise stated all cultures were grown at $24^{o}C \pm 2^{o}C$ under a 16 hour photoperiod. Light intensity was approximately 20µmoles $m^{-2}$ $s^{-1}$ of photosynthetically active radiation. Light was provided by a bank of 6 Philips fluorescent 125W warm white bulbs.

## 2.5 Sampling of Shoots from *Q. petraea* seedling clone JP3 and trunk sections of Phoenix park trees

Shoots exhibiting vigorous growth with visible closed buds were selected and removed with a sterile forceps and scalpel. Leaf material was excised and the shoot was cut into stem pieces of suitable length and placed into a sterile glass Beatson jar. Each stem piece contained at least one bud. Between each sample the forceps and scalpel were dipped for a 60 second period into a 75% Ethanol solution. Stem pieces were then sterilised as previously described

| *Ingredient* | *mg $l^{-1}$* |
|---|---|
| Sucrose | 20000 |
| Benzyladenine (1mg/ml solution). | 0.2 |
| Gelrite/Phytagel | 2500 |
| $NH_4NO_3$ | 400 |
| $Ca(NO3)_24H_2O$ | 556 |
| $K_2SO_4$ | 990 |
| $CaCl_22H_2O$ | 96 |
| $KH_2PO_4$ | 170 |
| $H_3BO_3$ | 6.2 |
| $Na_2MoO_42H_20$ | 0.25 |
| $MgSO_47H_2O$ | 370 |
| $MnSO_44H_2O$ | 22.3 |
| $ZnSO_47H_2O$ | 8.6 |
| $CuSO_45H_2O$ | 0.25 |
| Thiamine HCl | 1 |
| Nicotinic Acid | 0.5 |
| Pyridoxine Hcl | 0.5 |
| Glycine | 2 |
| Inositol | 100 |
| Fe 330 | 40 |

**Table 2.3.1 WPM3 media composition**

## 2.6 Micropropagation of cultures

### 2.6.1 Culture of sterilised buds

Sterilised stem pieces were placed into an empty petri- dish and trimmed of material displaying sterilisation damage. The sterilised stem pieces were placed horizontally onto Woody Plant Media (WPM3).

### 2.6.2 Subculturing of explants

Cultures were transferred to new media jars upon signs of phenolic exudation. Shoot tips and nodal explants about 0.5 - 1 cm long that developed on initial buds growing *in vitro*, free from fungal and bacterial contamination, were subcultured on fresh WPM3. Shoot tips were inserted vertically into the medium. Nodal explants were cultured horizontally on the medium. Resulting clones were subjected to successive subculturing every four weeks. Whenever possible five explants were placed into each jar.

## 2.7 Measurement of morphological and physiological characteristics.

### 2.7.1 Multiplication rates

At each successive subculture number of explants dead in jar, number of explants contaminated in jar, number of explants used for subculture and the number of new explants obtained was recorded. The multiplication rate was defined as the number of explants obtained per viable explant subcultured.

### 2.7.2 Rooting Studies

#### 2.7.2.1 Development of rooting protocol

The rooting protocol developed was adapted from that of Vieitez *et al.* (1985) and Manzanera and Pardos (1990). Initial studies were carried out using a *Q. robur* clone derived from stump sprouts (JR5).

#### 2.7.2.2 Preparation of rooting media solution

A range of rooting media (RM) containing various sucrose concentrations was prepared. Rooting media consisted of hormone free WPM3, supplemented with 20 $gl^{-1}$ (RM20), 30 $gl^{-1}$(RM30), 40 $gl^{-1}$(RM40), 50 $gl^{-1}$(RM50) of sucrose respectively. All rooting media contained 2.5 $gl^{-1}$ of Phytagel. Media was dispensed into 6oz Borosilicate glass Beatson jars (32 mls of medium per jar) and autoclaved as precviously described.

#### 2.7.2.3 Preparation of rooting hormone solution

A stock solution containing 1$gl^{-1}$ Indole Butyric Acid (IBA) was prepared by dissolving 10 mg of IBA in 10 ml of sterile distilled water and stored at 4$^{o}$C. This was then used to prepare a series of IBA concentrations (30 $mgl^{-1}$; 100 $mgl^{-1}$; 300 $mgl^{-1}$; 1000 $mgl^{-1}$) as required. IBA solutions were filter sterilised prior to use through 0.2µm Minisart NML disposable syringe filters. (Sartorius AG, Germany) to yield the rooting hormone solutions.

#### 2.7.2.4 Rooting of shoot tips

*Quercus* cultures were maintained on WPM3 media for a minimum of 18 subcultures, explants were randomly selected and examined 28 days after the most recent subculture. Explants were removed from culture and placed on a sterile Petri

Dish in the laminar flow cabinet. Shoot tips approximately 1-2 cm long were excised and excess leaves at the base of the shoot removed with a sterile scalpel blade. The shoot tips were then transferred to a sterile Petri-Dish containing 10 mls of sterile rooting hormone of concentration of either, 30 $mgl^{-1}$, 100 $mgl^{-1}$, 300 $mgl^{-1}$, 1000 $mgl^{-1}$ IBA respectively. Basal ends of shoot tips (0.5cm) were immersed for two minutes in one of the rooting hormone solutions. Care was taken to prevent the tips of the shoots being submerged in the solution. Shoots were then transferred to rooting media.. Each concentration of rooting hormone solution (four) was combined with each rooting medium (four) to yield sixteen treatments. 10 shoot tips were subjected to each combination. All rooting cultures were grown under conditions as previously described.

#### 2.7.2.4.1 Rooting of juvenile and mature clones

Rooting of shoot tips from two *Quercus robur* clones derived from stump sprouts (JR1, JR5); two *Q. robur* clones derived from seedlings (JR2, JR4); two *Q. petraea* clones derived from seedlings (JP1, JP3) and two *Q. petraea* clones derived from mature flowering trees (MP2, MP3) was examined. Varying numbers of shoot tips from each clone were subjected to a 2 minute treatment in a 300 $mgl^{-1}$ IBA solution. Treated shoot tips were placed onto RM20 and cultured as previously described. Controls consisted of untreated shoot tips.

#### 2.7.2.4.2 Rooting of Phoenix park clones

Rooting studies were carried out on cultured explants from Phoenix Park tree to examine rooting differences between different section clones. Varying numbers of shoot tips of clones derived from epicormic shoots from sections 5, 6, 7, 11, 13, 15, 18 and 23 were subjected to a 2 minute treatment in a 300 $mgl^{-1}$ IBA solution. Treated shoot tips were placed onto RM20 and cultured as previously described.

### 2.7.3 Explant morphology

After a minimum of 18 subcultures, explants were randomly selected and examined 28 days after the most recent subculture. For each clone a minimum of 5 explants were examined for morphological characteristics.

#### 2.7.3.1 Measurement of shoot angle

The angle of the shoot to the horizontal was measured in-situ to an accuracy of ± 2 degrees by placing a protractor behind the culture vessel in line with the shoot and horizontal to the medium.

#### 2.7.3.2 Measurement of stem length and stem diameters

Explants were removed from Beatson jars gently using a forceps to ensure leaf material did not become detached from stems. Stem length and stem diameters were measured using Vernier Calipers (Mututoyo, Japan) to an accuracy of ± 20 μm. Stem diameters were measured in internodes immediately above the basal callus, immediately below the whorl of leaves at the shoot tip, and in an internode midway between the basal and tip measurements.

#### 2.7.3.3 Measurement of leaf and shoot number

Photosynthetic leaves, defined as those that were green, expanded and firmly attached to the plant were counted by eye. The number of visible shoots, > 1mm in length, arising from each cultured explant was determined by eye. Scale leaves, defined as brown, small (<2mm in length) and easily detached from the shoot, were counted by eye. No attempt was made to characterise scale leaves.

### 2.7.4 Leaf morphology

Photosynthetic leaves were removed with a scalpel blade and attached using Sellotape to A4 sized white paper. A permanent record of the leaves was then produced by photocopying this sheet of paper. The area, breadth, length, perimeter length, roundness and aspect ratio of each leaf was then determined by image analysis of the photocopy using an Olympus BX10 TK 1280 E colour video camera with a Cosmica /Pentax 8-48mm TV zoom lens connected to a Quantimet 500 MC Image Processing and Analysing system with associated software. (Leica Cambridge Ltd., Cambridge, England).

## 2.8 Biochemical characteristics

### 2.8.1 Isolation of Protein Samples

Soluble and membrane proteins were extracted according to the method of Bon (1988). Leaves were excised from explants at the time of subculture and stored in a freezer until protein extraction. Approximately 400 mg of leaf material was ground in a cooled mortar and pestle with 2ml of 0.1M Tris-HCl buffer, pH 8 containing 1% 2-mercaptoethanol. The homogenate was transferred to a centrifuge tube and centrifuged in a Sorval Centrifuge (rotor SS-34) at 5000g (6,500 rpm) for 10 minutes. The pellet was discarded and the supernatant centrifuged at 100,000g (38,200 rpm) for 1 hour in a Beckmann L5-50B (rotor S-50) to yield a soluble protein enriched supernatant designated fraction S and a membrane enriched pellet

The membrane enriched pellet was rinsed with Tris-HCl buffer as above and centrifuged at 100,00g for 1 hour. The resulting pellet was resuspended in Tris buffer containing 1% deoxycholate, homogenized vigorously and stored overnight at $4^{\circ}$C. This homogenate was then centrifuged at 100,000g for 1 hour to yield a membrane -associated protein supernatant, designated fraction M.

Both membrane associated protein fraction (M) and soluble associated protein fraction (S) were precipitated with 5 volumes of cooled acetone for 2 hours at $-20^{\circ}$C. The precipitate was spun at 5000g for 10 minutes and the resultant pellet was washed with cooled acetone, air dried and resuspended in 25 mM Tris HCl buffer containing 0.2 M glycine adjusted to pH 8.3, 4% SDS, 15% glycerol and 1% 2-Mercaptoethanol and incubated at $100^{\circ}$C for 2 minutes.

### 2.8.2 Molecular weight determination of protein samples by SDS Polyacrylamide gel Electrophoresis

Protein concentration of samples was determined by the Bradford Assay (1976). Proteins samples were characterised in terms of molecular size by Polyacrylamide gel electrophoresis in the presence of Sodium Dodecyl Sulfate (SDS) according to Laemmeli (1970). A gel (5% stacking gel, 12% resolving gel)

was prepared fresh from stock solutions. Samples to be examined were diluted to a protein concentration of 1.5 $\mu g ml^{-1}$ in loading buffer, final concentration 25mM Tris-HCl, pH 8.0, 0.4M Glycine, 8% SDS 30% Glycerol, 1% mercaptoethanol. 12 µl of different leaf sample concentrations were applied to sample wells. Dalton Mark VI molecular weight markers ( Sigma chemicals, Poole, Dorset, UK) were used as protein reference standards.

Samples were boiled for 2-3 minutes, allowed to cool and loaded onto a SDS Polyacrylamide gel: 5% stacking gel; 12% resolving gel. Running buffer contained 25mM Tris H-Cl, pH 8.3; 0.192M Glycine; 0.1% w/v SDS. After electrophoresis gels were carefully removed and Protein bands were visualised by silver staining following an adapted version of Merril *et al.* (1981) as described below.

### 2.8.2.1 Silver Stain Technique

#### 2.8.2.1.1 Solutions for silver stain

Ultra pure water was used for all solutions (Mili U10, Water Purification System, Milipore, France)

***Fixation Solution***

400 mls Methanol plus 100 mls Acetic Acid were dissolved in 500mls of water

***Oxidation solution***

0.1g Potassium Dichromate plus 27 µl 65% Nitric Acid were dissolved in water and final volume made up to 100 mls in water

***Silver solution***

0.5g Silver Nitrate was dissolved in 100 mls water and final volume made up to 250 mls with water

***Developing Solution***

14.825g Sodium carbonate was dissolved in 400mls of water. 0.25 mls of 34% Formaldehyde (pH > 4.0) was then added and the final volume was made up to 500 mls with water

**2.8.2.1.2 Staining protocol**

Electrophoresed gels were placed in fixation solution and fixed overnight. After fixation they were then placed in oxidation solution for 5 minutes. The gels were then subjected to three five minute washes in ultra pure water. Gels were stained by immersion in silver stain solution for 20 minutes and then washed for 1 minute with ultra pure water. Protein bands were visualized by developer solutions. Developer solution was replenished with fresh solution when excess protein caused the developer solution to turn black. Development was terminated by the addition of a 5% acetic acid solution.

## 2.9 *In vitro* grafting of *Quercus* species

All grafting of *Quercus* species was carried out using an adapted version of the split agar Petri dish system devised by Parkinson *et al.* (1990)

### 2.9.1 Prepararion of Media combinations for *in vitro* grafting

#### 2.9.1.1 Preparation of Indole 3 Acetic Acid solution and 6 Benzylaminopurine

A 1 $mgml^{-1}$ stock solution of Indole 3 Acetic Acid (IAA) solution was prepared by dissolving 10 mg of Indole 3 Acetic Acid in 10 ml of distilled water. A commercial preparation of a $1mgml^{-1}$ solution of 6 Benzylaminopurine was used (Sigma Chemicals, Poole, Dorset, England).

#### 2.9.1.2 Preparation of Apical media

Nine different apical media combinations were prepared as in Table 2.9.1.2.1. These consisted of hormone free WPM3 to which was added a range of Indole 3 Acetic Acid (0.1, 1.0, 10.0 $mgl^{-1}$) concentrations and a range of 6 Benzylaminopurine concentrations ( 0.2, 0.02, 0.00 $mgl^{-1}$). The pH of the media preparations was adjusted and autoclaved as previously described.

#### 2.9.1.3 Preparation of Basal media

Two separate basal media combinations were prepared. These consisted of hormone free WPM3 to which was added 0.02 $mgl^{-1}$ or 0.2 $mgl^{-1}$ 6 Benzylaminopurine respectively. The pH of the media preparations was adjusted and autoclaved as previoulsy described.

| Treatment Code | APICAL MEDIA | | BASAL MEDIA |
|---|---|---|---|
| | Indole 3 Acetic Acid ($mgl^{-1}$) | 6 Benzylaminopurine ($mgl^{-1}$) | 6 Benzylaminopurine ($mgl^{-1}$) |
| Ab1 | 0.1 | 0.2 | 0.02 |
| Ab2 | 0.1 | 0.2 | 0.2 |
| Bb1 | 1.0 | 0.2 | 0.02 |
| Bb2 | 1.0 | 0.2 | 0.2 |
| Cb1 | 10.0 | 0.2 | 0.02 |
| Cb2 | 10.0 | 0.2 | 0.2 |
| Db1 | 0.1 | 0.02 | 0.02 |
| Db2 | 0.1 | 0.02 | 0.2 |
| Eb1 | 1.0 | 0.02 | 0.02 |
| Eb2 | 1.0 | 0.02 | 0.20 |
| Fb1 | 10.0 | 0.02 | 0.02 |
| Fb2 | 10.0 | 0.02 | 0.20 |
| Gb1 | 0.1 | 0.00 | 0.02 |
| Gb2 | 0.1 | 0.00 | 0.20 |
| Hb1 | 1.0 | 0.00 | 0.02 |
| Hb2 | 1.0 | 0.00 | 0.20 |
| Ib1 | 10.0 | 0.00 | 0.02 |
| Ib2 | 10.0 | 0.00 | 0.20 |

**Table 2.9.1.2.1 Apical and Basal media concentrations and combinations**

### 2.9.2 Preparation of culture dish

Autoclaved media and perspex box sections (dividers) (86mm by 10mm by 10mm) were allowed to cool for 10 minutes in a laminar air flow cabinet. The lower half of a 90mm sterile plastic Petri-dish was divided into two halves with a piece of perspex box sections section and was marked at the base of the dish to act as an indication of orientation for apical and basal media. The appropriate apical and basal media was then poured into marked section of petri dish and allowed to solidify. Perspex dividers were then removed using a sterile forceps.

### 2.9.3 Graft assembly

Cultured clones to be grafted were transferred to laminar flow cabinet where the exterior of each Beatson jar was sprayed with 75% Ethanol to minimise contamination. Explants to be used in grafting were removed with a sterile forceps and placed in a sterile petri-dish. Autografts, grafts formed from the same internode were constructed by removing approximately 12 mm length of stem from the middle of the internode. Preliminary studies carried out in the laboratory investigating the horizontal and vertical incision of graft site indicated that a diagonal incisions resulted in easier manipulation and higher graft take, therefore, to facilitate graft assembly and graft development, a diagonal incision at approximately $45^{o}$ was made across the stem piece. Homografts, grafts constructed from plants of the same species, and heterografts, grafts constructed from plants of different species were constructed by removing internodes of approximately 12 mm in length, with stems of similar diameters and placed side by side. A diagonal incision was made across the stem pieces as above. To further enhance graft assembly and promote graft development grafts were assembled, as shown in Figure 2.9.3.1, inside a 5mm long sterile cylinder of 'Esco' translucent silicone rubber tubing with a 0.5mm thick wall and a 1.0mm internal diameter (Bibby Sterilin Ltd, England).

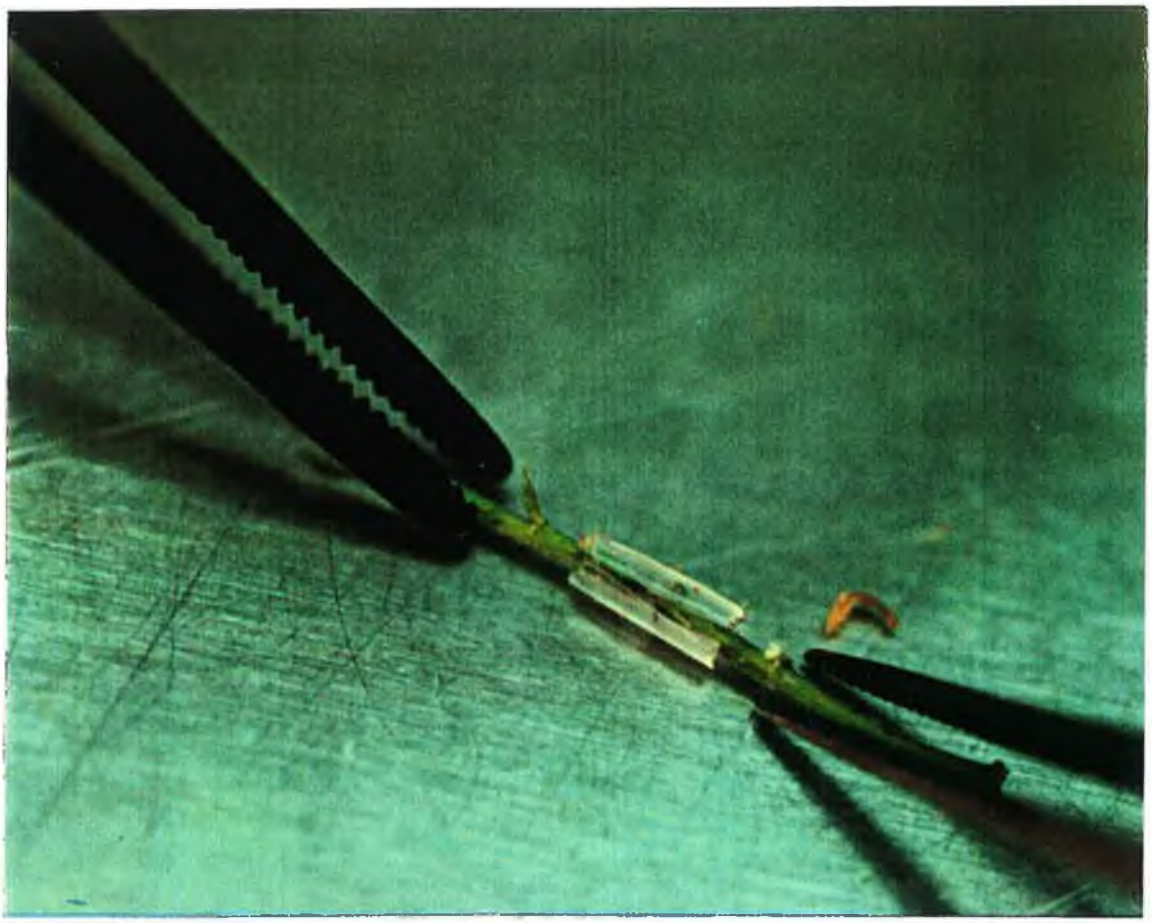


**Figure 2.9.3.1. Assembly of graft partners within silicone tubing**

### 2.9.4 Culture of grafted partners

The grafted internodes were placed between the two agar surfaces of the culture dish as shown in Figure 2.9.4.1. A minimum of two and a maximum of five grafted partners were placed into each dish to allow for support of media. Dishes were sealed with parafilm and placed vertically on the wall of the growth room with the aid of autoclave tape to ensure that the original polarity of the plant tissue was maintained during culture.

### 2.9.5 Optimisation of Media combinations for *in vitro* grafting

In order to optimise the media combinations 18 different media preparations were assessed as in Table 2.9.1.2.1. Optimisation studies were carried using a juvenile *Quercus robur* clone (JR5) derived from stump sprouts. Ten autografts were assembled per combination.

Grafts were cultured as previously described. After 28 days culture grafts were examined and the diameter of the scion tip, the scion base, the stock tip, the stock base, and the length of shoots growing from stock or scion were measured using Vernier Calipers (Mututoyo, Japan) to an accuracy of ± 20 μm. Union success, presence of callus at union, bud number, leaf number, condition of leaf and presence of callus on stock or scion was also recorded.

### 2.9.6 Grafting of *in vitro* cultured *Quercus* clone in a vertical plane

Grafting assembly was carried out as before except grafted partners were vertically placed into a Beatson jar containing WPM3. Grafts were cultured under the same condition as for split agar Petri dish grafts.

### 2.9.7 Initial grafting studies

Unless otherwise stated all grafts were cultured on medium combination Cb1for 28 days under conditions as previously described.

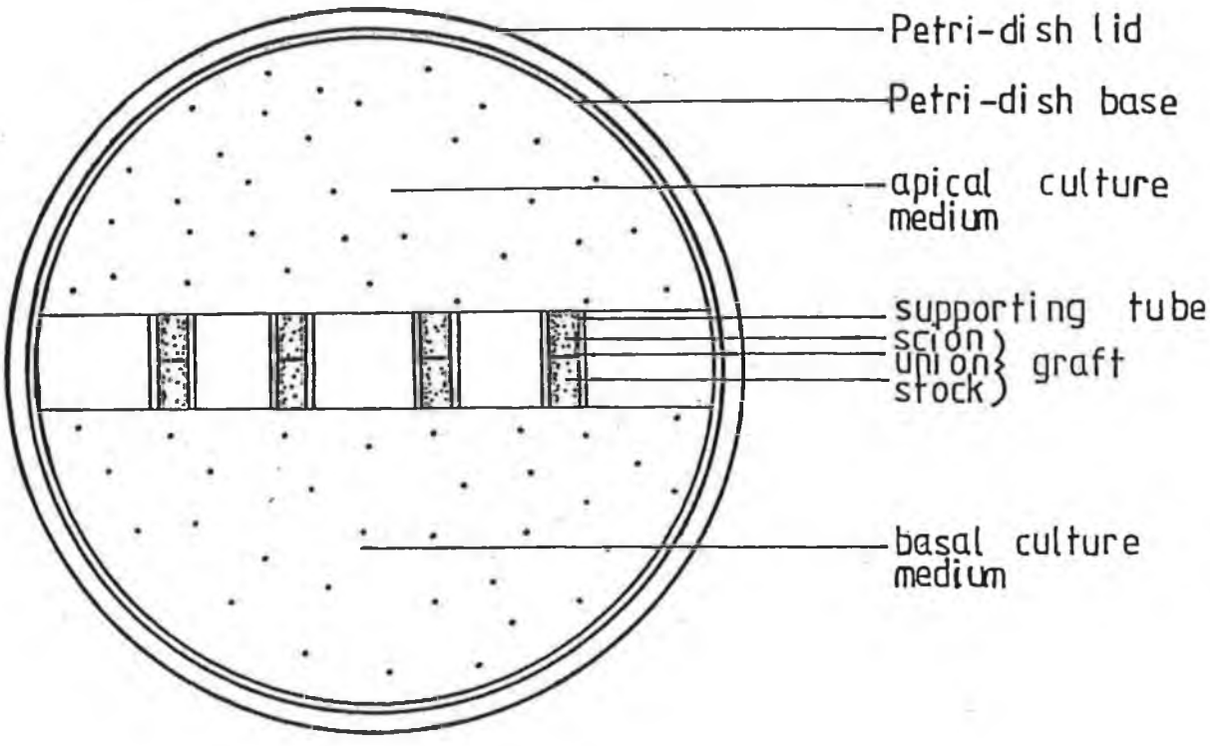



**Figure 2.9.4.1 Set up of culture dish for *in vitro* grafting**

### 2.9.7.1 Assesment of of the effect of scion and stock buds on graft development

Scion and stocks were isolated from a seedling derived *Q. robur* clone (JR4). All except two leaves were removed, with a sterile scalpel, from the scion and stock respectively. Auto grafts were assembled with varying bud numbers on scion and stock as outlined in Table 2.9.7.1 and cultured under conditions as previously described.

### 2.9.7.2 Assesment of of the effect of scion leaves on graft development

Scion and stocks were isolated from a seedling derived *Q. robur* clone (JR4). All leaf material was removed from the stock with a sterile scalpel blade whilst 1, 2, or 4 leaves were left remaining on individual scions. Two autografts were assembled per separate leaf number examination as outlined in Table 2.9.7.2 and cultured conditions as previously described.

### 2.9.7.3 Assesment of of the effect of leaves on stock on graft development

Scion and stocks were isolated from a seedling derived *Q. petraea* clone (JP3). All leaf material was removed from the scion with a scalpel whilst two leaves where left remaining on separate stocks. Ten autografts were assembled and cultured under conditions as previously described.

### 2.9.7.4 Examination of potential for hetrografting juvenile *Quercus robur* and juvenile *Quercus petraea* clone

Initial studies were carried out to examine the feasibility of grafting *Q. robur* clones to *Q. petraea* clones. Scion and stocks were isolated from a seedling derived *Q. robur* (JR4) and *Q. petraea* clone (JP3). All except two leaves were removed with a sterile from the scion and stock respectively. Heterografts were assembled with varying bud numbers on scion and stocks, numbers not being recorded.

| Scion bud number | Stock bud number |
|---|---|
| 1 | 1 |
| 1 | 2 |
| 1 | 3 |
| 2 | 1 |
| 2 | 2 |
| 2 | 3 |
| 3 | 1 |
| 3 | 2 |
| 3 | 3 |

**Table 2.9.7.1 Set up of scion and stock bud numbers**

| Scion leaf number | Stock leaf number |
|---|---|
| 1 | 0 |
| 2 | 0 |
| 3 | 0 |
| 4 | 0 |

**Table 2.9.7.2 Set up of Leaf numbers on scion and stock**

Five hetrografts consisting of *Q. robur* scions and *Q. petraea* stocks were assembled whilst five heterografts consisting of *Q. petraea* scions and *Q. robur* stocks were assembled. All grafts were cultured under conditions as previously described.

#### 2.9.7.5 Examination of potential for heterografting of mature *Q. petraea* clone to juvenile *Q. petraea* clone

Scion material was isolated from clone MP2 and stocks were isolated from juvenile clone JP3 and prepared for grafting as previously described. Both scions and stocks had two leaves each. Scion and stock bud numbers were not recorded. Ten heterografts were assembled, supported on graft media Cb1 and cultured for 28 days under conditions as previously described.

#### 2.9.7.6 Grafting of cultures derived from epicormic shoots of mature *Q. robur* tree to seedling derived juvenile *Q. robur* clones

Scions were isolated from clones obtained from the epicormic shoots growing from sections 6, 11, and 15 of the branch from the Phoenix Park *Q. robur* tree. Stocks were isolated from a seedling derived *Q. robur* clone (JR4). Due to large leaf size evident on scion material all leaves were excised except for leaf whorl at tip of scion. Stocks contained two leaves. In total five homografts of clones from section 11, four homografts consisting of clones from section 6 and one homograft of clones from section 15, grafted to clone JR4 were assembled. Graft media and culture conditions were as previously described.

### 2.9.8 Optimising the grafting of mature *Quercus* clones to juvenile *Quercus.* clones

Grafting of *Quercus* clones was further optimized by examining the effect of foliage presence on mature and juvenile stock and scion and the positioning of juvenile and mature material within the graft assembly, following an adapted

method from a study of Doorenbos(1953). A clone (MP2) derived from a mature flowering *Q. petraea* tree acted as the source for mature material with a seedling derived *Q. petraea* clone (JP3) being used as juvenile material. Grafts were assembled using either juvenile scions and mature stocks, or vice versa, that were with or without leaf material as outlined as in Table 2.9.8.1. Where necessary, leaf material was excised with a sterile scalpel blade. Scions and Stocks that contained leaf material contained only two leaves. Due to constraints on mature material only four separate graft combinations could be set up. Five homografts were assembled per combination and cultured under conditions as previously described.

### 2.9.9 Grafting of Mature *Q. petraea* clone to *in vitro* rooted Juvenile *Q. petraea* clone

Scion material was isolated from a *Q. petraea* clone derived from a mature flowering tree (MP2). A seedling derived *Q. petraea* clone (JP3) acted as source for juvenile rootstocks and were isolated from a 28 day old culture of an *in vitro* rooted clones. Using a sterile scalpel blade rootstocks were prepared that consisted of approximately 1 cm of lower explant with root material attached. To facilitate graft assembly and support, scions contained leaf whorls only , stocks were defoliated and root stocks were trimmed to approximately 2cm in length. Due to constraints on mature material availability, only two grafts could be assembled for this combination of mature scion and juvenile rootstock. Two control grafts were also established consisting of scions (isolated from the mature clone MP2) with leaf whorls still remaining and unrooted defoliated stocks isolated from seedling clone JP3. All grafts were placed between the two agar surfaces of a culture dish as previously described. Apical media consisted of apical media from graft medium combination Cb1 whilst basal media consisted of WPM3 without added hormones. Grafts were cultured under conditions as previously described.

| Plate | Stock | Scion |
|---|---|---|
| 1 | M +L | J + L |
| 2 | M - L | J + L |
| 3 | J + L | M - L |
| 4 | J - L | M - L |

**Key**

**M + L** **Mature Leafy**

**M - L** **Mature Leafless**

**J + L** **Juvenile Leafy**

**J - L** **Juvenile Leafless**

**Table 2.9.8.1 Set up of graft combinations to investigate foliage presence and position of mature explant in mature to juvenile grafts**

### 2.9.10 Serial grafting of mature *Q. petraea* material to juvenile *Q. petraea* material

A mature clone (MP2) derived form a mature flowering *Q. petraea* tree was previously used in a study to assess the effect of the presence of foliage on mature to

juvenile graft assembly. The successful grafts from this study were isolated and shoots that had developed from mature material were grafted to juvenile material isolated from a seedling derived clone (JP3). In total ten homografts were placed into culture. Five grafts were assembled that consisted of Juvenile scions with two leaves grafted to Mature stocks absent of leaves. Five grafts were assembled that consisted of Mature scions with two leaves grafted to Juvenile Stocks with two leaves. Culture and media conditions were as previously described. After 28 days results were recorded.

### 2.9.11 Cascade grafting of material isolated from epicormic shoots derived from a mature *Q. robur* tree to juvenile *Q. robur* clone

Scions were isolated from cultures obtained from the branch sections 6, 7, 11, 13, 15 and 23 from the Phoenix park *Q. robur* tree. Stocks were isolated from a *Q. robur* clone (JR5) derived from stump sprouts. Leafy mature scions containing two leaves were grafted to juvenile leafy stocks containing two leaves. The following mature clones were grafted to the seedling derived juvenile clone: Four homografts of the clones from branch section 6; Five homografts of the clones from branch section 7; Five homografts of the clones from branch section 11; One homograft of the clones from branch section 13; Four homografts of the clones from branch section 15; Three homografts of the clones from branch section 23. Graft media and culture conditions were as previously described.

After 28 days result were recorded and successful grafts, free from contamination, were removed (Homografts of clones from branch sections 7 and 11) and set aside for regrafting. Shoots that grew from scions were excised, trimmed of

all but two leaves and re-grafted to juvenile stocks, containing two leaves, sourced from the seedling derived clone. Graft media combinations and culture conditions were as previously described.

To compare the effect of serial grafting versus single graft clones isolated form epicormic shoots on branch section 7 and 11, a second series of grafts were set up. Mature leafy scions were grafted to juvenile leafy stocks. A seedling derived *Q. robur* clone (JR5) acted as the source for juvenile clones. Five homografts from clones of bracnh section7 and ten homografts from branch section 11 were assembled. Culture and media conditions were as previously described.

### 2.9.12 Examination to see effect of grafting of mature material on rooting ability

After 28 days grafted material from above was examined. Successful grafts free from contamination were removed to sterile Petri dishes and shoots growing from mature matrial were excised. Shoot tips approximately 15 mm long were excised and excess leaves removed. The tips were then subjected to rooting treatment and cultured as for rooting protocol. In total three shoots of regrafted clones from branch section 7 and three shoots from regrafted clones of branch sections 11 were suitable for use. After 28 days the cultures were examined for rooting success.

### 2.9.13 Grafting of a mature *Q. robur* clone MR1 to juvenile *Q. robur* clone JR5

To examine if grafting of mature *Q. robur* to juvenile *Q. robur* was feasible and if leaf presence would influence graft development, a range of mature to juvenile grafts were assembled. Mature material was isolated from a *Q. robur* clone MR1 derived form a mature flowering tree. Juvenile material was isolated from a seedling derived *Q. robur* clone (JR5). Where necessary leaf material was excised with a sterile scalpel blade. Grafts were assembled using either juvenile scions and stocks or mature scions and stocks with or without leaf material as outlined as in Table 2.9.13.1. Due to severe constraints on mature material only five graft

| Graft Combination | Scion | Stock |
|---|---|---|
| 1 | J-L | M+L |
| 2 | J+L | M-L |
| 3 | M+L | M+L |
| 4 | M+L | J-L |
| 5 | M-L | J+L |

Key:

**M + L** **Mature Leafy**

**M - L** **Mature Leafless**

**J + L** **Juvenile Leafy**

**J - L** **Juvenile Leafless**

**Table 2.9.13.1 set up of grafting of mature *Q. robur* to juvenile *Q. robur***

combinations could be set up. One homograft was assembled per combination, and graft media and culture conditions were as previously described.

### 2.9.14 Grafting of Mature *Q. petraea* clone MP2 to juvenile clone JP3

Mature *Q. petraea* clones were grafted to juvenile *Q. petraea* clones according to combination which yielded best graft development as in section 2.9.10. Where necessary leaf material was excised with a sterile scalpel blade. Mature material was isolated from a *Q. petraea* clone derived from a mature flowering tree (MP2) and juvenile material was isolated from a seedling derived clone (JP3). Grafts were assembled as outlined as in Table 2.9.14.1. Due to severe constraints on mature material four homografts were assembled for combination 1, four homografts were assembled for combination 2 and two control autografts were assembled as in combination 3. Graft media and culture conditions were as previously described.

| *Graft Combination* | *Scion* | *Stock* |
|---|---|---|
| 1 | M+L | J+L |
| 2 | J+L | M-L |
| 3 | M+L | M+L |

**Key:**

**M + L** **Mature Leafy**

**M - L** **Mature Leafless**

**J + L** **Juvenile Leafy**

**Table 2.9.14.1: Set up of grafting of mature *Q. petraea* to juvenile *Q. petraea***

### 2.9.15 Clearing of Grafts

Successful grafts were cleared according to the method of Fushs (1963), to elicit if xylem vessels had connected across the graft union and therefore act as a transport mechanism for water.

#### 2.9.15.1 Preparation of clearing solution

Basic fuschin (Pararosanilin)(C.I. No. 42500) was added to distilled water at 80 $^{o}$C to make a 1% solution (w/v). Potassium hydroxide pellets were then slowly added to give an overall concentration of 6% (w/v). This solution was then filtered through two layers of whatman No.1 filter paper and stored in the dark at 4 $^{o}$C until required.

#### 2.9.15.2 Clearing Procedure

Successfully grafted explants were removed from the silicone tubing and the plant stem was trimmed to 5 mm on either side of the graft union. The graft was very carefully cut in half lengthwise. The two halves of the graft were then placed in a test-tube with 10 ml of clearing solution and incubated overnight at 60 $^{o}$C. The clearing solution was then replaced by 50% ethanol and left for 10 minutes. This step was repeated four times. The 50% ethanol was then replaced by 10 ml of 70% ethanol and left for ten minutes. This step was repeated five times. 5 ml of concentrated Hydrochloric acid were added to the last 10 ml of 70% ethanol. The graft pieces were left in this mixture for 30-90 seconds or until the vascular tissue had turned purple. The acid/alcohol solution was replaced by 10 ml of 70% ethanol and left for 10 minutes. This step was repeated once.

The 70% ethanol was replaced by 10 ml of absolute ethanol and left for thirty minutes. This step was repeated every 30 minutes for six hours. The graft pieces were then left in absolute ethanol overnight.

The absolute ethanol was replaced by 10 ml of xylene for 60 minutes. The xylene was replaced by a further 10 ml of xylene and the graft pieces were left overnight.

The graft pieces were removed from the xylene, placed on a microscope slide and flattened by covering with a coverslip and applying pressure. Surplus xylene was removed. The graft pieces were mounted in immersion oil (temporary mount) , then covered with a cover-slip and viewed under a microscope.

## 2.10 Statistical analysis

All statistical analysis was carried out using SPSS ® for Windows ™ (SPSS inc., Chicago, Illinois.). One way analysis of variance (ANOVA) was carried out on shoot cultures of juvenile and mature origin and for results on grafting studies. For discriminant analysis, the shoots were divided into those of seedling and stump sprouts origin, and those derived from mature, flowering trees, excluding shoots derived from 20-25 year old trees, and those of the severely hedged stock plant. All factors (shoot angle, mid, base and tip stem diameter, number of green leaves, number of scale leaves, number of shoots, and shoot length) were included and the analysis was carried out on the data using stepwise selection of factors using Wilks' Lambda.

# Chapter 3

## Results

### 3.1 Examination of Multiplication rates of *in vitro* grown shoot cultures

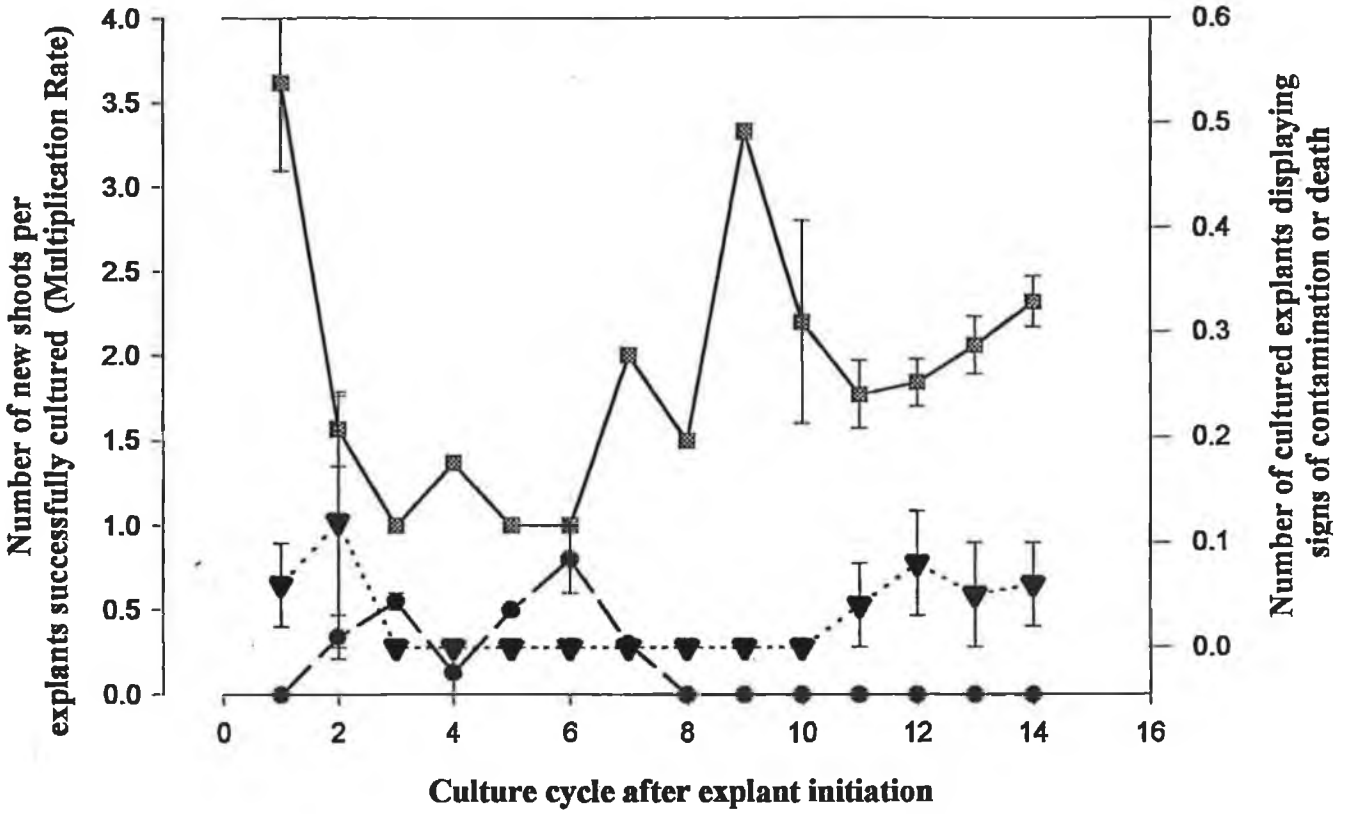
Several authors have argued that material of juvenile status is more amenable to tissue culture, less subject to contamination and displays higher shoot vigour than material diplaying mature status. The data on culture rates, contamination rates and death rates of clones that was monitered in establishing *in vitro* grown cultures of *Quercus* was examined to assess this hypothesis and establish useful criteria that may distiguish between juvenile amd mature material. Multiplication rates were observed for selected *Q. robur* and *Q. petraea* clones. Mean multiplication rates obtained over varying culture cycles are shown in Table 3.1.1. Juvenile clones tend to have a higher multiplication rate than mature clones. There was a tendency for there to be a higher number of shoots per explant in the seedling and stump sprout derived shoots than in the shoots derived from mature and adolescent trees. What is evident is that multiplication rates for clones derived from both mature and juvenile material tended to be highest in the subcultures immediately following explant initiation and then tended to decrease to a relatively stable value. However there is a high variability in the multiplication rate both within a clone and between clones of a similar maturity state as shown in Figures 3.1.1 - 3.1.9. Two of the seedling derived *Q. petraea* clones had low mean multiplication rates consistent with clones derived from mature trees.

Propagation rates were generally low, displaying within clonal varaibilty and did not provide a suitable means for discriminating between explants from juvenile or mature origin. Hence it was clear that other physiological means were required to distinguish between such clones.

| Species | Clone | Source | Mean number of explants per explant subcultured | Number of subculture cycles | Total number of shoots recorded |
|---|---|---|---|---|---|
| ***Q. robur*** | JR2 | Seedling | 2.05 ±0.13 | 14 | 54 |
| | JR4 | Seedling | 1.92 ±0.05 | 14 | 127 |
| | JR5 | Stump sprouts | 2.21 ±0.11 | 14 | 132 |
| | MR2 | Hedged stockplant | 1.68 ±0.19 | 7 | 26 |
| ***Q. petraea*** | JP1 | Seedling | 1.69 ±0.06 | 12 | 166 |
| | JP2 | Seedling | 1.74 ±0.24 | 9 | 43 |
| | JP3 | Seedling | 2.34 ±0.12 | 7 | 39 |
| | AP1 | Adolescent | 1.49 ±0.1 | 13 | 50 |
| | MP3 | Mature flowering | 1.67 ±0.14 | 12 | 40 |

**Table 3.1.1 Mean propagation rates of *Quercus* clones over a range of subculture cycles**

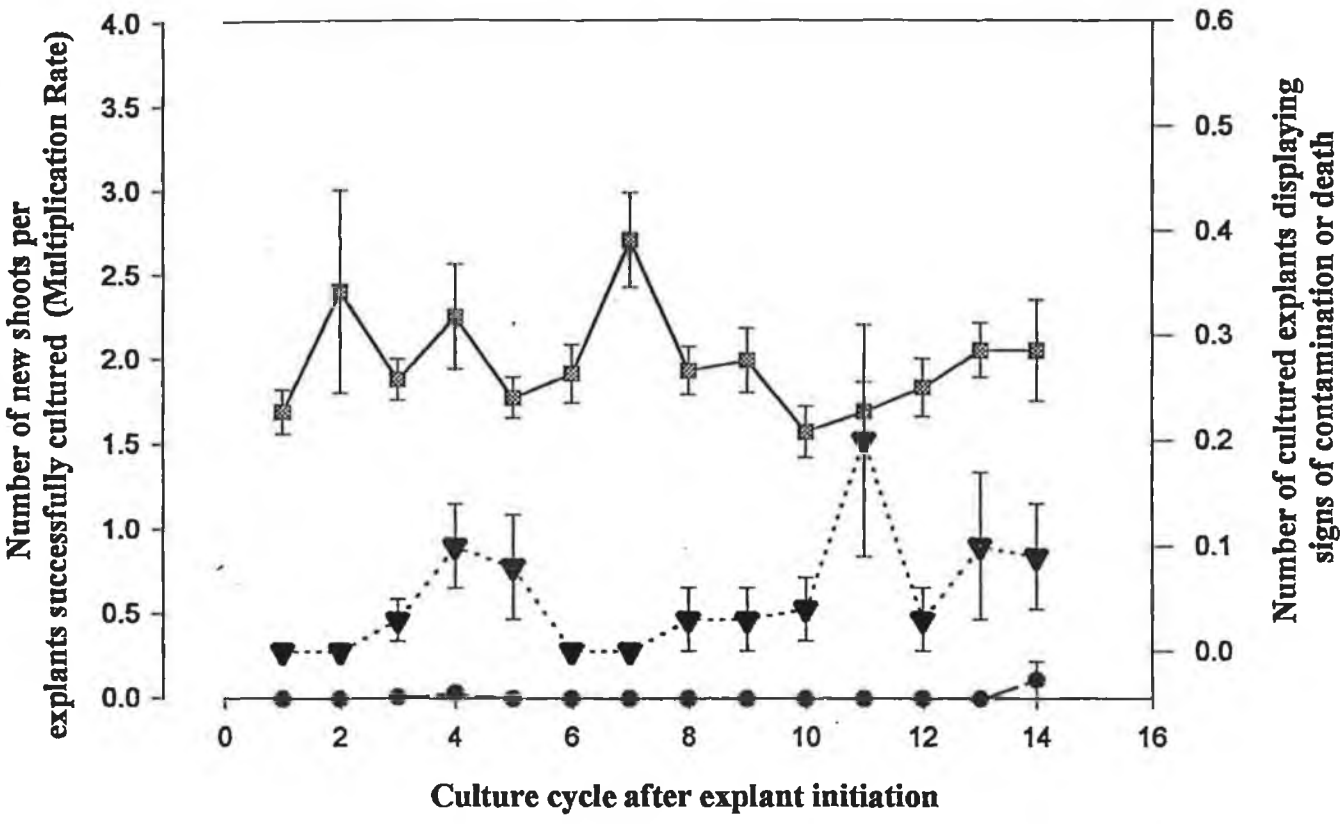
**Multiplication, Death and Contamination Rates for a seedling derived *Q.robur* clone (JR2)**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.1**

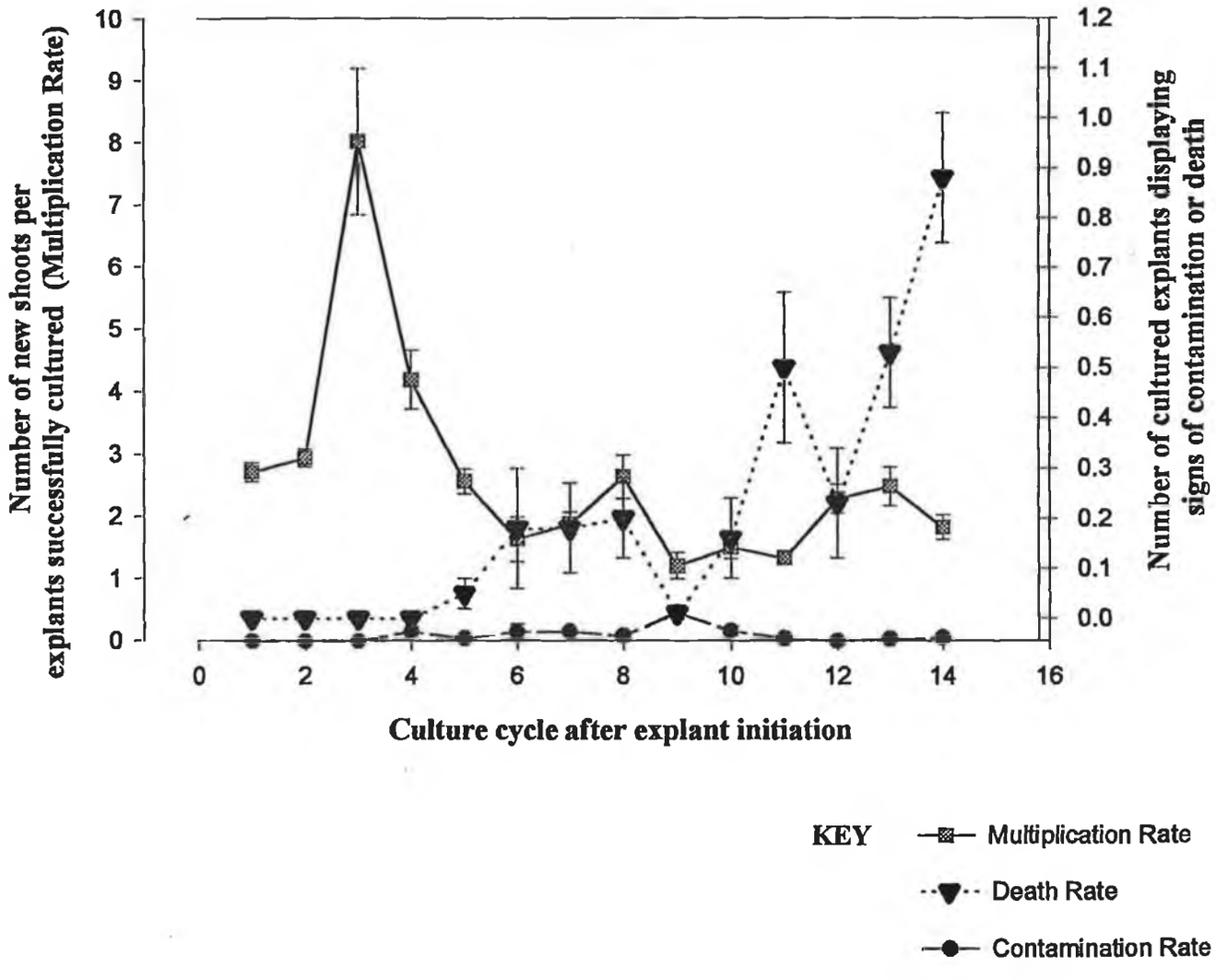
**Multiplication, Death and Contamination Rates for a seedling derived *Q.robur* clone (JR4)**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.2**

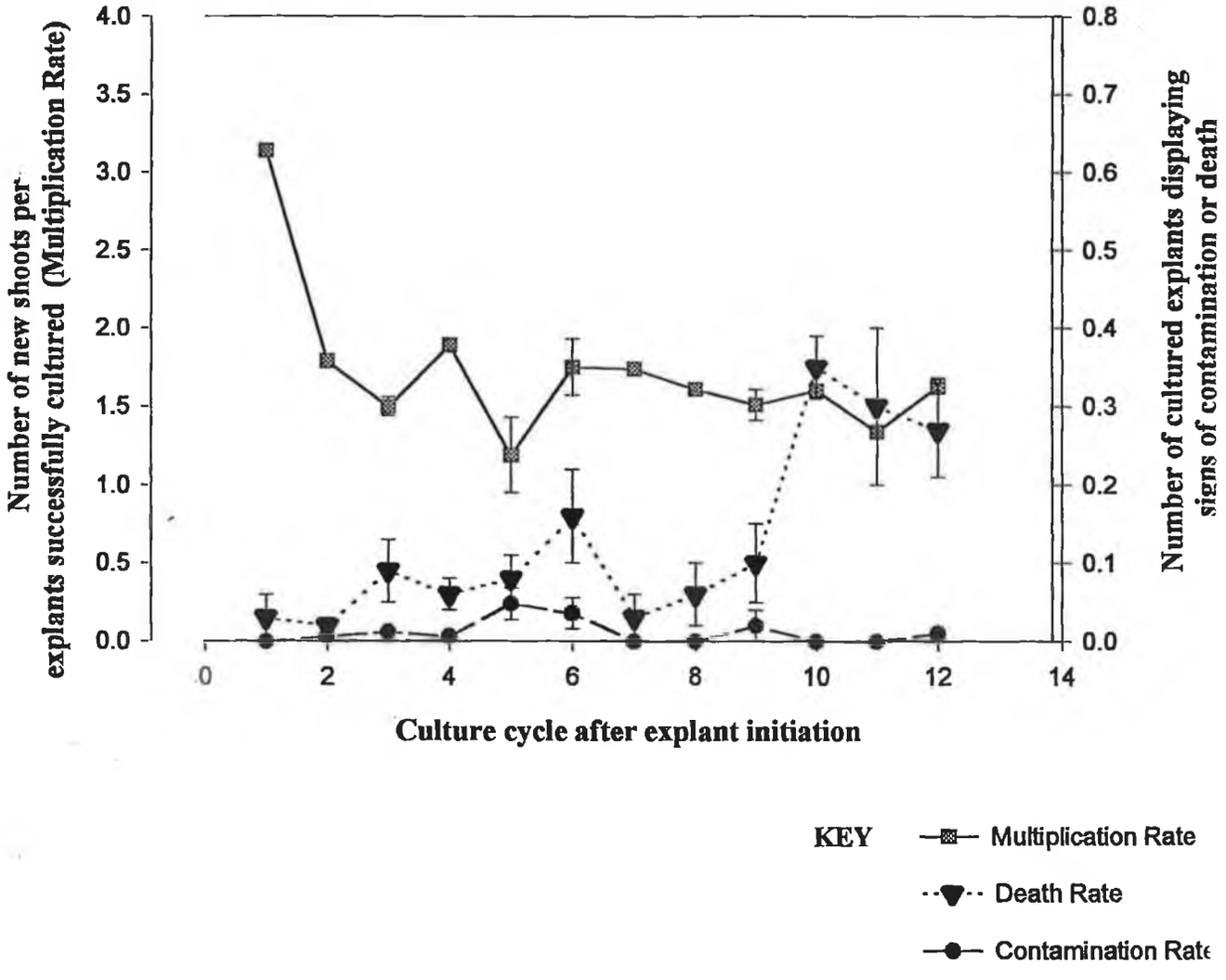
**Multiplication, Death and Contamination Rates for a *Q.robur* clone (JR5) derived from stump sprouts**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.3**

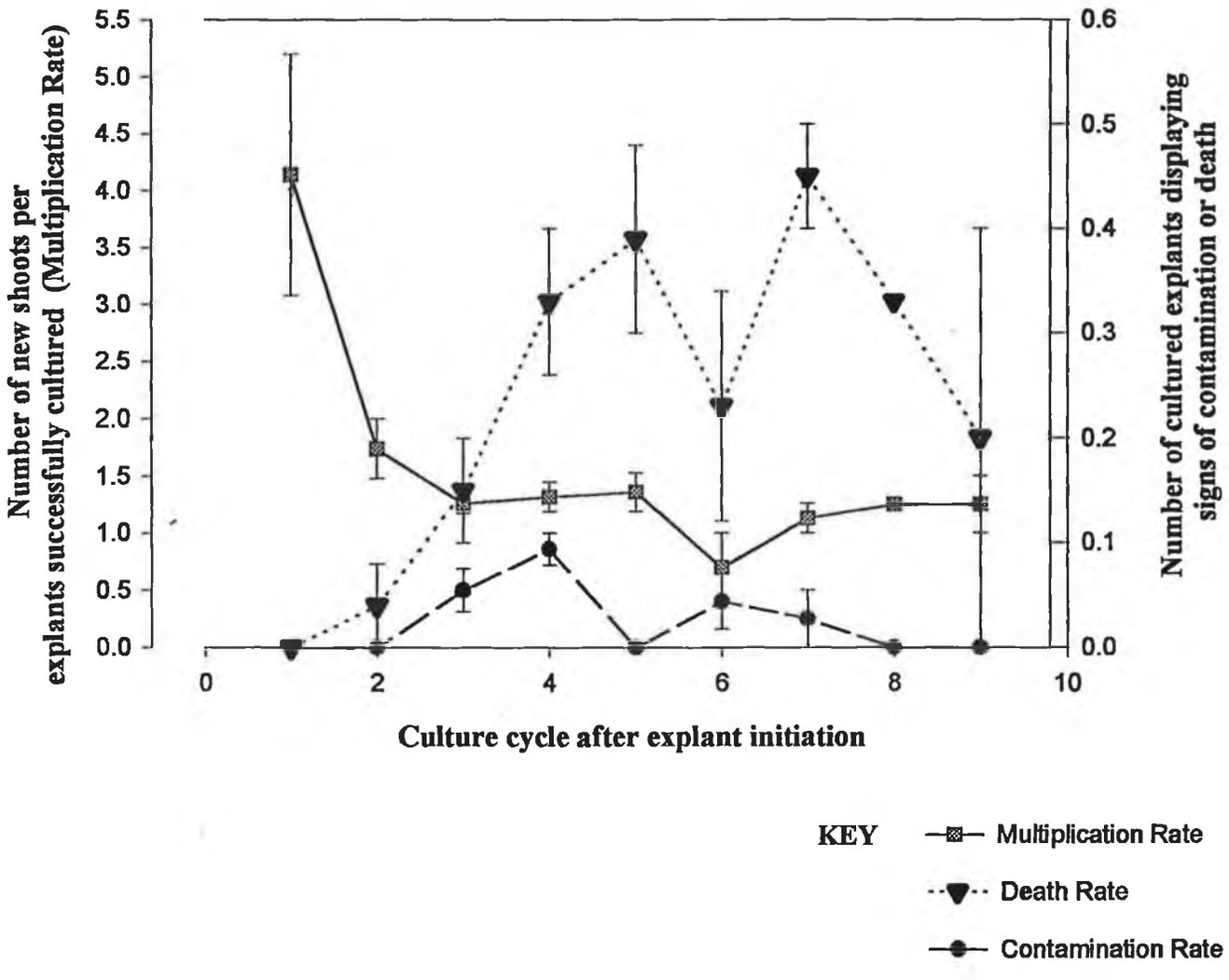
**Multiplication, Death and Contamination Rates for a seedling derived *Q.petraea* clone (JP1)**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.4**

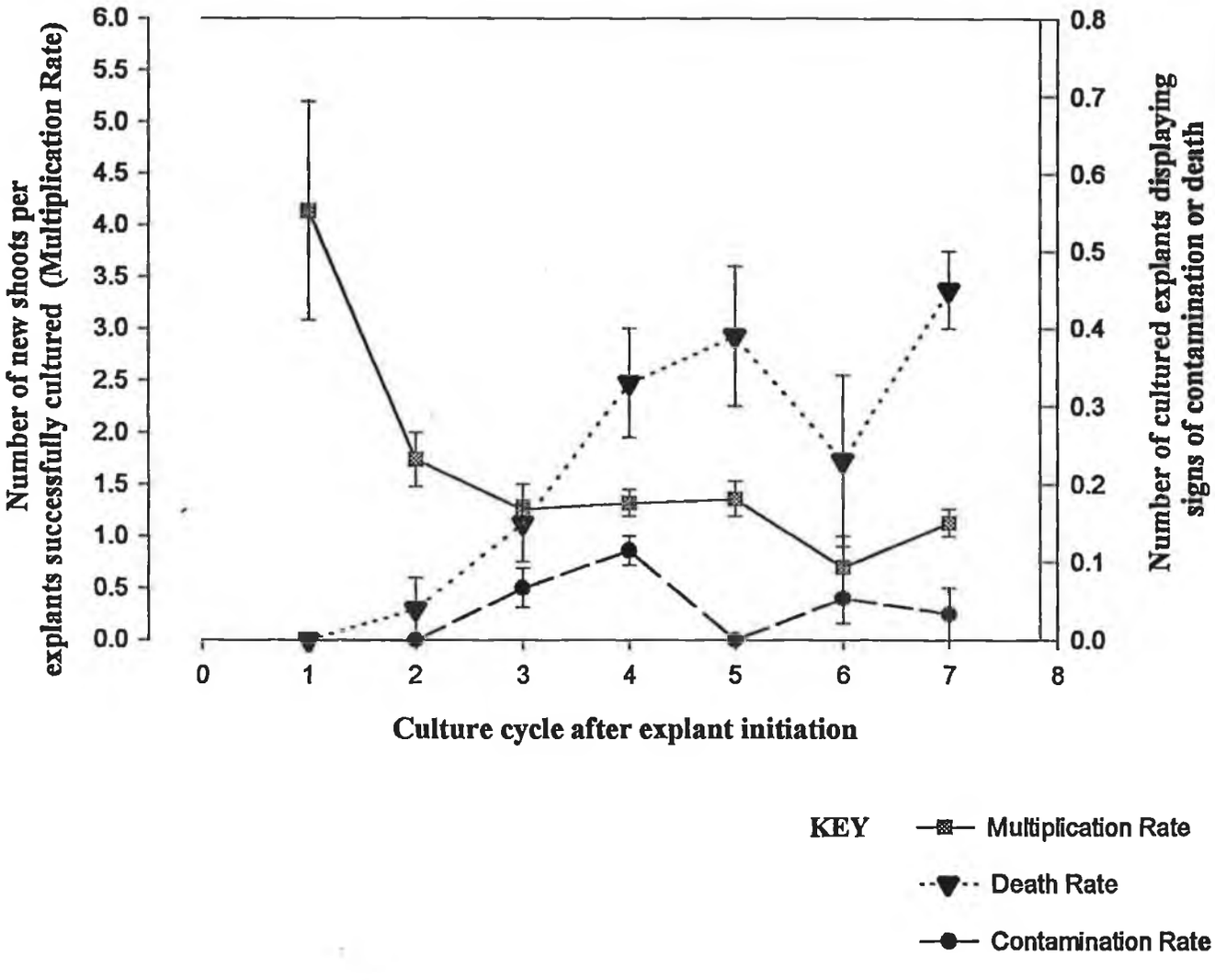
**Multiplication, Death and Contamination Rates for a seedling derived *Q.petraea* clone (JP2)**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.5**

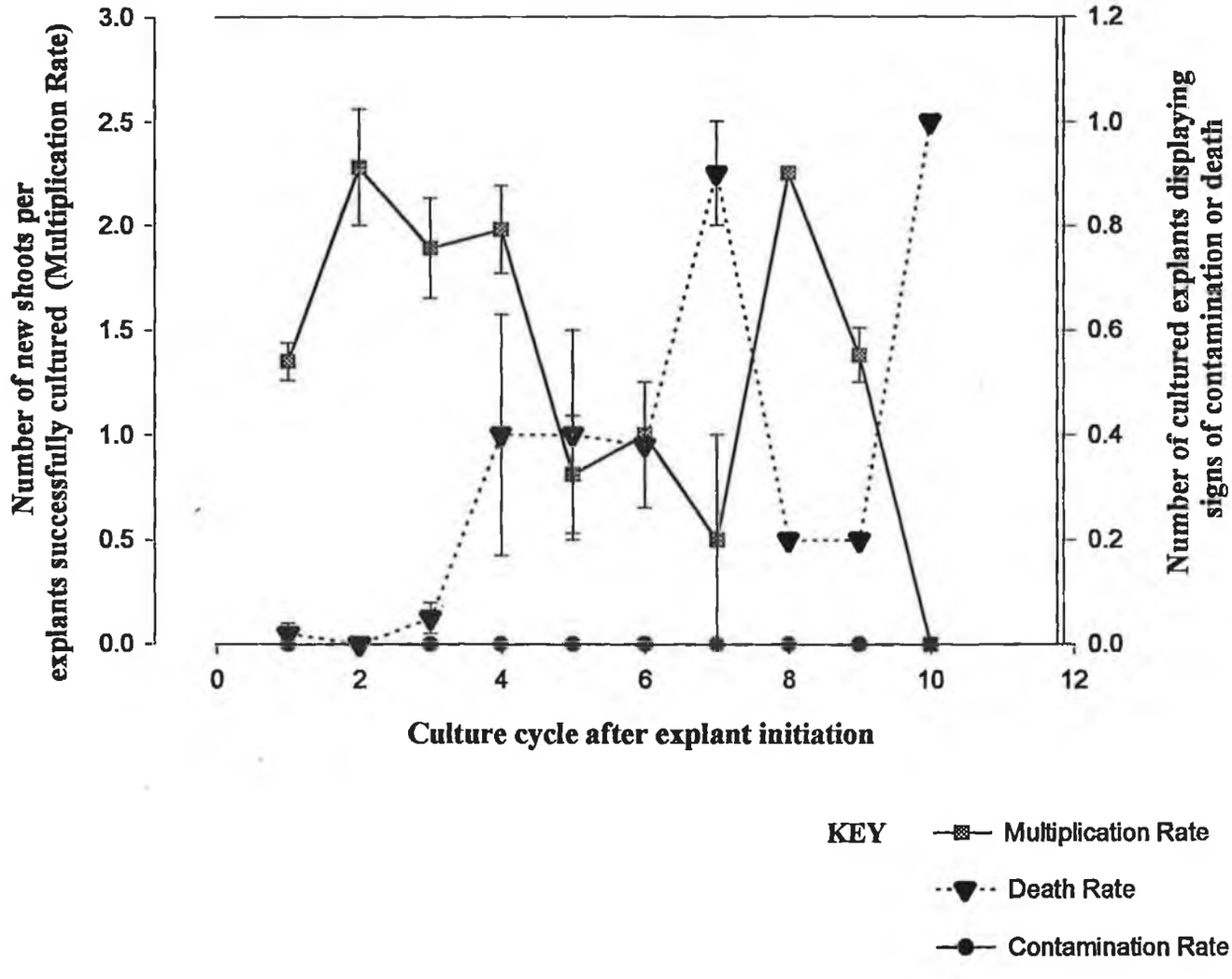
**Multiplication, Death and Contamination Rates for a seedling derived *Q.petraea* clone (JP3)**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.6**

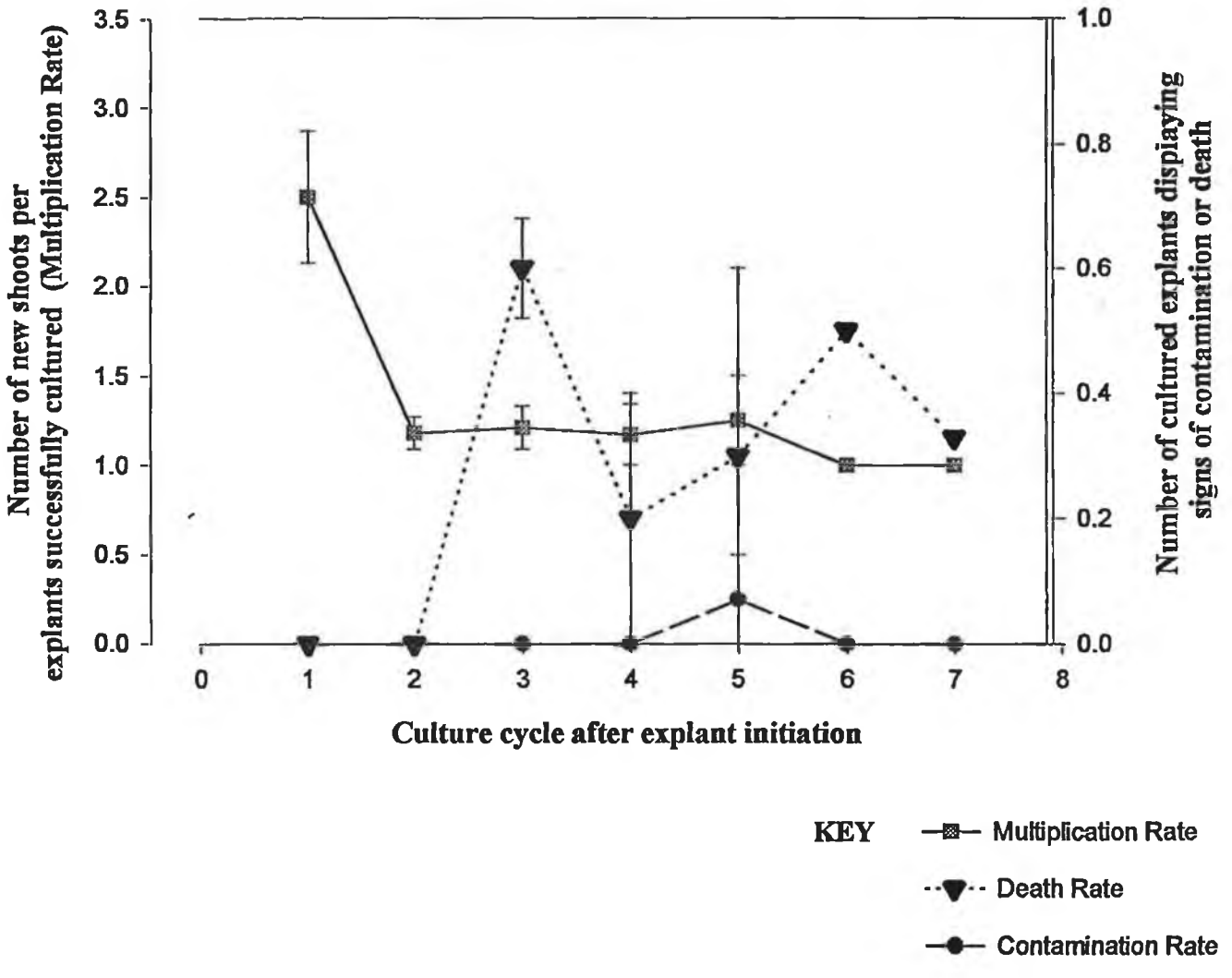
**Multiplication, Death and Contamination Rates for a *Q.petraea* clone (AP1) derived from an Adolescent tree**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.7**

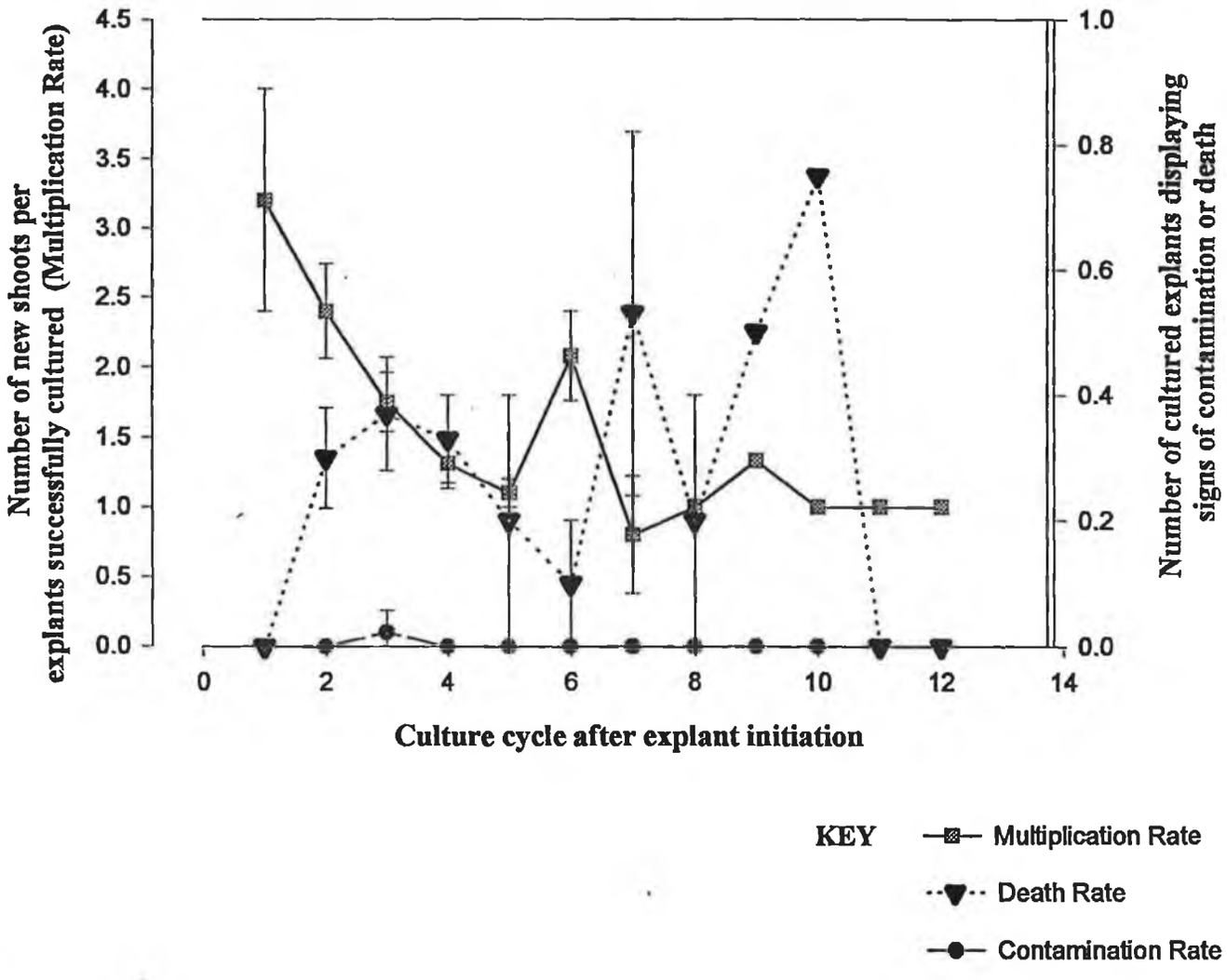
**Multiplication, Death and Contamination Rates for a *Q.robur* clone (MR2) derived from adult budwood of a pruned hedged plant**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.8**

**Multiplication, Death and Contamination Rates for a *Q.petraea* clone (MP3) derived from a Mature Flowering Tree**




**Legend**
**All values are the mean of at least 5 replicates and are shown with the standard error of the mean**

**Figure 3.1.9**

### 3.2 Development of rooting protocol

In the previous experiments it was demonstrated that multiplication rates do not provide a suitable means to differentiate between shoots of juvenile or mature origin. It was therefore necessary to examine another physiological character as a possible marker of juvenile and mature status. Potential to form roots has been used in several woody plants as a marker for juvenility/ maturity (Hess, 1959; Pliego Alfaro and Murashige,1987; Huang *et al.*, 1992a).

*In vitro* grown shoots of *Quercus* have been successfully rooted (Vieitez *et al.*, 1985; Manzanera and Pardos, 1990) and this trait may offer a viable method to determine the juvenile or mature status of *Quercus* clones.

After 21 days rooting success, root number, shoot tip necrosis, and callus formation at base of shoot were recorded. The percentage of rooting success, shown in Table 3.2.1, was greatest at 70% for treatments of a 300 $mgl^{-1}$ IBA dip cultured on 20 $gl^{-1}$ Sucrose and for 1000 $mgl^{-1}$ IBA dip cultured on 50 $gl^{-1}$ Sucrose. Number of roots which grew from each treated shoot tip was low except for the treatment which showed the highest rooting % (Table 3.2.2). Only two treatments yielded a low percentage shoot tip necrosis as shown in Table 3.2.3. Treatment of a 30 $mgl^{-1}$ IBA dip cultured on 20 $gl^{-1}$ sucrose and a 100 $mgl^{-1}$ IBA dip cultured on 50 $gl^{-1}$ sucrose. Shoots rooted under the following conditions: dip in IBA concentration of 100 $mgl^{-1}$, cultured on 40 $gl^{-1}$ sucrose; dip in IBA concentration of 300 $mgl^{-1}$, cultured on 30 $gl^{-1}$ sucrose; dip in IBA concentration of 1000 $mgl^{-1}$, cultured on 30 $gl^{-1}$ or 50 $gl^{-1}$ sucrose root length was less than 1 mm and proved extremely difficult to record. For those roots that were greater than 1 mm the length of root was recorded. Average root lengths suggest a treatment of 300 $mgl^{-1}$ IBA cultured on 20 $gl^{-1}$ sucrose gave rise to roots of greatest length (Table 3.2.4). It is evident that juvenile clones can be rooted. Rooting percentage tends to be highest at higher levels of IBA but independent of sucrose concentration. Shoot tip necrosis is a major problem leading to the deaths of many clones.

| IBA Concentration $mgl^{-1}$ | Sucrose Concentration $gl^{-1}$ | | | |
|---|---|---|---|---|
| | *20* | *30* | *40* | *50* |
| 30 | 10 | 0 | 0 | 0 |
| 100 | 10 | 0 | 20 | 0 |
| 300 | 70 | 20 | 0 | 30 |
| 1000 | 0 | 20 | 0 | 70 |

**Table 3.2.1 % Rooting in shoot tip cultures after 21 days following a 2 minute dip in various IBA concentrations and culturing on hormone free WPM with a varying range of sucrose concentrations**

| IBA Concentration $mgl^{-1}$ | Sucrose Concentration $gl^{-1}$ | | | |
|---|---|---|---|---|
| | *20* | *30* | *40* | *50* |
| 30 | *1.0 ± 0.10* | *0.0 ± 0.00* | *0.0 ± 0.00* | *0.0 ± 0.00* |
| 100 | *1.0 ± 0.10* | *0.0 ± 0.00* | *0.2 ± 0.13* | *0.0 ± 0.00* |
| 300 | *1.5 ± 0.40* | *0.3 ± 0.21* | *0.0 ± 0.00* | *0.5 ± 0.26* |
| 1000 | *0.0 ± 0.00* | *0.2 ± 0.13* | *0.0 ± 0.00* | *2.2 ± 0.60* |

**Table 3.2.2 Average number of roots emanating from shoot tip cultures after 21 days following a 2 minute dip in various IBA concentrations and culturing on hormone free WPM with a varying range of sucrose concentrations. All values are the mean of 10 replicates and are shown with the standard error of the mean**

| IBA Concentration $mgl^{-1}$ | Sucrose Concentration $gl^{-1}$ | | | |
|---|---|---|---|---|
| | *20* | *30* | *40* | *50* |
| 30 | 10 | 70 | 40 | 40 |
| 100 | 30 | 80 | 20 | 10 |
| 300 | 30 | 80 | 0 | 30 |
| 1000 | 60 | 50 | 40 | 20 |

**Table 3.2.3 Percentage necrosis of shoot tip cultures after 21 days following a 2 minute dip in various IBA concentrations and culturing on hormone free WPM with a varying range of sucrose concentrations**

| Rooting treatment | | | Average length of roots per rooted shoot (mm) |
|---|---|---|---|
| *IBA concentration* $mgl^{-1}$ | *Sucrose Concentration* $gl^{-1}$ | *Number of roots recorded* | |
| 30 | 20 | 1 | 10.62 ± 0.00 |
| 100 | 20 | 1 | 6.93 ± 0.00 |
| 300 | 20 | 15 | 8.94 ± 1.12 |
| 300 | 50 | 5 | 7.45 ± 2.41 |

**Table 3.2.4 Average root length per rooted shoot after 21 days culture following a 2 minute dip in various IBA concentrations and culturing on hormone free WPM with a varying range of sucrose concentrations**

### 3.2.1 Rooting of Juvenile and Mature *Quercus* clones

In order to assess if rooting ability was a suitable critieria to distinguish *Quercus* clones of juvenile and mature origin the established rooting protocol was applied to several *Quercus* clones of juvenile and mature status. Clonal variability affecting the rooting bioassay was also examined by subjecting shoots to rooting treatment at differing time periods. Results of rooting, displayed in Table 3.2.1.1, suggest that there are differences between juvenile and mature *Q. petraea* clones in ability to root under an applied treatment. It appears that mature material does not root. Although one seedling clone showed an ability to form roots there was a wide variation in rooting percentage between rooting treatments applied to shoots at differing time periods suggesting a within clonal variabilty.

Variation between clones in rooting ability was evident in juvenile *Q. robur* clones isolated from seedlings and stump sprouts and shoot tip necrosis of all shoots was high for all clones contributing to the death of many clones in this rooting study. The results of rooting studies suggest that rooting is an unreliable and unsuitable marker for the phase status of the donor clone. Furthermore it results in the destruction of clonal material. What is required is the use of clearly distinguishable and highly quantitative characteristics that are non destructive of valuable mature and juvenile material.

| Origin of Clone | Treatment | Rooting % | Shoot Tip Necrosis % |
|---|---|---|---|
| ***Q. robur*** | | | |
| *Stump sprouts* | 1 | 10 | -- |
| *Seedling* | 1 | 40 | 100 |
| | 2 | 10 | 80 |
| *Seedling* | 1 | 45 | -- |
| | 2 | 10 | 90 |
| | 3 | 40 | 80 |
| *Stump sprouts* | 1 | 70 | 30 |
| | 22 | 22 | 22 |
| ***Q.petreaa*** | | | |
| *Seedling* | 1 | 0 | -- |
| | 2 | 0 | 100 |
| *Seedling* | 1 | 33 | 66 |
| | 2 | 70 | 0 |
| *Mature flowering* | 1 | 0 | 100 |
| *Mature flowering* | 1 | 0 | 100 |

**Table 3.2.1.1 Rooting and Shoot tip percentages of selected clones over varying periods of time**

### 3.3 Morphological examination of *in vitro* grown shoot cultures

The physiological criteria examined above proved to be unsuitable markers to differentiate between shoots of juvenile and mature status. Morphological characteristics of plant material such as stem diameter, leaf morphology (Lyrene, 1981; Brand and Lineberger, 1992) and orthotropic or plagiotropic growth (Arnauld *et al.*, 1993) have been used to describe the maturity/juvenility of shoots in many species. A range of morphological criteria were evaluated, in shoot cultures from plants of proven juvenile, adolescent (20-25 yrs) and mature status to assess their value as indicators of juvenility or maturity, the objective being to characterise mature and juvenile *in vitro* morphotypes and determine if intermediate forms could be characterised using material of known chronological ages.

The morphological characteristics of shoot cultures of seedling, stump sprout, mature and hedged stockplant clones of *Q. robur* are summarised in Table 3.3.1. Results for seedling, adolescent and mature clones of *Q. petraea* are summarised in Table 3.3.2 respectively. For *Q. robur*, the characteristics of two clones derived from stump sprouts were similar to three clones from juvenile seedlings and these were distinguishable from cultures derived from mature trees and hedged stock plants by angle of shoot growth and stem tip and mid - stem diameter. Similarly two seedling clones of *Q. petraea* were distinguishable from three mature clones by these morphological traits. Cultures derived from a 20 - 25 year old (adolescent) tree of *Q. petraea* had greater similarities to the mature clones of their species than to juvenile material. The cultures could therefore be grouped according to the maturity of the initial explants. A comparison of all cultures with juvenile characteristics (five *Q. robur* and two *Q. petraea*) with those showing mature characteristics (two *Q. robur* and four *Q. petraea*) revealed that angle of shoot growth to the horizontal was greater for juvenile clones than for mature or adolescent cultures. Similarly, the tip diameter and mid-stem diameter were also smaller for the juvenile clones than for the mature or adolescent clones with the exception of one *Q. petraea* clone. Number of green leaves per shoot, number of scale leaves per shoot, base stem diameter and shoot length were highly variable between clones for both oak species and there were no striking differences for these

| Origin of Clone | Shoot Angle to horizontal | Green leaves per Shoot | Scale leaves per shoot | Shoot Tip Diamete (mm) | Mid-Stem Diameter (mm) | Stem Base Diameter (mm) | Shoots per explant | Shoot Length (mm) |
|---|---|---|---|---|---|---|---|---|
| *Seedling* | 66.77 ±5.99 | 5.00 ± 0.62 | 1.56 ± 0.75 | 0.66 ±0.04 | 0.74 ±0.05 | 1.11 ± 0.07 | 1.8 ±0.37 | 26.47 ± 3.00 |
| | 62.28 ± 4.15 | 6.45 ± 1.21 | 2.73 ± 0.37 | 0.78 ± 0.11 | 0.79 ± 0.12 | 1.16 ± 0.15 | 2.0 ±0.36 | 22.64 ± 5.21 |
| | 66.25 ± 3.21 | 6.88 ± 1.17 | 1.19 ± 0.34 | 0.79 ± 0.08 | 0.69 ± 0.07 | 0.99 ± 0.07 | 2.83 ±0.47 | 15.06 ± 2.76 |
| *Stump sprouts* | 68.50 ± 7.79 | 6.29 ± 1.35 | 0.29 ± 0.29 | 0.72 ± 0.04 | 0.66 ± 0.04 | 0.82 ±0.05 | 1.60 ±0.24 | 22.05 ± 5.79 |
| | 75.0 ± 3.16 | 8.43 ± 2.08 | 1.57 ± 1.07 | 0.77 ± 0.10 | 0.71 ± 0.07 | 1.05 ± 0.10 | 1.75 ± 0.75 | 23.75 ± 5.81 |
| *Mature Flowering* | 55.0 ± 7.42 | 8.6 ±3.07 | 0.00 ±0.0 | 1.12 ±-0.54 | 1.12 ±0.22 | 1.15 ±0.19 | 1.25 ±0.25 | 21.21 ± 4.60 |
| *Hedged stockplants* | 60.29 ± 4.35 | 7.14 ±2.13 | 1.57 ±1.27 | 1.61 ±0.14 | 1.23 ±0.23 | 1.59 ±0.15 | 1.6 ±0.24 | 21.39 9±2.84 |

**Table 3.3.1. Morphological characteristics of shoot cultures of *Q. robur* derived from seedlings, stump sprouts, mature crown scions on grafted plants and old hedged stock plants.**

| Origin of Clone | Shoot Angle to horizontal | Green leaves per Shoot | Scale leaves per shoot | Shoot Tip Diameter (mm) | Mid-Stem Diameter (mm) | Stem Base Diameter (mm) | Shoots per explant | Shoot Length (mm) |
|---|---|---|---|---|---|---|---|---|
| *Seedling* | 60.0 ± 2.58 | 5.12 ± 0.57 | 1.00 ± 0.28 | 0.77 ± 0.02 | 0.70 ± 0.03 | 1.06 ± 0.06 | 3.40 ± 0.62 | 23.64 ± 3.19 |
| | 71.11 ± 5.45 | 1.44 ± 0.38 | 2.22 ± 0.72 | 0.93 ± 0.07 | 0.92 ± 0.10 | 1.28 ± 0.13 | 1.50 ± 0.22 | 22.38 ± 3.64 |
| *Adolescent* | 40.28 ± 5.29 | 7.90 ± 1.23 | 3.67 ± 0.52 | 1.49 ± 0.49 | 1.00 ± 0.04 | 1.19 ± 0.08 | 2.00 ± 0.44 | 15.77 ± 1.85 |
| *Mature* | 55.0 ± 3.78 | 6.57 ± 0.53 | 0.86 ± 0.59 | 1.01 ± 0.07 | 1.10 ± 0.14 | 1.34 ± 0.04 | 1.75 ± 0.48 | 22.25 ± 2.5 |
| *Flowering* | 32.17 ± 12.52 | 4.17 ± 1.4 | 1.33 ± 0.99 | 0.81 ± 0.04 | 0.74 ± 0.05 | 0.81 ± 0.04 | 1.5 ± 0.28 | 12.79 ± 1.24 |
| | 43.52 ± 4.65 | 4.85 ± 0.75 | 1.89 ± 0.49 | 1.04 ± 0.614 | 0.99 ±0.66 | 1.19 ±0.07 | 1.8 ± 0.29 | 18.23 ± 2.00 |

**Table 3.3.2. Morphological characteristics of *in vitro* shoot cultures of *Q. petraea* derived from seedlings, scions from a 20 - 25 year old tree grafted to seedlings (adolescent) and scions from a 150 year old tree grafted to seedlings (mature)**

characteristics between the juvenile and rejuvenated clones and the mature and adolescent clones. The number of shoots per explant tended to be higher for the juvenile and rejuvenated clones than for the adolescent and mature clones. It was however highly variable. Scale leaf number was difficult to assess in both species due to the tendency of scale leaf material to detach easily from the explant stems and this may have contributed to the variability in this characteristic. The diameter at the base of the stem was in all cases greater than elsewhere on the shoot.

A one way Factorial Analysis of Variance (ANOVA) on juvenility status was carried out on all raw data for *Q. petraea* and *Q. robur* clones with the exception of *Q. robur* clone derived from hedged plants which could not be unambiguously assigned to either juvenile or mature categories. In the case of *Q. petraea*, very highly significant differences were obtained for angle of shoot to horizontal ($p = 0.0001$), stem-tip diameter $p = 0.0006$), and mid stem diameter ($p = 0.0003$) between juvenile and mature explants. Results obtained for ANOVA of *Q. robur* clones indicated a highly significant difference in Mid-Stem Diameter ($p = 0.0031$) and shoot tip diameter ($p = 0.0046$) between clones assigned as either juvenile or mature.

Discriminant analysis was carried out on raw data for *Q. petraea* and *Q. robur* clones with the exception of the *Q. robur* clone derived from hedged plants. The analysis yielded coefficients associated with morphological traits that allow us to propose the following formulae to assign a Discriminant Score (D) to *Q. robur* and *Q. petraea* clones when morphologically assessed:

*Q. petraea*

**D = - 1.308 - 0.0351*Angle($^0$) + 2.41*Tip diameter(mm) + 1.435*Mid - Stem diameter(mm)**

*Q. robur*

**D = - 3.546 + 2.418*Tip diameter(mm) + 2.202*Mid-Stem Diameter(mm)**

Negative values returned in the formula suggest that the material examined is juvenile while positive values suggest material is mature.

Discriminant scores for clones of *Q. robur* and *Q. petraea* are shown in Figure 3.3.1 and Figure 3.3.2 respectively. For *Q. robur*, all clones of seedling origin or which have been derived from stump sprouts are closely grouped with an average negative discriminant score. Three *Q. robur* clones of seedling origin score negatively as expected. *Q. robur* clones cultured from stump sprouts of a mature tree, and which have previously been shown to be highly rejuvenated yield a negative score indicative of juvenile material. The mature *Q. robur* clone derived from a mature tree has a strongly positive discriminant score. The *Q. robur* clone cultured from a severely pruned hedge of mature origin, which was not included in the discriminant analysis, scores positive ($1.497 \pm 0.748$) reflecting its' mature origin.

For *Q. petraea*, both clones of seedling origin are closely grouped with an average negative discriminant score. All clones of mature or adolescent origin are closely grouped with an average positive discriminant score. The 20 year old adolescent clone scores strongly positive suggesting a mature status. Available data therefore suggests that despite being non flowering the remaining morphological characters are mature.

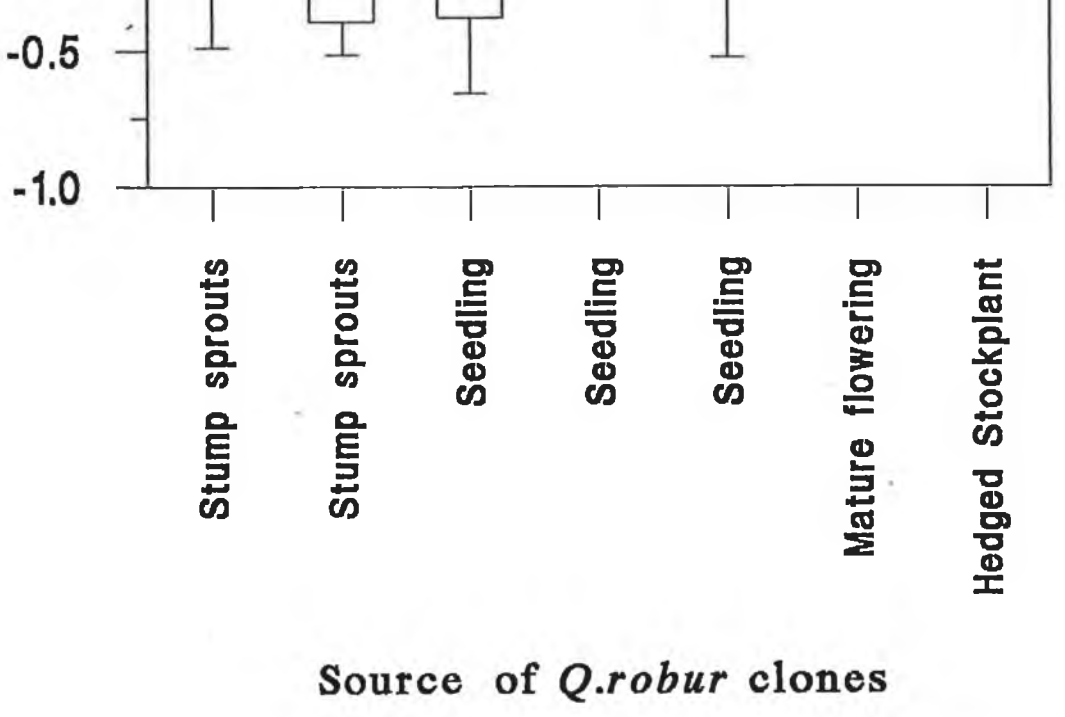
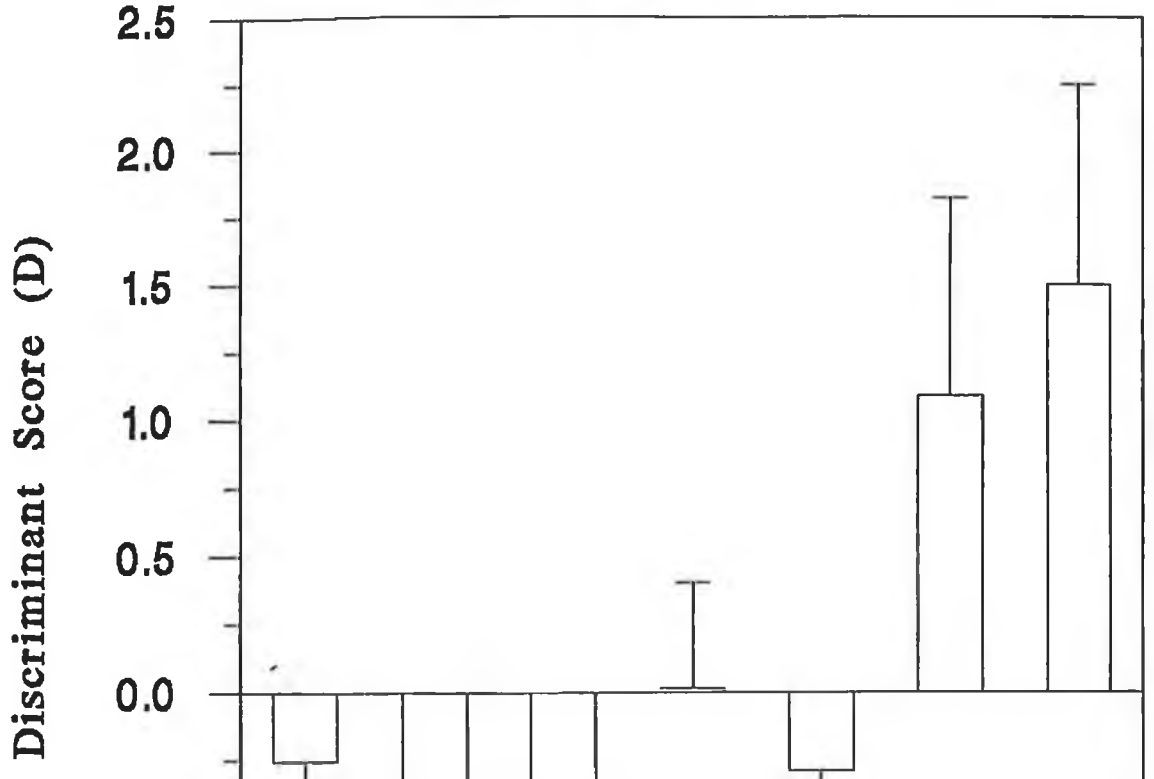

-0.5
-1.0
Stump sprouts
Stump sprouts
Seedling
Seedling
Seedling
Mature flowering
Hedged Stockplant
Source of Q.robur clones

**Figure 3.3.1**

# Discriminant Scores obtained for *Q.robur* clones



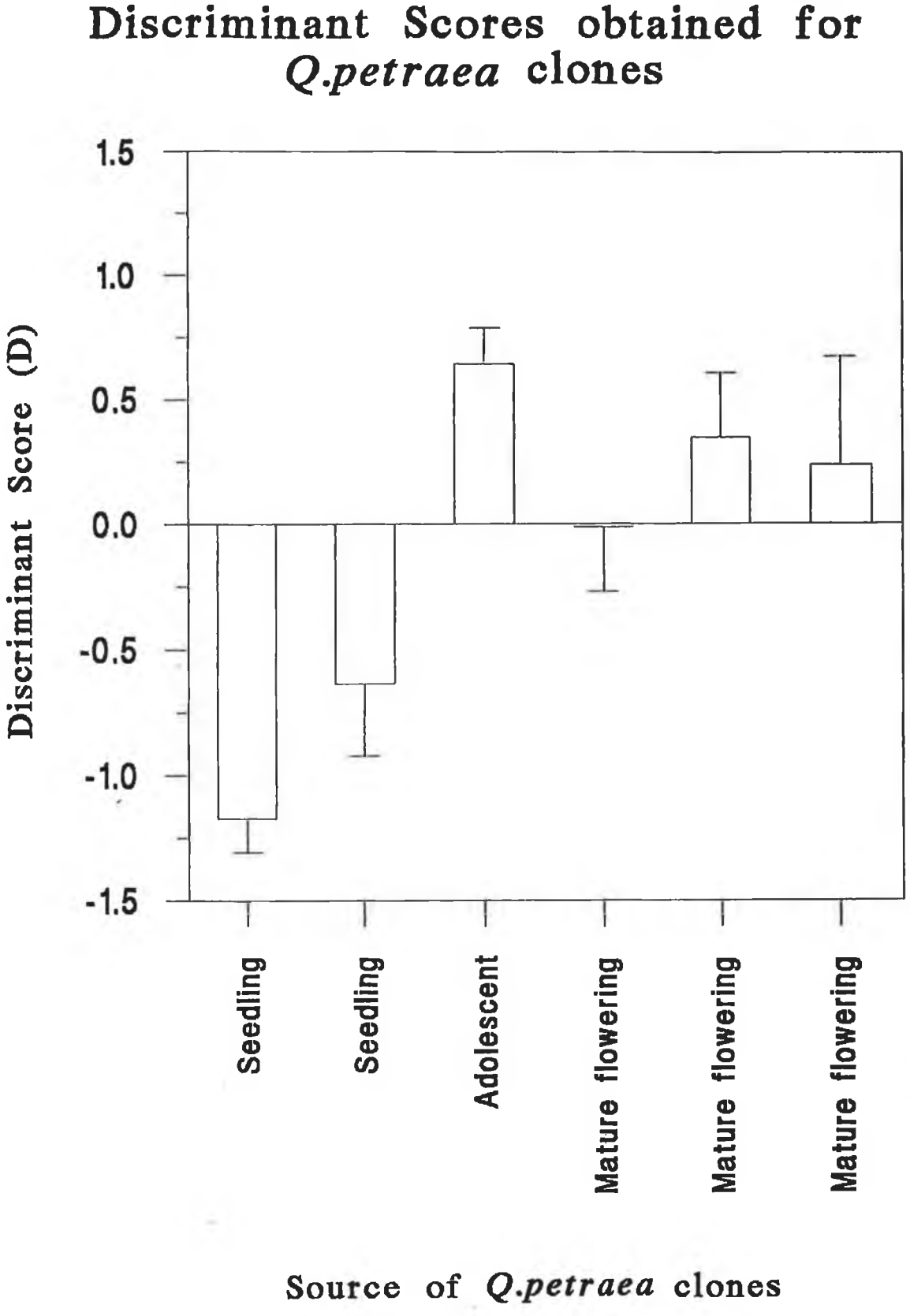

Discriminant Scores obtained for
Q.petraea clones
Discriminant Score (D)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Seedling
Seedling
Adolescent
Mature flowering
Mature flowering
Mature flowering
Source of Q.petraea clones

**Figure 3.3.2**

### 3.4 Morphological examination of leaf samples from *in vitro* grown shoot cultures

The morphological characteristics of shoots examined above provide us with easily measured qualitative and quantitative criteria of juvenility and maturity. However the measurement of these parameters is destructive of these shoots. The subculture of oak material under *in vitro* conditions allows for the removal of leaf material whilst maintaining both the sterility and the integrity of shoots. The use of image analysis offers a powerful tool for the morphological assessment of leaf material and is non destructive of shoots. To assess the feasibility of applying leaf morphology as a means to quantify differences between juvenile and mature shoots a range of morphological characteristics of leaves excised from shoots were examined by image analysis. Results from the image analysis of leaf material (Table 3.4.1) suggest differences within species between mature and juvenile clones in surface area of leaves, perimeter, lamina length of leaves and width of leaves. In the case of *Quercus robur* clones, surface area, width, perimeter and lamina length of leaves are all greater in the mature clones. The opposite is the case for *Q. petraea* clones where it was found that surface area, width, perimeter and laminar length of leaves are greater in the juvenile clones. The results are however based on large numbers of leaves (452 leaves from juvenile shoots and 312 leaves from mature shoots) and the technique may therefore not be useful for routine monitoring of shoot cultures.

These initial findings suggest a possible means to distinguish between *Q. robur* and *Q. petraea* clones grown *in vitro* based on leaf characteristics. However there is wide variability in data within each measured parameter. Data obtained was examined by discriminant analysis and results indicated a considerable overlap between juvenile and mature leaf morphology.

| Leaf character and maturity state | SPECIES | |
|---|---|---|
| | *Quercus petraea* | *Quercus robur* |
| *Area ($mm^2$)* | | |
| Juvenile | 67.73 ± 4.59 | 43.58 ± 2.62 |
| Mature | 32.74 ± 2.09 | 57.82 ± 7.81 |
| *Length/Width* | | |
| Juvenile | 2.36 ± 0.03 | 2.32 ±0.03 |
| Mature | 2.38 ± 0.03 | 2.22 ± 0.06 |
| *Width (mm)* | | |
| Juvenile | 6.06 ± 0.2 | 4.98 ± 0.17 |
| Mature | 4.27 ± 0.15 | 5.96 ± 0.41 |
| *Length (mm)* | | |
| Juvenile | 14.33 ± 0.52 | 11.42 ± 0.48 |
| Mature | 9.78 ± 0.32 | 13.25 ± 0.96 |
| *Perimeter (mm)* | | |
| Juvenile | 36.32 ± 1.28 | 30.12 ± 1.46 |
| Mature | 25.27 ± 0.85 | 33.6 ± 2.47 |
| *Roundness* | | |
| Juvenile | 1.7 ± 0.02 | 1.86 ± 0.09 |
| Mature | 1.8 ± 0.02 | 1.69 ± 0.04 |

**Table 3.4.1 Leaf characterisitcs for juvenile and mature *Q. robur* and *Q. petraea* clones**

## 3.5 Morphological and Physiological investigation of cultures derived from Epicormic shoots obtained from *Q. robur* tree at Phoenix Park

In order to provide material sourced from adult stock to allow further investigations of morphological and physiological characteristics of oak and to examine clonal variability, cell lines where initiated from epicormic shoots of sections from a branch of a single 100 year old *Q. robur* tree.

### 3.5.1 Micropropagation of cultures derived from Epicormic Shoots

Shoots that survived initiation were cultured for a minimum of 28 weeks. The results of the micropropagation of *in vitro* grown shoots isolated from the sections of a single branch are shown in Table 3.5.1.1. Multiplication rates tended to be higher for cell lines isolated from epicormic shoots that were on sections closest to the trunk (Sections 5, 6, 7). A decrease in the multiplication rate can be observed as cell line origin moves from the trunk There is no distinguishable trend for rates of death or contamination. Contamination rates were consistently low throughout culture but still posed a significant problem due to the low propagation rate.

### 3.5.2 Rooting of cultures derived from Epicormic Shoots

Results for rooting of cultures derived from epicormic shoots (Table 3.5.2.1) suggest that these cultures do not form roots easily. Only one cell line, cultures isolated from section 27, displayed an ability to form roots and this was at a low percentage (20%). Shoot tip necrosis was high for all cell lines and contributed to the death of many of the cultures in this rooting study.

### 3.5.3 Morphological examination of *in vitro* grown shoot cultures derived from Epicormic Shoots

The morphological characteristics of individual cell lines of a mature *Q. robur* tree are summarised in Table 3.5.3.1

| Section Number | Multiplication Rate | Death Rate | Contamination Rate | N |
|---|---|---|---|---|
| 5 | 1.55 ± 0.26 | 0.33 ± 0.07 | 0.09 ± 0.06 | 23 |
| 6 | 1.83 ± 0.22 | 0.26 ± 0.08 | 0.03 ± 0.03 | 32 |
| 7 | 1.53 ± 0.15 | 0.22 ± 0.05 | 0.00 ± 0.00 | 42 |
| 11 | 1.45 ± 0.12 | 0.38 ± 0.041 | 0.03 ± 0.02 | 81 |
| 13 | 0.84 ± 0.12 | 0.30 ± 0.08 | 0.10 ± 0.07 | 20 |
| 15 | 1.36 ± 0.13 | 0.40 ± 0.05 | 0.00 ± 0.00 | 48 |
| 18 | 1.20 ± 0.18 | 0.39 ± 0.07 | 0.00 ± 0.00 | 23 |
| 23 | 1.28 ± 0.15 | 0.41 ± 0.05 | 0.03 ± 0.02 | 58 |
| 27 | 1.05 ± 0.10 | 0.30 ± 0.06 | 0.00 ± 0.00 | 43 |

**Table 3.5.1.1 Multiplication rate, death rate and contamination rate for *in vitro* grown cell lines isolated from the epicormic shoots of sections from the branch of a *Q. robur* tree**

| Section Number | Treatment Number | Rooting % | Shoot Tip Necrosis % |
|---|---|---|---|
| *5* | 1 | 0 | 100 |
| *6* | 1 | 0 | 71 |
| | 2 | 0 | 100 |
| *7* | 1 | 0 | 100 |
| | 2 | 0 | 33 |
| *11* | 1 | 0 | 100 |
| | 2 | 0 | 100 |
| | 3 | 0 | 100 |
| *13* | 1 | 0 | 66 |
| *15* | 1 | 0 | 100 |
| | 2 | 0 | 87 |
| *18* | 1 | 0 | 83 |
| *23* | 1 | 0 | 66 |
| | 2 | 20 | 33 |

**Table 3.5.2.1 Percentage of shoots rooting and percentage of shoots showing shoot tip necrosis for cultures of cell lines isolated from the epicormic buds of a *Q. robur* tree**

| Section Number | Shoot Tip Diameter (mm) | Mid-Stem Diameter (mm) | Stem Base Diameter (mm) | Shoot Length (mm) | Shoot Angle to Horizontal ($^0$) | Discriminant Score | *N* |
|---|---|---|---|---|---|---|---|
| 5 | 1.65 ± 0.15 | 1.35 ± 0.05 | 1.47 ± 0.17 | 19.85 ± 4.45 | 57.5 ± 22.50 | 3.42 ± 0.47 | 2 |
| 6 | 0.74 ± 0.07 | 1.37 ± 0.13 | 1.43 ± 0.19 | 27.40 ± 5.71 | 40.6 ± 5.22 | 1.26 ± 0.37 | 5 |
| 7 | 0.76 ± 0.04 | 0.86 ± 0.13 | 1.49 ± 0.33 | 39.20 ± 8.14 | 60.2 ± 8.24 | 0.19 ± 0.35 | 5 |
| 11 | 1.04 ± 0.18 | 1.14 ± 0.23 | 1.48 ± 0.25 | 54.60 ± 6.01 | 56.0 ± 9.19 | 1.49 ± 0.94 | 5 |
| 13 | 1.03 ± 0.10 | 1.39 ± 0.19 | 1.62 ± 0.22 | 25.00 ± 3.44 | 43.6 ± 7.77 | 2.02 ± 0.57 | 7 |
| 15 | 1.39 ± 0.06 | 1.49 ± 0.15 | 1.94 ± 0.16 | 21.34 ± 1.59 | 66.7 ± 6.01 | 3.20 ± 0.45 | 3 |
| 18 | 1.53 ± 0.11 | 1.27 ± 0.11 | 1.51 ± 0.13 | 27.91 ± 2.91 | 64.0 ± 6.78 | 2.95 ± 0.12 | 5 |
| 23 | 0.99 ± 0.13 | 1.10 ± 0.14 | 1.43 ± 0.12 | 25.49 ± 1.92 | 63.0 ± 9.30 | 1.29 ± 0.59 | 5 |
| 27 | 1.62 ± 0.02 | 1.64 ± 0.04 | 2.10 ± 0.50 | 16.91 ± 7.69 | 45.0 ±0 20 | 3.98 ± 0.14 | 2 |

**Table 3.5.3.1 Morphological characteristics of shoot cultures derived from epicormic shoots growing on separate sections from a single branch of a 100 year old *Q. robur* tree**

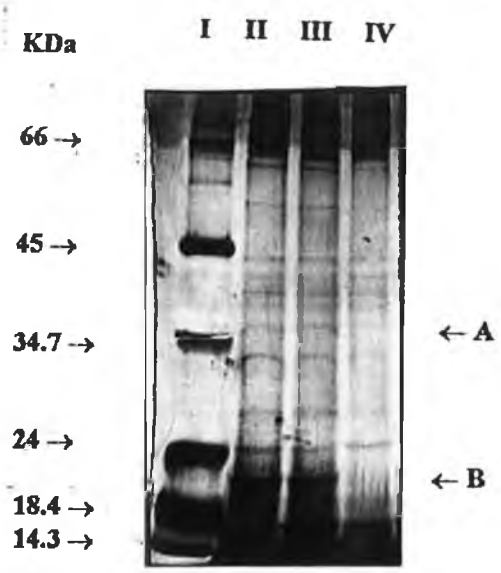
Shoots from all sections tended to display a growth habit consistent with the findings for mature material in Section 3.3. In cultures of all clones derived from epicormic shoots the angle of shoot growth to the horizontal was less than for juvenile clones in the morphological examination in Section 3.3. Similarly, the tip diameter and mid-stem diameter were also smaller for the juvenile clones than for the clones derived from epicormic shoots. The base stem diameter and shoot length were highly variable between all epiormic shoot clones however both these parameters were greater when compared to the clones in the previous morphological study. The diameter at the tip and mid stem of Epicormic shoots clones appeared to be consistent with those of mature material. With the exception of clones derived from section 7 of the branch, the scores obtained when using the Discriminant Score formula suggests that these clones are still mature in phase state. Results obtained also suggest that there is no trend between phase state and distance the original shoot was from trunk of tree.

### 3.6 Molecular weight determination of protein samples by SDS Polyacrylamide Gel Electrophoresis

Morphological characteristics have been demonstrated to be a useful means of defining juvenility and maturity in many species. All of these parameters could allow a quantitative and qualitative approach to defining the phenomenon. Factors such as the physiological state of the plant, the concentration or type of active substances of plant metabolism are affected by environmental factors and the levels of substances differ between juvenile and adult plants and differing woody species (Haffner *et al.*, 1991). It is possible that protein differences between *Quercus* material of juvenile and mature origin could provide both the means to discriminate mature and juvenile material and also an understanding in the events surrounding the maturation phenomenon.

Soluble and membrane protein samples were isolated from Juvenile *Q. robur* clones JR4 (seedling clone), JR5 (stump sprouts clone) and *Quercus petraea* seedling clones JP1 and JP3. Protein studies were also carried out on mature *Q .petraea* clones MP1, MP2, MP3 and mature *Q. robur* clone MR1. Figure 3.6.1 shows results of running soluble membrane protein samples isolated from clones JR4 and JR5 on denaturing SDS Polyacrylamide gels. Membrane proteins were also run but banding was too faint to capture image via UVP photography system. Table 3.6.1 outlines results for bands obtained for membrane bound proteins. Differences existed in protein banding between these two clones in both soluble and membrane proteins. In gels examining soluble protein clone JR4 displays bands of approximately 20 and 39 KDa which are absent from clone JR5. Likewise for gels examining membrane protein, for clone JR5 proteins that stain at approximately 46.2, 32 and 15 KDa are similar to those of clone JR4 with bands at 62 and 30 KDa not being present in JR4.

Several gels were run on denaturing SDS Polyacrylamide gels of the remaining protein samples isolated. Protein staining was erratic and yielded gels with inconsistent banding. No conclusion could be drawn on differences in protein banding between mature or juvenile material.




**Key:**

| | |
|---|---|
| *Lane I* | Molecular weight marker |
| *Lane II & III* | Seedling derived clone (JR4) |
| *Lane IV* | Stump sprout derived clone (JR5) |

**Figure 3.6.1 SDS Page of soluble protein isolated from clones JR4 and JR5**

| Seedling | | Stump Sprout | |
|---|---|---|---|
| *Membrane* | *Soluble* | *Membrane* | *Soluble* |
| 67520 | 58205 | 62105 | 58205 |
| 53220 | 54435 | 46241 | 54435 |
| 46184 | 47714 | 31178 | 47714 |
| 43362 | 45002 | 29958 | 45002 |
| 41474 | 43999 | 15504 | 43999 |
| 39606 | 41454 | | 41454 |
| 37222 | 38930 | | 35552 |
| 32513 | 35552 | | 34017 |
| 25934 | 34017 | | 28286 |
| 21091 | 28286 | | 24021 |
| 18651 | 24021 | | 17386 |
| 17049 | 20166 | | 14350 |
| 14913 | 17386 | | 11876 |
| 10738 | 14350 | | |
| | 11876 | | |

**Table 3.6.1 Molecular weight bands (KDa) of soluble and membrane bound protein isolated from *Q. robur* clones derived from seedling (JR4) and stump sprouts (JR5)**

### 3.7 *In vitro* grafting of *Quercus* explants

The previous investigations have shown that morphological criteria may be used to differentiate material of juvenile and mature origin. It is now possible to follow the progress of rejuvenation and examine if the technique of *in vitro* grafting of mature *Quercus* explants may yield such rejuvenated material.

#### 3.7.1 Optimisation of media combination for *in vitro* grafting

From results for successful grafts combinations (Table 3.7.1.1) treatments that consisted of a basal media containing 0.2 $mgl^{-1}$ Benzyladenine tended to give best graft success (i.e. 100% graft union). All of the successful grafts were strong and could resist parting when pulled with a tweezers. The effects of treatments on bud number, length of buds and number of leaves on scion and stock were measured and the results are summarised in Table 3.7.1.2a and Table 3.7.1.2b. The effect of each treatment on tip and base diameter, of scion and stock were measured and results are summarised in Table 3.7.1.3a and 3.7.1.3b. At a Benzyladenine concentration of 0.02 $mgl^{-1}$ in the basal media, as increase in IAA concentrations in the apical media resulted in an increase in the mean scion bud number. The effect being most noticeable when apical media contained 0.2 $mgl^{-1}$ Benzyladenine. This observation did not occur for apical media containing 0.00 $mgl^{-1}$ Benzyladenine where scion bud numbers decreased then increased with increasing IAA concentrations. A similar trend was observed at the higher concentration of 0.2 $mgl^{-1}$ in the basal media. At this level it appeared that at each Benzyladenine concentration in apical media (0.00, 0.02, and 0.002 $mgl^{-1}$), as the IAA level increased a decrease in scion bud number occurred followed by a. increase except at 0.02 $mgl^{-1}$ Benzyladenine in the apical media where the opposite occurred. Scion bud lengths displayed increasing values as IAA concentration increased at levels of 0.02 $mgl^{-1}$ and 0.2 $mgl^{-1}$ in the apical media when basal media contained 0.02 $mgl^{-1}$ and for 0.02 $mgl^{-1}$ in the apical media when the basal media contained 0.2 $mgl^{-1}$. At all other Bezyaladenine concentrations the trend observed was a large decrease in scion bud lengths followed by an increase. It would appear that overall

| Apical Media | | Basal Media | |
|---|---|---|---|
| BA Concentration $mgl^{-1}$ | IAA Concentration $mgl^{-1}$ | 0.02 $mgl^{-1}$ Benzyladenine | 0.2 $mgl^{-1}$ Benzyladenine |
| | | *% Graft Success* | |
| ***0*** | *0.1* | 60 | 100 |
| | *1.0* | 40 | 100 |
| | *10.0* | 100 | 100 |
| ***0.02*** | *0.1* | 60 | 60 |
| | *1.0* | 80 | 100 |
| | *10.0* | 100 | 100 |
| ***0.2*** | *0.1* | 60 | 100 |
| | *1.0* | 80 | 100 |
| | *10.0* | 80 | 60 |

**Table 3.7.1.1 The effect of apically applied auxin and cytokinin on graft success, with 0.02 $mgl^{-1}$ Benzyladenine or 0.2 $mgl^{-1}$ Benzyladenine applied to the basal media**

| Apical Media | | Basal Media (0.02 $mgl^{-1}$ Benzyladenine) | | | | | |
|---|---|---|---|---|---|---|---|
| BA Concentration $mgl^{-1}$ | IAA Concentration $mgl^{-1}$ | *Scion Bud Number* | *Scion Bud Length (mm)* | *Scion Leaf Number* | *Stock Bud Number* | *Stock Bud Length (mm)* | *Stock Leaf Number* |
| 0 | 0.1 | 1.20 ± 0.4 | 6.48 ± 1.7 | 1.43 ± 0.7 | 0.20 ± 0.2 | 1.41 ± 1.4 | 0.20 ±0.2 |
| | 1.0 | 0.60 ± 0.4 | 1.23 ± 0.7 | 0.00 ± 0.0 | 0.00 ± 0.0 | 0.00 ± 0.0 | 0.00 ±0.0 |
| | 10.0 | 1.20 ± 0.6 | 7.34 ± 4.0 | 0.75 ± 0.4 | 0.00 ± 0.0 | 0.00 ± 0.0 | 0.00 ± 0.0 |
| 0.2 | 0.1 | 0.60 ± 0.4 | 1.89 ± 0.9 | 0.17 ± 0.1 | 0.60 ± 0.4 | 3.65 ± 1.8 | 0.20 ± 0.2 |
| | 1.0 | 0.80 ± 0.4 | 2.95 ± 0.9 | 0.50 ± 0.4 | 0.00 ± 0.0 | 0.00 ± 0.0 | 0.00 ± 0.0 |
| | 10.0 | 0.80 ± 0.4 | 3.42 ± 2.3 | 1.83 ± 1.2 | 0.80 ± 0.4 | 12.26 ± 5.1 | 0.00 ± 0.0 |
| 0.02 | 0.1 | 1.20 ± 0.2 | 9.18 ± 1.9 | 3.0 ± 0.7 | 0.16 ± 0.1 | 8.26 ± 8.2 | 0.20 ± 0.2 |
| | 1.0 | 1.8 ± 0.7 | 11.74 ± 4.3 | 2.40 ± 0.9 | 0.40 ± 0.2 | 0 71 ± 0.4 | 0.00 ± 0.0 |
| | 10.0 | 2.20 ± 0.4 | 12.51 ± 3.7 | 1.91 ± 0.7 | 0.60 ± 0.2 | 16.38 ± 6.7 | 0.00 ± 0.0 |

**Table 3.7.1.2a Measurements of leaf number, bud number and bud length on scion and stock as effected by apically applied auxin and cytokinin with 0.02 $mgl^{-1}$ Benzyladenine applied to the basal media. All values are the mean of 10 replicates and are shown with the standard error of the mean**

| Apical Media | | Basal Media (0.2 $mgl^{-1}$ Benzyladenine) | | | | | |
|---|---|---|---|---|---|---|---|
| BA Concentration $mgl^{-1}$ | IAA Concentration $mgl^{-1}$ | *Scion Bud Number* | *Scion Bud Length (mm)* | *Scion Leaf Number* | *Stock Bud Number* | *Stock Bud Length (mm)* | *Stock Leaf Number* |
| 0 | 0.1 | 1.75 ± 1.1 | 10.55 ± 5.4 | 1.87 ± 1.0 | 0.25 ± 0.2 | 4.525 ± 4.5 | 1.00 ± 1.0 |
| | 1.0 | 0.20 ± 0.2 | 3.43 ± 3.4 | 0.00 ± 0.0 | 0.00 ± 0.0 | 0.00 ± 0.0 | 0.00 ± 0.0 |
| | 10.0 | 0.60 ± 0.6 | 3.89 ± 1.9 | 0.57 ± 0.4 | 0.40 ± 0.2 | 7.24 ± 4.4 | 1.60 ± 1.0 |
| 0.02 | 0.1 | 0.80 ± 0.5 | 2.86 ± 1.1 | 0.57 ± 0.3 | 0.40 ± 0.2 | 4.76 ± 4.1 | 1.0 ± 0.8 |
| | 1.0 | 2.80 ± 0.5 | 7.12 ± 1.9 | 1.43 ± 0.3 | 0.20 ± 0.2 | 0.468 ± 0.5 | 0.00 ± 0.0 |
| | 10.0 | 1.00 ± 0.3 | 15.89 ± 6.8 | 3.00 ± 1.5 | 0.00 ± 0.0 | 1.196 ± 1.2 | 0.00 ± 0.0 |
| 0.2 | 0.1 | 1.2 ± 0.4 | 19.58 ± 7.8 | 5.57 ± 2.4 | 0.20 ± 0.2 | 0.42 ± 0.4 | 0.00 ± 0.0 |
| | 1.0 | 0.80 ± 0.4 | 4.14 ± 1.7 | 1.17 ± 0.7 | 0.80 ± 0.5 | 13.4 ± 7.2 | 2.43 ± 1.7 |
| | 10.0 | 1.00 ± 0.5 | 6.13 ± 4.2 | 1.2857 ± 1.3 | 0.40 ± 0.4 | 2.09 ± 1.4 | 0.17 ± 0.1 |

**Table 3.7.1.2b Measurements of leaf number, bud number and bud length on scion and stock as effected by apically applied auxin and cytokinin with 0.2 $mgl^{-1}$ Benzyladenine applied to the basal media. All values are the mean of 10 replicates and are shown with the standard error of the mean**

| Apical Media | | Basal Media 0.02 $mgl^{-1}$ Benzyladenine | | | |
|---|---|---|---|---|---|
| BA Concentration $mgl^{-1}$ | IAA Concentration $mgl^{-1}$ | *Scion Tip Diameter (mm)* | *Scion Base Diameter (mm)* | *Stock Tip Diameter (mm)* | *Stock Base Diameter (mm)* |
| 0 | 0.1 | 1.10 ± 0.076 | 1.19 ± 0.143 | 1.24 ± 0.277 | 2.04 ± 0.388 |
| | 1.0 | 1.44 ± 0.369 | 1.06 ± 0.096 | 0.98 ± 0.086 | 2.09 ± 0.42 |
| | 10.0 | 2.19 ± 0.137 | 1.31 ± 0.199 | 1.24 ± 0.156 | 1.45 ± 0.079 |
| 0.02 | 0.1 | 0.84 ± 0402 | 0.94 ± 0.104 | 0.9 ± 0.082 | 1.99 ± 0.236 |
| | 1.0 | 1.76 ± 0.413 | 1.12 ± 0.292 | 1.29 ± 0.251 | 2.00 ± 0.366 |
| | 10.0 | 1.63 ± 0.145 | 1.11 ± 0.122 | 1.3 ± 0.143 | 3.40 ± 0.268 |
| 0.2 | 0.1 | 2.15 ± 0.497 | 1.01 ± 0.073 | 1.03 ± 0.091 | 3.32 ± 0.279 |
| | 1.0 | 2.68 ± 0.623 | 1.88 ± 0.459 | 1.84 ± 0.325 | 3.98 ± 0.287 |
| | 10.0 | 1.21 ± 0.052 | 1.15 ± 0.202 | 1.25 ± 0.081 | 2.81 ± 0.236 |

**Table 3.7.1.3a Measurements of scion and stock tip and base diameters as effected by apically applied auxin and cytokinin with 0.02 $mgl^{-1}$ Benzyladenine applied to the basal media. All values are the mean of 10 replicates and are shown with the standard error of the mean**

| Apical Media | | Basal Media | | | |
|---|---|---|---|---|---|
| | | 0.2 $mgl^{-1}$ Benzyladenine | | | |
| BA Concentration $mgl^{-1}$ | IAA Concentration $mgl^{-1}$ | *Scion Tip Diameter (mm)* | *Scion Base Diameter (mm)* | *Stock Tip Diameter (mm)* | *Stock Base Diameter (mm)* |
| **0** | 0.1 | 1.15 ± 0.098 | 1.03 ± 0.032 | 1.13 ± 0.076 | 7.45 ± 0.561 |
| | 1.0 | 1.99 ± 0.221 | 1.04 ± 0.257 | 1.09 ± 0.141 | 2.64 ± 0.093 |
| | 10.0 | 1.22 ± 0.157 | 1.02 ± 0.187 | 1.14 ± 0.209 | 3.4 ± 0.739 |
| **0.02** | 0.1 | 0.97 ± 0.075 | 0.98 ± 0.056 | 0.92 ± 0.204 | 3.67 ± 0.251 |
| | 1.0 | 1.51 ± 0.291 | 1.05 ± 0.075 | 1.21 ± 0.052 | 3.5 ± 0.470 |
| | 10.0 | 1.5 ± 0.140 | 1.00 ± 0.0343 | 1.08 ± 0.064 | 4.37 ± 0.702 |
| **0.2** | 0.1 | 2.66 ±0.533 | 1.1 ± 0.056 | 1.30 ± 0.054 | 3.64 ± 0.365 |
| | 1.0 | 1.16 ± 0.331 | 0.68 ± 0.101 | 1.08 ± 0.071 | 2.43 ± 0.289 |
| | 10.0 | 1.31 ± 0.218 | 1.07 ± 0.032 | 1.07 ± 0.195 | 4.20 ± 0 605 |

**Table 3.7.1.3b Measurements of scion and stock tip and base diameters as effected by apically applied auxin and cytokinin with 0.2 $mgl^{-1}$ Benzyladenine applied to the basal media. All values are the mean of 10 replicates and are shown with the standard error of the mean**

for both basal media treatments, as the level of BA in the apical media increases along with IAA levels, a corresponding increase occurs in both scion bud numbers and scion bud length. Scion bud leaf numbers on the otherhand, show an opposing trend in that, at both BA concentrations in the basal media an increasing IAA concentration in the apical media results in a decrease in the number of leaves. However the observation is compounded by the fact at a 0.02 $mgl^{-1}$ BA concentration in the apical media for both basal media, an increasing IAA concentration in the apical media resulted in an observed increase in leaf numbers on scion buds.

Where basal media contained a Benzyladenine concentration of 0. 02 $mgl^{-1}$ there appears to be a relationship between the level of Benzyladenine in the apical media interacting with the IAA in the apical media. At the lowest BA concentration in apical media stock bud numbers appear to be inhibited by increasing levels of IAA. However as the level of BA in apical media increases the inhibitory effect of IAA concentrations appears to be reversed. At a higher level of BA in basal media the opposite appears to occur. For stock bud length at both basal media concentrations no discernible trend seems apparent.

Results obtained for stock leaf number at the lower basal media BA concentration suggest that an increasing IAA concentration inhibits leaf growth at all BA concentrations in the apical media. At the higher BA concentration in the basal media there appears to be an interaction between the higher IAA and BA concentration in the apical media effecting the leaf numbers on stock buds.

When basal media contains 0.02 $mgl^{-1}$, the level of BA in the apical media effects the scion tip diameter. At low levels of BA in apical media (0.00 $mgl^{-1}$ and 0.02 $mgl^{-1}$) an increase in IAA causes a corresponding increase in the tip diameter. At the higher BA concentration, an increasing IAA level results in an inhibition of scion tip development.

In the case of basal media containing 0.2 $mgl^{-1}$ Ba, then at each separate IAA concentration in the apical media, an increase in BA in the apical media results in a decrease in tip diameter except at 0.1 $mgl^{-1}$ IAA where a decrease occurs at 0.1 $mgl^{-1}$ BA followed by a doubling of the tip diameter at 0.2 $mgl^{-1}$ BA.

Examining scion base diameter it appears that at the level of 1 $mgl^{-1}$ IAA an interaction occurs with BA in both the apical and basal media. At the lower BA concentration in the basal media a level of 0.2 $mgl^{-1}$ BA in the apical media results in an 80% increase in the scion base diameter whilst at the higher basal media BA concentration the scion base diameter decreases almost 30% at the same level of BA and IAA in the apical media.

Stock tip diameters appear not to be influenced by any treatment in the apical and basal media whilst there is a noticeable difference in the stock base diameters between the two different basal media BA concentrations.

Analysis of variance (ANOVA) was carried out to assess the effect of media combinations on growth of measured parameters from scions and stocks. Parameters that were significantly affected are outlined in Table 3.7.1.4. As this study was concerned with the examination of possible rejuvenation through cascade grafting, the combination that yields the greatest bud numbers and lengths on both stocks and scions to allow further grafting would be most desirable.

***Scion bud numbers***

ANOVA showed a significant effect on scion bud numbers by auxin level in the apical media interacting with BA level in the apical media (the apical media itself) and an interaction between the basal media and BA levels in apical media. A graph of the means of scion bud numbers against Auxin level in apical media interacting with BA level in apical media (Figure 3.7.1.1) shows that the combination of 1 $mgl^{-1}$ IAA interacting with 0.02 mg $l^{-1}$ BA gives the highest bud numbers (1.8). Apical media containing 10 $mgl^{-1}$ IAA and 0.2 $mgl^{-1}$ BA gives second highest bud number (1.6). The combination of 0.1 $mgl^{-1}$ IAA and 0 $mgl^{-1}$ BA gives third highest value (1.444). The graph of scion bud numbers as affected by BA level in apical media interacting with basal media (Figure 3.7.1.2) indicates that a combination of 0.2 $mgl^{-1}$ BA in apical media interacts with 0.02 $mgl^{-1}$ BA in the Basal media to give the highest numbers of buds (1.7333). The second highest mean bud number for BA level in apical media interacting with basal media is that of 0.02 $mgl^{-1}$ BA level in Apical media with 0.2 $mgl^{-1}$ BA in Basal media.

| Scion and Stock Parameter | Interaction of Media combinations | *p* value |
|---|---|---|
| *Scion bud number* | Aux_level with BA_level. | 0.027 |
| | BA_level with Basal media. | 0.023 |
| *Scion bud length* | BA_level. | 0.032 |
| *Scion leaf number* | BA_level. | 0.003 |
| | Aux_level with BA_level. | 0.013 |
| *Scion base diameter* | Basal media. | 0.016 |
| | Aux_level with BA_level with Basal media. | 0.022 |
| *Scion tip diameter* | BA_level. | 0.020 |
| | Aux_level with BA_level | 0.001 |
| | Aux_level with BA_level with Basal media. | 0.007 |
| *Stock bud length* | Aux_level with BA_level with Basal media | 0.046 |
| *Stock leaf number* | Aux_level with Basal media | 0.047 |
| | Aux_level with BA_level with Basal media | 0.023 |
| *Stock base diameter* | Basal media | 0.003 |

**Key**

Basal media represents all basal media.

BA_level represent BA level in Apical media

Aux_level represents IAA level in Apical media

**Table 3.7.1.4 Measured parameters significantly effected by apically applied auxin and cytokinin with 0.02 $mgl^{-1}$ Benzyladenine or 0.2 $mgl^{-1}$ Benzyladenine applied to the basal media**

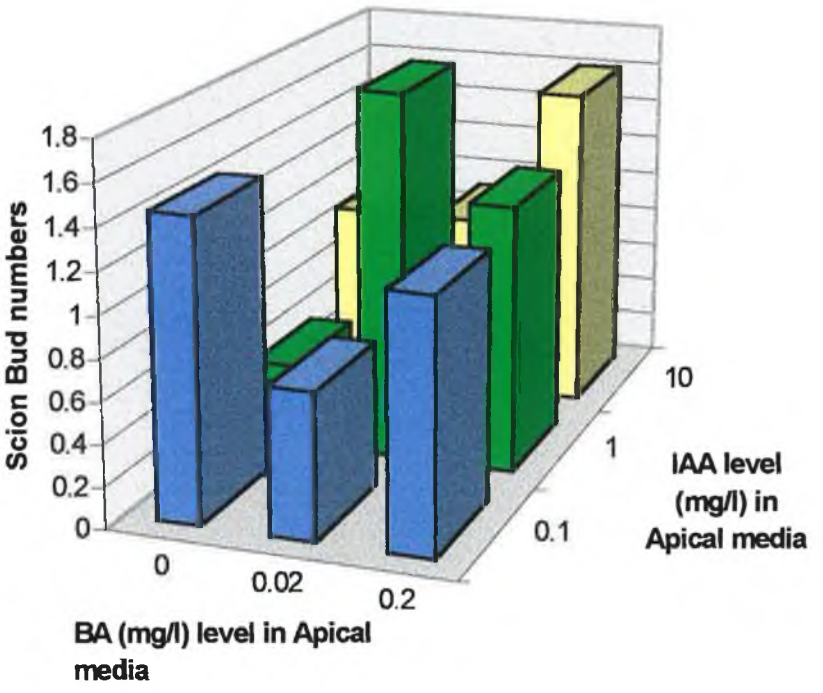



**Figure 3.7.1.1 Scion bud number as influenced by interaction of IAA concentration in apical media with BA concentration in apical media**

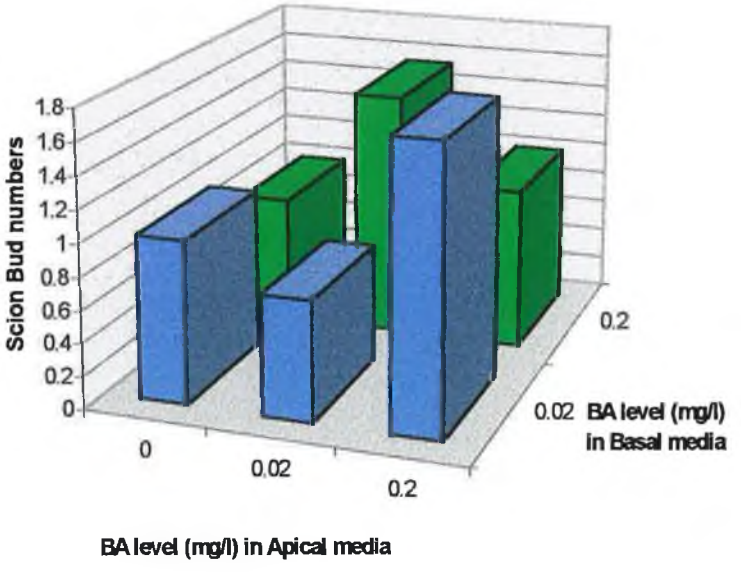



**Figure 3.7.1.2 Scion bud numbers as influenced by interaction between BA level in apical media with BA level in basal media**

***Scion bud Length***

ANOVA indicated that BA level in apical media had a significant effect on the scion bud length. Figure 3.7.1.3 indicates that bud length is greatest influenced by a BA level of 0.2 $mgl^{-1}$ in apical media which gives highest mean scion bud length at 10.957 mm. Also of note is that there is no significant difference between bud length at 0 $mgl^{-1}$ BA and 0.02 $mgl^{-1}$ BA.

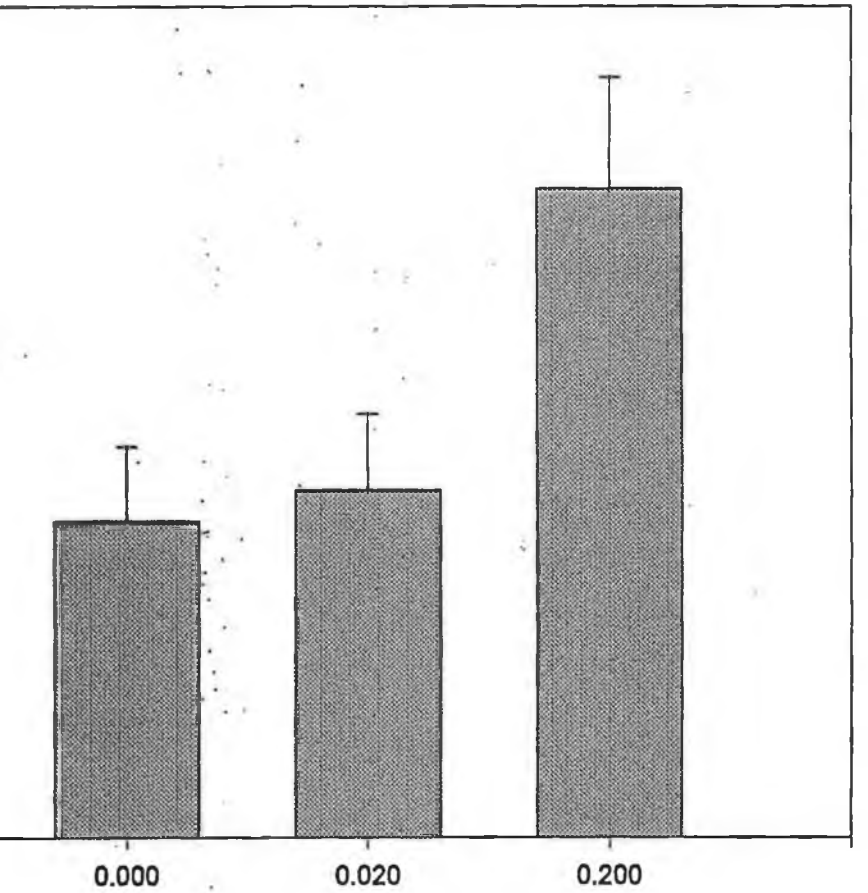
***Stock bud lengths***

Length of buds on stock was significantly influenced by an overall interaction between the apical media and the basal media. Graphs of effects on stock bud lengths by different apical and basal media combinations are displayed in Figure 3.7.1.4 and 3.7.1.5. From Figure 3.7.1.4 it is visible that apical media containing a BA level of 0.2 $mgl^{-1}$ and IAA concentration of 10 $mgl^{-1}$ combines with a basal media containing 0.02 $mgl^{-1}$ to give highest stock bud length (16.388 mm). Second highest is that of 0.2 $mgl^{-1}$ BA and 1 $mgl^{-1}$ IAA in apical media interacting with basal media containing 0.2 $mgl^{-1}$ displaying a mean bud length of 13.3457 mm. BA concentration of 0.02 $mgl^{-1}$ BA and IAA concentration of 10 $mgl^{-1}$ in apical media interacts with Basal media containing 0.2 $mgl^{-1}$ to give a mean bud length of 12.26 mm.

To determine the treatment which yielded grafts, that would allow the greater number of shoots coupled with greatest shoot length, the mean of shoot length was multiplied by bud number for each individual treatment. The results for each treatment are displayed in Table 3.7.1.5a and Table 3.7.1.5b. Considering that ANOVA indicated that scion bud length, scion bud number and stock bud length were all significantly effected by treatments, it is therefore possible to ignore the effect of stock bud number. Hence the best treatment for both scion and stock development is a Basal media containing 0.02 $mgl^{-1}$ and an Apical media containing 10 $mgl^{-1}$ IAA with 0.2 $mgl^{-1}$ BA.

**Figure 3.7.1.3**

**Scion Bud length (mm) as influenced by BA concentration in apical media**




**BA level ($mgl^{-1}$) in apical media**

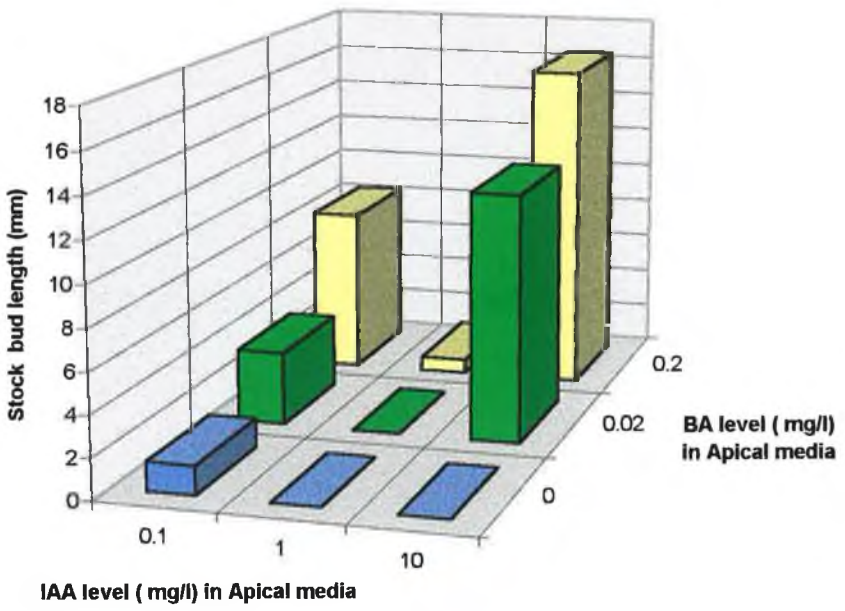



**Figure 3.7.1.4 Stock bud length (mm) as influenced by interaction between apical media and 0.02 $mgl^{-1}$ BA in basal media.**

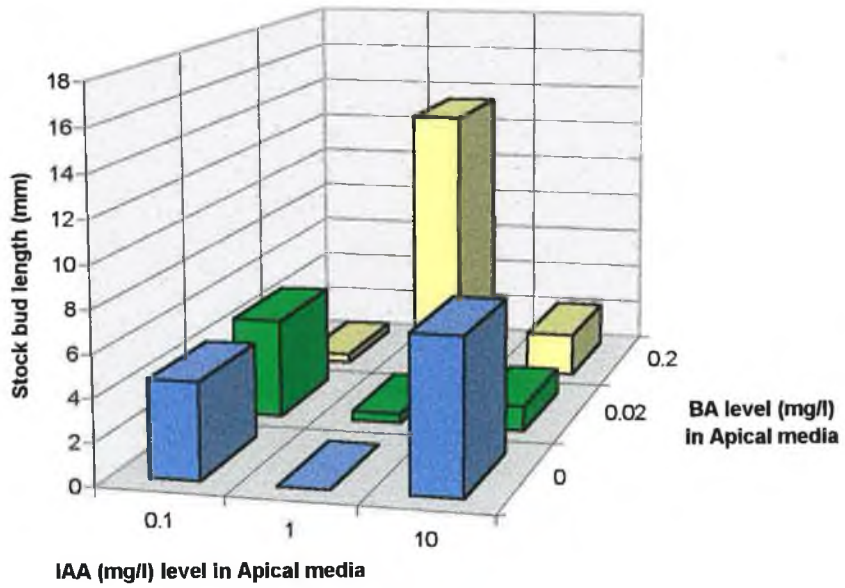



**Figure 3.7.1.5 Stock bud length (mm) as influenced by interaction between apical media and 0.2 $mgl^{-1}$ BA in basal media**

| Apical Media | | Basal Media 0.02mgl$^{-1}$ Benzyladenine | | | | | |
|---|---|---|---|---|---|---|---|
| | | Scion | | | Stock | | |
| BA Concentration mgl$^{-1}$ | IAA Concentration mgl$^{-1}$ | Mean bud length (mm) | Mean bud number | Combined Bud number multiplied by bud length | Mean bud length (mm) | Mean bud number | Combined Bud number multiplied by bud length |
| 0 | 0.1 | 6.48 | 1.2 | 7.78 | 1.42 | 0.2 | 0.28 |
| | 1.0 | 1.23 | 0.6 | 0.74 | 0.00 | 0.0 | 0.00 |
| | 10.0 | 7.34 | 1.2 | 8.81 | 0.00 | 0.0 | 0.00 |
| 0.02 | 0.1 | 1.84 | 0.6 | 1.10 | 3.65 | 0.6 | 2.19 |
| | 1.0 | 2.50 | 0.8 | 2.00. | 0.00 | 0.0 | 0.00 |
| | 10.0 | 3.42 | 0.8 | 2.74 | 12.26 | 0.8 | 9.81 |
| 0.2 | 0.1 | 9.18 | 1.2 | 11.02 | 8.27 | 0.2 | 1.65 |
| | 1.0 | 11.74 | 1.8 | 21.13 | 0.71 | 0.4 | 0.28 |
| | 10.0 | 12.51 | 2.2 | 27.52 | 16.39 | 0.6 | 9.83 |

**Table 3.7.1.5a Measurement of mean bud number and bud length with value of mean bud number X bud length for scion and stock as effected by apically applied auxin and cytokinin with 0.02 mgl$^{-1}$ Benzyladenine applied to the basal media. All values are the mean of 10 replicates and are shown with the standard error of the mean**

| Apical Media | | Basal Media 0.2 $mgl^{-1}$ Benzyladenine | | | | | |
|---|---|---|---|---|---|---|---|
| | | Scion | | | Stock | | |
| BA Concentration $mgl^{-1}$ | IAA Concentration $mgl^{-1}$ | Mean bud length (mm) | Mean bud number | Bud number X Bud Length (mm) | Mean bud length (mm) | Mean bud number | Bud number X Bud Length (mm) |
| 0 | 0.1 | 10.55 | 1.75 | 18.46 | 4.53 | 0.25 | 1.13 |
| | 1.0 | 3.43 | 0.20 | 0.69 | 0.00 | 0.00 | 0.00 |
| | 10.0 | 3.88 | 0.60 | 2.33 | 7.24 | 0.40 | 2.90 |
| 0.02 | 0.1 | 2.85 | 0.80 | 2.28 | 4.76 | 0.40 | 1.90 |
| | 1.0 | 7.12 | 2.80 | 19.94 | 0.47 | 0.20 | 0.09 |
| | 10.0 | 15.89 | 1.00 | 15.89 | 1.20 | 1.00 | 1.20 |
| 0.2 | 0.1 | 19.59 | 1.20 | 23.50 | 0.42 | 0.20 | 0.08 |
| | 1.0 | 4.14 | 0.80 | 3.31 | 13.35 | 0.80 | 10.68 |
| | 10.0 | 6.13 | 1.00 | 6.13 | 2.09 | 0.40 | 0.84 |

**Table 3.7.1.5b Measurement of mean bud number and bud length with value of mean bud number X bud length for scion and stock as effected by apically applied auxin and cytokinin with 0.2 $mgl^{-1}$ Benzyladenine applied to the basal media. All values are the mean of 10 replicates and are shown with the standard error of the mean**

### 3.7.2 Influence on graft development due to presence of buds on scion and stock material

After 28 days of culture, grafts were examined. Number of shoots, length of shoots, number of leaves and presence of callus on scion and stock were recorded (Table 3.7.2.1). Grafts were also tested for failure rate. Failed grafts were those that came apart at tugging with tweezers. Examining effect of bud number on shoot development was restricted due to necrosis of scions and stocks. Grafts that failed displayed scion material that suffered necrosis whereas that of successful grafts suffered only one case of necrosis. Results suggest that either failure of grafting was a result of necrosis of the scion or that necrosis of scion occurs following failure of the graft union. It would appear that bud number had no effect on stock or scion shoot development. Increasing bud numbers on scion and stock led to no associated increase in shoot number on stock or scion. Likewise there appeared to be no discernible effect of bud number on scion and stock in relation to graft success. What is apparent is a possible influence on callusing of grafts due to increasing numbers of buds on scion and stock. This is expressed most where scions contain three buds and stocks contain two and three buds.

### 3.7.3 Influence on graft development due to presence of leaves on scion material

In total sixteen autografts were assembled to examine leaf effect. Table 3.7.3.1 outlines results of graft success, leaf presence, shoot development and callusing on scion and stock of *in vitro* grafts. Overall initial leaf number on scion had no influence on the success of graft union with 100% graft success for all combinations. Once again, successful grafts were deemed those that resisted tugging. No shoot development on scion or stock occurred in all combinations with stock being engulfed by callus. The absence of leaf material on stock may have been a contributing factor in callus development of stock. Further leaf growth was evident in two combinations with loss of leaf material from scion with original leaf number of four leaves. Callusing of graft union may be associated with presence of leaf material on scion.

| Initial bud number | | Graft Union | | Scion condition | | | Stock Condition | | |
|---|---|---|---|---|---|---|---|---|---|
| Scion | Stock | *Success Rate* | *Callus* | *Shoot development* | *Leaf Number* | *Callus* | *Shoot development* | *Leaf Number* | *Callus* |
| 1 | 1 | Success | SC | 1 (30 mm) | 5 | SCT | --- | --- | Engulfing Stock |
| 1 | 1 | Success | --- | 1 (15 mm) | 3 | SCT | --- | --- | MCB |
| 1 | 2 | Success | --- | --- | --- | SCT | --- | --- | SCB |
| 1 | 2 | Success | --- | --- | --- | SCT | --- | --- | SCB |
| 1 | 3 | Failed | --- | Necrotic Scion | --- | --- | 1 | 3 | SCB |
| 1 | 3 | Failed | --- | Necrotic scion | --- | --- | 2 | --- | Contaminated |
| 2 | 1 | Failed | --- | Necrotic scion | | | 1 (10 mm) | 4 | MCB |
| 2 | 1 | Success | --- | Contaminated | --- | --- | Contaminated | --- | --- |
| 2 | 2 | Failed | --- | Necrotic scion | --- | --- | 1 (25 mm) | 7 | LCB |
| 2 | 2 | Success | SC | 1 (10 mm) | 4 | SCT | --- | --- | SCB |
| 2 | 3 | Success | SC | --- | --- | --- | 1(10 mm) | 4 | SCB |
| 2 | 3 | Success | SC | Necrotic scion | --- | --- | 1 (10 mm) | 2 | MCB |
| 3 | 1 | Failed | --- | Necrotic scion | --- | --- | dead | --- | --- |
| 3 | 1 | Failed | --- | Necrotic scion | --- | --- | 1 (10 mm) | 1 | SCB |
| 3 | 2 | Success | SC | --- | --- | SCT | 1 | 10 | MCB |
| 3 | 2 | Success | SC | 1 (10 mm) | 4 | SCT | --- | --- | Engulfing Stock |
| 3 | 3 | Success | SC | --- | --- | --- | 1 (5 mm) | --- | LCB |
| 3 | 3 | Success | SC | 1 (5 mm) | 1 | SCT | --- | --- | MCB |

**Table 3.7.2.1 Influence on graft development due to presence of buds on scion and stock material**
**SC:** *Small callus*; **SCT:** *Small callus at tip*; **SCB:** *Small callus at base*; **MCB:** *Medium callus at base*; **LCB:** *Large callus at base*

| Original Leaf number | | Graft Union | | Scion | | | Stock | | |
|---|---|---|---|---|---|---|---|---|---|
| *Scion* | *Stock* | *Success/ Fail* | *Callus* | *Shoot number* | *Leaf number* | *Callus* | *Shoot number* | *Leaf number* | *Callus* |
| 1 | 0 | Success | SC | --- | 1 | SCT | --- | --- | ESt |
| 1 | 0 | Success | SC | --- | 1 | ESc | --- | --- | Est |
| 2 | 0 | Success | SC | --- | 6 | SCT | --- | --- | ESt |
| 2 | 0 | Success | --- | --- | 0 | ESc | --- | --- | MCB |
| 3 | 0 | Success | MC | --- | 2 | LCT | --- | --- | ESt |
| 3 | 0 | Success | SC | --- | 4 | SCT | --- | --- | MCB |
| 4 | 0 | Success | SC | --- | 0 | ESc | --- | --- | Est |
| 4 | 0 | Success | SC | Several buds | 1 | --- | --- | --- | MCB |

**Table 3.7.3.1 Influence on graft development due to presence of leaves on scion material.**

**SC:** *Small callus*; **MC:** *Medium callus*; **SCT:** *Small callus at tip*; **LCT:** *Large callus at tip*; **MCB:** *Medium callus at base*; **ESc:** *Engulfing the scion*; **ESt:** *Engulfing the stock*

### 3.7.3.1 Examination of graft development as influenced by presence of leaves on stock

Results obtained for the examination of presence of leaves on stocks are summarised in Table 3.7.3.1.1. Graft take was excellent with all ten auto-grafts being strong and successful grafts. All grafts displayed very slight callus formation at the graft union point. Development of buds and shoots from scions was good with six grafted scions producing shoots of 10 mm or more. Leaf growth on scions was good with several leaves being visible on growing shoots. In all but one graft there was no callus visible on scion material. One scion was necrotic. Stocks tended to have slight bud development with little or no shoot growth. Three stocks gave rise to shoots of 15 mm or more and leaf growth occurred on these shoots only. Remaining stocks had only one leaf remaining. Callusing was evident at base of stocks only. Overall it appeared that grafting of scion with no leaves to stocks with two leaves resulted in good graft development, with shoot development on the scion, some additional leaf growth and no callusing of scion. Stocks developed well with good leaf growth and slight to medium callusing of the base.

### 3.7.4 Examination of potential for heterografting of juvenile *Quercus robur* clone to juvenile *Quercus petraea* clone

Results of grafting of *Q. robur* to *Q.petreaea* are displayed in Table 3.7.4.1. Only one of the five heterografts of *Q. robur* scions grafted to *Q. petraea* stocks failed. Growth from scions of the successful grafts was poor with slight shoot. Callusing of the scion occurred in only one graft. Stocks displayed slight or medium sized callus at base and in one case stock was engulfed by callus. All grafts of the combination of *Q. petraea* scions grafted to *Q. robur* were successful. Scion and stock development was poor with two scions suffering necrosis and two producing small shoots. No callusing of the scion was evident. There was no leaf growth on scions.

| Original leaf number | | Graft Union | | Scion condition | | | Stock condition | | |
|---|---|---|---|---|---|---|---|---|---|
| *Scion* | *Stock* | *Success/ Fail* | *Callus* | *Shoot number* | *Leaf number* | *Callus* | *Bud/Shoot number* | *Leaf number* | *Callus* |
| 0 | 2 | Success | SC | --- | --- | ---- | 1 (30 mm) | 7 | MCB |
| 0 | 2 | Success | SC | 1 (15 mm) | 9 | --- | 2 | 2 | SCB |
| 0 | 2 | Success | SC | 1 (10 mm) | 1 | --- | 1 (5 mm) | 1 | MCB |
| 0 | 2 | Success | SC | 2 (15 mm) | 2 | --- | 1 | --- | SCB |
| 0 | 2 | Success | MC | 1 (10 mm) | --- | SCT | 1 (15 mm) | 1 | SCB |
| 0 | 2 | Success | SC | 1 (5 mm) | 1 | --- | 1 (15 mm) | 7 | SCB |
| 0 | 2 | Success | SC | 1 (10 mm) | 1 | --- | 2 | 1 | --- |
| 0 | 2 | Success | SC | 2 (NSc) | --- | --- | 2 | --- | SCB |
| 0 | 2 | Success | SC | 2 | --- | --- | 1 | 0 | MCB |
| 0 | 2 | Success | SC | 1(10 mm) | --- | 1 | 2 | 1 | SCB |

**Table 3.7.3.1.1 Influence on graft development due to presence of leaves on stock material with length of shoots in brackets.** **SC:** *Small callus*; **MC:** *Medium callus*; **NSc:** Necrotic at tip; **SCT:** *Small callus at tip*; **SCB:** *Small callus at base*; **MCB:** *Medium callus at base*;

| Set up of Scion and Stock | | Graft Union | | Scion condition | | Stock condition | |
|---|---|---|---|---|---|---|---|
| *Scion* | *Stock* | *Success/ Fail* | *Callus* | *Shoot number* | *Callus* | *Shoot number* | *Callus* |
| *Q. robur* | *Q. petraea* | Success | SC | 1 (NSc) | --- | 4 | -- |
| *Q. robur* | *Q. petraea* | Success | SC | 1 | STC | 1 | MCB |
| *Q. robur* | *Q. petraea* | Success | SC | --- | --- | --- | ESt |
| *Q. robur* | *Q. petraea* | Success | SC | 1 (NSc) | --- | 2 | MCB |
| *Q. robur* | *Q. petraea* | Failed | SC | NSc | --- | 1 | SCB |
| *Q. petraea* | *Q. robur* | Success | SC | 1(NSc) | STC | 1 | -- |
| *Q. petraea* | *Q. robur* | Success | SC | 1 | --- | -- | --- |
| *Q. petraea* | *Q. robur* | Success | SC | 1 | --- | --- | SCB |
| *Q. petraea* | *Q. robur* | Success | SC | 1 | STC | --- | SCB |
| *Q. petraea* | *Q. robur* | Success | SC | 2 | --- | 1 | MCB |

**Table 3.7.4.1 Influence on graft development due to presence of leaves on stock material with length of shoots in brackets. SC:** *Small callus*; **MC:** *Medium callus*; **NSc:** Necrotic scion; **SCT:** *Small callus at tip*; **SCB:** *Small callus at base*; **MCB:** *Medium*

Shoot and leaf development on stocks was poor with one stock producing a shoot which contained three leaves. A medium sized callus grew at the base of this stock. Results for the heterografting of *Q. robur* to *Q.petreaea* suggest that the technique tends to retard the growth of *Q. robur* material either when in scion or stock the effect on growth most emphasised when *Q. robur* acts as stock. There appears to be no discernible difference between the growth of *Q. petraea* in either stock or scion position.

### 3.7.5 Examination of potential for grafting of mature Quercus material to juvenile Quercus material

In the previous experiments grafting of *Q. robur* and *Q. petraea* material was examined and shown to be feasible for juvenile material. However the ability to graft mature clones to juvenile clones has not been established. a range of experiment were devised to investigate such grafting

#### 3.7.5.1 Examination of potential for homografting of mature *Quercus petraea* to juvenile *Quercus petraea* clone

10 homografts were assembled and cultured. All grafts were successful and strong. Shoot growth from mature *Q. petraea* scions tended to be good with good leaf and bud development. Stock material isolated from juvenile *Q. petraea* displayed good shoot development along with bud and leaf growth. Callus at base of stock was medium to large. No callus was evident on scion material. Results showed that mature material could be grafted *in vitro* to juvenile material.

### 3.7.5.2 Optimising the grafting of mature Quercus clones to juvenile Quercus clones

After 4 weeks all graft combinations displayed 80% union success rate (Table 3.7.5.2 1) except for that of plate 4 (Juvenile leafless scion to mature leafless stock) which yielded 100 % union success.

Grafts consisting of Mature leafy scions and Juvenile leafy stock displayed good bud growth with good leaf development on scions. Leaf material on juvenile stocks had grown poorly. Little bud development was evident with only one stock showing good shoot development (15 mm long shoot). Grafts consisting of mature leafless scions grafted to leafy juvenile stock yielded scions with poor leaf development, no callus and poor bud growth. The failed graft gave good leaf development and good bud growth. (2 buds). Stocks had good growth of buds and good growth of leaf material. Combinations consisting of Juvenile leafy scion, Mature leafless scion produced scions with good growth of material coupled with good bud development, good leaf development. Shoots produced on the scion grew to 35 mm is some cases as would be expected for juvenile material. Mature stocks in this combination gave in one case a shoot of 30 mm with 7 leaves with a second grafts giving a stock with 2 buds only. The remaining stocks were healthy in appearance. In total this plate gave two stocks that had developed somewhat. Graft combination of juvenile leafless scion to mature leafless stock yielded a 100% union success. Scions produced reasonable shoot growth with several leaves and slight callus at tip. Mature stocks did not develop well. In fact one stock was necrotic whilst only one stock produced any bud development. Remaining stocks showed no bud, shoot or leaf development.

| Combination | | Success/ Numbers grafted | Graft Condition | |
|---|---|---|---|---|
| *Scion* | *Stock* | | *Condition of scion* | *Condition of Stock* |
| M + L | J + L | 5/5 | Good bud and leaf development. 1 healthy shoot (10 mm) | Poor bud and leaf development. 1 stock displays good shoot growth, slight callusing of base |
| M - L | J + L | 4/5 | Poor development of scion, no shoot growth, poor bud development | Good leaf development, poor shoot developed |
| J + L | M - L | 4/5 | good growth of leaves and long healthy shoots | 2 shoots healthy, remainder of stocks poor with callusing of stocks. |
| J - L | M - L | 5/5 | Excellent leaf, bud, shoot development | Poor shoot leaf, bud, development. Slight callus of stock |

**Table 3.7.5.2.1 Summary of optimisation of grafting of mature Quercus clones to juvenile Quercus clones**

### 3.7.5.3 Grafting of Mature *Q. petraea* clone to *in vitro* rooted Juvenile *Q. petraea* clone

After 28 days in culture only one of the two grafts of MP1 grafted to rooted stocks succeeded. Scions of both grafts became necrotic at tips with no shoot, leaf and bud development. Roots of stocks of both grafts had elongated and in the case of the successful graft secondary roots had emerged. No bud, leaf or shoot development was evident on the rooted stocks. Control grafts were successful with bud and leaf development on scion and stock.

### 3.7.5.4 Serial grafting of mature material to juvenile material

Results for re-grafting of mature *Q. petraea* clone MP2 grafted to juvenile *Q. petraea* JP3 clone are summarised in Table 3.7.5.4. Three out of five graft combinations consisting of Leafy Juvenile scion grafted to Leafless Mature stock, graft were successful. However development of both scion and stock was poor with only slight bud development occurring on both the scion and stock. Good growth of leaf material was evident on one scion of successful grafts but all scions suffered necrosis. Stocks displayed no leaf development. Grafting of Mature Leafy scions to Juvenile Leafy scions was unsuccessful with all grafts failing. Leaf development on scions was slight with only one scion producing a shoot of approximately 5 mm. Stocks were also poorly developed

| Combination | | Success/ Numbers grafted | Graft Condition | |
|---|---|---|---|---|
| *Scion* | *Stock* | | *Condition of scion* | *Condition of Stock* |
| J + L | M - L | 3/5 | Poor bud, shoot development. Necrosis of scion tip | Poor bud, shoot development. Necrosis of scion tip |
| M + L | J + L | 0/4 | Poor bud, shoot development. Necrosis of tip | Poor bud, shoot development |

**Table 3.7.5.4 Summary of results of re-grafting of mature *Q. petraea* material to juvenile *Q. petraea* material**

### 3.7.5.5 Cascade grafting of material isolated from epicormic shoots derived from a mature *Q. robur* tree to juvenile *Q. robur* clone

There was a wide variation in the results of grafting shoots isolated from epicormic buds. Cultures isolated from branch section number 6 (pp6) gave rise to grafted scions and stocks that were fully necrotic although graft take was successful. Cultures isolated from branch section number 23 (pp23), close to crown of branch, yielded scions that displayed poor growth after grafting. Of the three grafted scions, two grafted successfully but both were overcallussed and necrotic. Stocks displayed similar traits. For graft of pp13 to juvenile stocks all grafts failed and suffered from complete callusing. Shoot growth, bud and leaf development was good from scions of grafts from clones pp7 and pp11. Stock development particularly of shoot and leaf was also good. Graft take was good for these clones with all five homografts of pp11 and all five homografts of clone pp7 being successful. All homografts of Cel line pp15 grafted successfully. However shoot, leaf and bud development was poor with only one scion producing a small shoot. Re-grafts or serial grafting was carried out with shoots isolated form scions of cultures of pp7 and p 11 as these yielded shoots with best growth and form. In total five homografts of shoots from pp7 were assembled. Five homografts were assembled from shoots that grew from the scions of pp 11 grafts. After 28 days of culture grafts were examined. All the grafts assembled for clone pp11 had failed and both scion and stock material had become necrotic. Of the five serial grafts from cultures pp7 all were successful and strong. Overall shoot, bud and leaf development was poor with two scions being completely engulfed by callusing. Two scions did produce shoots that had good leaf form. In total three shoots were obtained and these were subjected to a rooting treatment to examine potential to form roots *in vitro*. All three shoots failed to root but did show good growth *in vitro* without necrosis of shoot tips.

### 3.7.5.6 Grafting of mature *Q. robur* clone MR1 to juvenile *Q. robur* clone JR5

Grafts were examined after 28 days of culture. All grafts were successful and resisted tugging. The combination of Mature leafless scion grafted to Juvenile

leafy stock yielded a mature scion that was poorly developed with no bud, leaf or shoot growth. The juvenile stock was healthy displaying good shoot development (5 mm) with healthy leaves. Grafts of mature leafy scion to juvenile leafless stock resulted in a healthy scion with good leaf growth and slight bud development. The leafless stock had developed a shoot approximately 40 mm in length with healthy leaves and several buds developing. The control homograft of mature leafy scion to mature leafy stock gave rise to a very strong, neat graft union. Bud development was slight and no shoot or leaf development was evident on scion. The stock also displayed similar traits. Juvenile scions from graft of leafy juvenile scion to leafless mature scion were healthy with good shoot development. Shoots were approximately 30 mm and 10 mm respectively with healthy leaves. The mature stock contained leaf material but this had become necrotic. Bud development was poor. For juvenile leafless scion grafted to mature leafy stock, development of scion was poor with no bud, leaf or shoot development evident. Stock retained original leaves which were healthy. A shoot approximately 10 mm in length with healthy leaves developed.

### 3.7.6 Clearing of grafts

Previous studies have shown that the grafting of Quercus material in vitro is possible. The transmission of juvenile factors across the graft union have been cited as possible agents in the rejuvenation of plant material. Verification of the existence of vascular connections between grafted partners would enforce the possibility of such translocation occurring.

Successful grafts were cleared in order to visualise the formation of vascular connections between grafted partners. Figure 3.7.6.1 displays a 10X magnification image of a stained section of a cleared auto-graft of *Q. petraea* derived seedling clone(JP3). Vascular connections can be clearly seen crossing the graft union (GU) between the scion (SC) and the stock (St) across the graft section (Labeled A). Formation of new Xylem vessels can be seen on the right hand side of the stained graft section (Labeled B). Callus formed at the graft union can be seen in the form of sponge like cells or parenchyma. Figure 3.7.6.2 displays a 40 X magnified image of the area labeled A. Although difficult to see it is possible to visualise strands of vascular connections (VC) traversing the graft union (G.U.). Figure 3.7.6.3 displays 100X magnified image of area labeled A. New Xylem formation (X) is visible within the sponge like parenchyma layer between the graft union (GU). Clearly formation of vascular elements has occurred and in some cases has traversed the graft union.

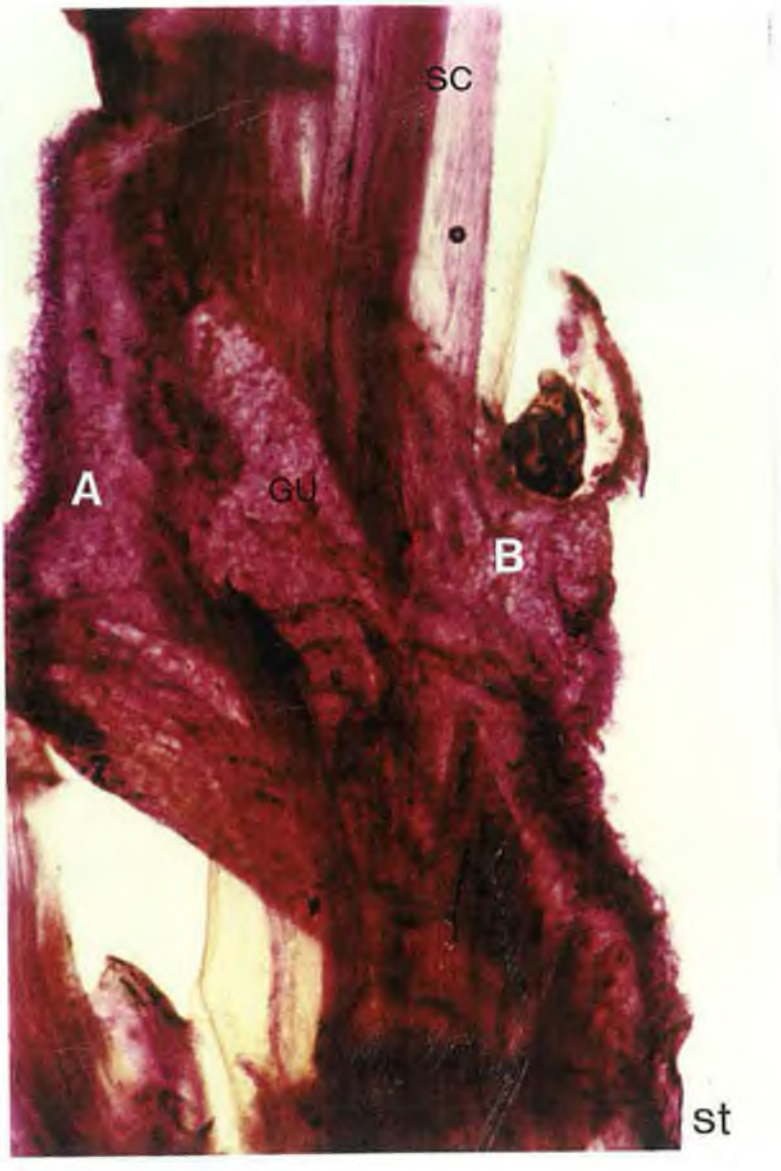



**Figure 3.7.6.1 10X magnification of the graft union (GU) of whole cleared graft union of *Q. petraea* auto-graft with scion (SC) orientated at the top and stock (St) orientated at bottom of plate**

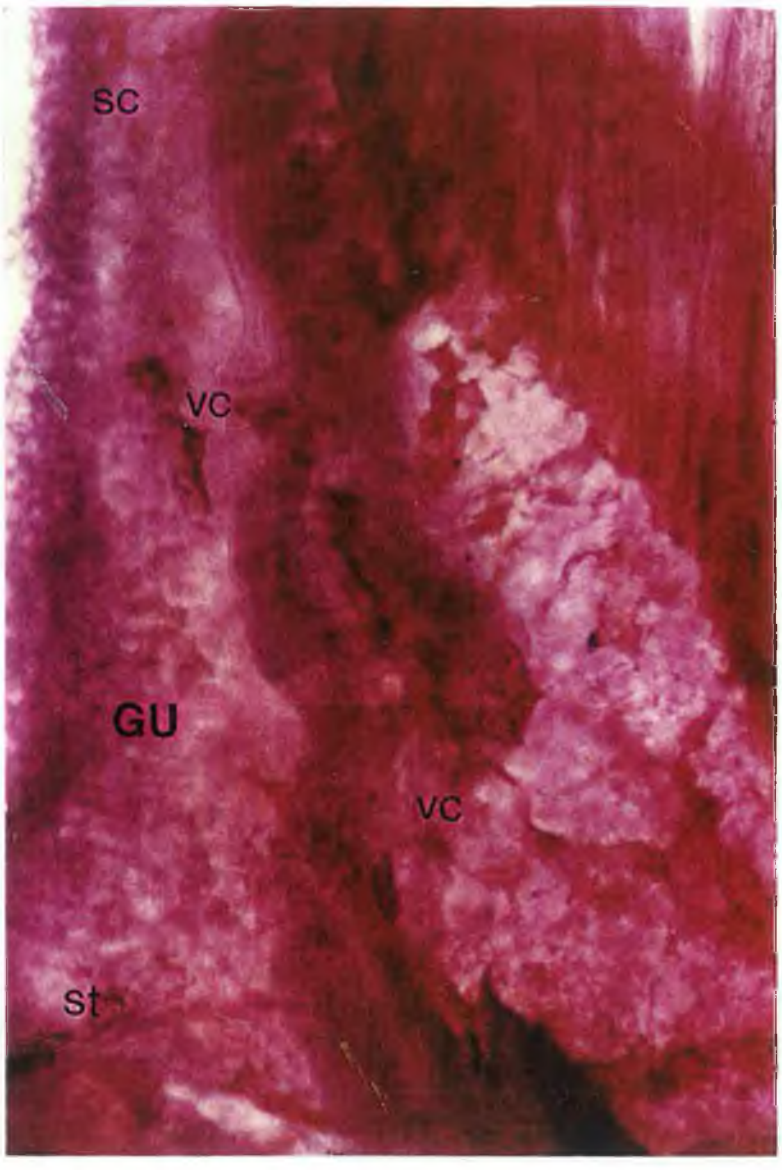



**Figure 3.7.6.2 40X magnification of graft union (GU) of whole cleared graft union of *Q. petraea* auto-graft. Vascular connections (VC) can be seen crossing the graft union between the (SC) orientated at the top of plate and stock (St) orientated at bottom of plate**


**Figure 3.7.6.3 100X magnification of graft union (GU) of whole cleared graft union of *Q. petraea* auto-graft. Formation of new Xylem vessels (X) can be seen at the graft union between the (SC) orientated at the top of plate and stock (St) orientated at bottom of plate**

# Chapter 4

# Discussion

Preliminary investigations of growth parameters in cultures of oak cultures (Ballester and Meir-Dinkel, 1992) have suggested that clones sourced from mature material are more difficult to initiate into culture, are less amenable to micropropagation, exhibit recalcitrant tendencies and are more difficult to root than juvenile sourced clones. Several authors (Arnauld, 1993; Franclet, 1983 ; Hackett, 1985) have proposed that such differences in physiological characters form the basis for a suitable system to distinguish between clones of juvenile and mature origin in woody species. However, the findings in this study do not suport this claim. Multiplication rates did not provide a suitable means to differentiate between juvenile and mature material since this was highly variable between successive subcultures for all clones. A similar finding was observed in studies on the *in vitro* propagation of *Q. robur* (San Jose *et al.,* 1988) where between each successive subculture, multiplication coefficients varied. Differences in both multiplication coefficients and shoots per explants were also visible between cultures of juvenile origin. San Jose *et al.* (1988) argued that shoot proliferation through *in vitro* culture depended on 'both the identity of the clone and the source of the explant'. Shoot cultures utilised in this study were isolated from several sources and by varying means which may account for the variability in multiplication rate. Meir - Dinkel *et al.* (1993) reported a large variation in shoot productivity between 52 juvenile *Q. robur* clones propagated under the same multiplication conditions and felt that between clone heterogeneity caused problems. Junker and Favre (1989) also found important between clone differences concerning the *in vitro* growth behaviour of 16 clones derived from juvenile seedlings. Evers *et al.* (1996) reported difficulties in assessing effects of mother tree age on *in vitro* micropropagation of *Q. robur* due to high variations. Propagation of shoot cultures entailed the use of nodal explants and shoot tips. Shoot tips were cultured with tips remaining and hence the influence of apical dominance may play a role in the variability of multiplication rates. Previous research has shown that in *Quercus*, nodal explants produce more new shoots and segments than apical explants (Vieitez *et al.*, 1985; San Jose *et al.,* 1988; Volkaert *et al.,* 1990). Hutchinson (1984) similarly observed that shoot tips, though commonly used, are not the best type of explant for routine maintenance of apple cultures due to the poor shoot production. The effect of episodic growth may also

have resulted in variability of data. Favre and Junker (1987) reported the episodic growth in *Q. robur* and this may lead to poor understanding of growth patterns in oak. The genetic stability of material under the various methods of micropropagation is poorly understood but it is evident that the axillary shoot system is genetically the most stable one (Tormala *et al.*, 1990). The amount of variation arising *in vitro* depends on several factors such as clone type, age of culture, selection of material or stresses in culture (Skirvin *et al.*, 1993). The question of variation occurring in the culture of clones in this study is speculative but it may contribute to the variability obtained in propagation data. Endemic contamination of *in vitro* cultured clones, both juvenile and mature, posed a significant problem in this study and resulted in either death or material that could not be used for further subculturing. The clonal propagation of *Quercus* material was further disrupted by the recalcitrant nature of mature clones which resulted in low vigour and propagation rates and lead to difficulties in attaining reasonable quantities of cultures for examination. It is clearly evident that, due to culture problems of oak material, the use of multiplication rates is not a suitable marker in assessing the rejuvenation of oak material.

Although a successful rooting protocol, based on those of Vieitez *et al.* (1985) and Manzanera and Pardos (1990), was developed, rooting results for shoot cultures were highly variable. Rooting was generally poor for all mature clones with variation in rooting ability of juvenile clones being evident for both *Q. robur* and *Q. petraea* species. This contrasts with the findings of Juncker and Favre (1989) where shoot tips of juvenile *Q. robur* cultures rooted at levels of 90% and higher. Clones from adult trees displayed strong within provenance variations in rooting ability from 20 to 95%. Vieitez *et al.* (1985) achieved rooting rates as high as 63.3% for adult material and 83.3% for juvenile material of *Q. robur*. In a further study Vieitez *et al.* (1989) further enhanced rooting of *Q. robur* juvenile clones to 83%. San Jose *et al.* (1988) obtained 100% rooting of cultures isolated from epicormic shoots of a 75 year old tree. Differences in rooting abilities between studies on rooting lies in the use of varying media and rooting treatments. Juncker and Favre (1989) established *in vitro* grown shoots on a reference media based on quarter strength Murashige and Skoog macronutrients supplemented with IBA (4.9μM) and

20 $gl^{-1}$ Sucrose, then rooted the shoot cultures on reference media containing 1$gl^{-1}$ activated charcoal instead of IBA. Vieitez *et al.* (1985) favoured dipping the basal ends of shoots in IBA solution of either 0.5 $gl^{-1}$ or 1 $gl^{-1}$ for a 2 minute period and then transferring shoots to an auxin free modified Heller media. In the enhanced study (Vieitez, 1989) shoots were dipped in 0.5 $gl^{-1}$ IBA for 8 minutes and transferred to a modified Greshoff and Doy media. San Jose *et al.* (1988) subjected shoot tips to a 2 minute dip in 1 $gl^{-1}$ IBA solution followed by a transfer to a modified auxin free Greshoff and Doy media. Along with variation in the rooting ability between juvenile clones, shoot tip necrosis presented considerable problems in this study. Material that suffered necrosis tended to develop poorly and in many cases died. Loss of such material affected the rooting studies and led to inconclusive results. Necrosis has been reported as a major disorder in the *in vitro* rooting of oak and chestnut species (Vieitez *et al.*, 1989) and is believed to be caused by a lack of cytokinin linked with the root inducing auxin treatment. Calcium deficiency in potato, birch, apple, elm, redwood and rhododendron cultures (Sha *et al.*, 1985) is also believed to be responsible for shoot tip necrosis. Treatments to prevent necrosis occurring include applications of BA to decapitated rooting shoot tips (Vieitez *et al.*, 1989), increased Calcium levels in rooting medium and increased vessel air exchange to facilitate translocation (Sha *et al.*, 1985). It is clear, that a rooting strategy which results in necrosis of material under study, does not lend itself to a successful discrimination between material of juvenile and mature origin. It is generally accepted that juvenile wood roots much more readily than mature wood (Leopold and Kriedmann, 1975) and several researchers have argued that rooting is a suitable bioassay for juvenility and rejuvenation (Bonga, 1987; Franclet, 1987; Hackett, 1985; Juncker and Favre 1989; Pliego Alfaro *et al.*, 1987). Considering that medium and rooting treatments vary from material of mature or juvenile origin and that necrosis disrupts rooting results, then findings cannot be fully conclusive. In addition, the variation in rooting ability of juvenile material between cultures isolated from the same clone and the ability of mature *Quercus* cultures to display 100% rooting (San José *et al.*, 1988), suggests that *in vitro* rooting of oak cultures is not a definitive parameter to define maturity or juvenility. Clearly further studies

of physiological traits are required in order to fully understand the events underlining their role played in maturation and provide better physiological markers for juvenility/maturity.

The morphological examination undertaken in this study provide useful parameters in following rejuvenation. Overall results indicate that some morphological traits may be used to differentiate juvenility and maturity in both *Q. robur* and *Q. petraea* but that different traits apply to each species. Most criteria examined for juvenility/maturity were highly variable. In the case of *Q. petraea* , shoot angle to the horizontal was the most clear-cut and least variable of the parameters. In addition, the characters of stem diameter at the apex, leaf number and stem length showed significant differences between juvenile and mature clones. The stem diameter at the mid point was the most useful criterion, whereas plagiotropic growth was observed *in-vitro* but was not a reliable character for the status of juvenility/ maturity.

Stump sprouts have been used as a means for the multiplication of mature trees. Their origin is uncertain, and it has been suggested (Hackett 1985) that they may arise either from quiescent axillary buds which were produced during the juvenile phase or may arise adventitiously by *de novo* meristem formation. In either case, buds from stump sprouts are regarded as having juvenile characteristics (Hackett, 1985; Franclet *et al.*, 1987; Greenwood, 1987). *Q. robur* clones which originated from stump sprouts of a 100 year old *Q. robur* have *in vitro* growth characteristics suggestive of juvenile status (shoot production and vigour) and in our quantitative analysis of their traits they yielded a negative discriminant score, for both clones, indicative of a juvenile status. This is consistent with the belief that stump sprouts have some juvenile morphological characteristics.

Severe pruning as a means of rejuvenation has been investigated in several plant species (Hatcher 1959, Garner and Hatcher 1962, Black 1972, Franclet 1979). From the work of Franclet and Garner and Hatcher, Hackett (1985) felt it unlikely that such a treatment yielded fully rejuvenated plants. The clone of *Quercus robur* derived from a hedge from which scions were grafted displayed mature characteristics *in vitro* (Marks and Simpson 1993), however the hedge showed some leaf retention in winter suggesting some juvenile traits *in situ*. Marks and Simpson

(1993) found that the cultures did not grow as quickly *in vitro* as material from a seedling which may suggest they possess a less than juvenile characteristic. This clone displayed a large mid stem diameter, a large diameter at the apex and gave a discriminant score that indicated a mature status. However this may be an indicator to a partial rejuvenation of this clone and it is possible that this clone may be further rejuvenated by *in vitro* serial grafting. The partial rejuvenation which can occur upon grafting is a factor which must be taken into account in examination of the discriminant scores for the mature and adolescent shoots. It proved impossible to culture shoot explants directly from the field, and all mature and adolescent clones were grafted on to juvenile rootstocks prior to culture. Results presented show that grafting (single grafting to 2 year old rootstocks) of mature and adolescent material did not give juvenile characters *in vitro*. However some variation in characters *in vitro* may be due to a partial rejuvenating influence of the grafting step. Despite this caveat, there were clear visible differences between the shoots of juvenile and mature origin.

Full maturation occurs upon attainment and maintenance of flowering and therefore is seen as the best indicator of maturity in woody species. In oak species the length of the juvenile period is estimated to be around 25-30 years (Clark, 1983). In this study, for the shoot culture of *Quercus petraea* derived from a 20 year old tree, a positive discriminant score was obtained suggesting a mature physiological status. In addition, traits of angle to the horizontal, length of shoots and tip diameter were consistent with those for mature material. Although flowering is the true indicator of sexual maturity it is possible that the 20 - 25 year old *Quercus petraea* had attained a morphological maturity prior to entering into sexual maturity. Unlike three clones of *Q. petraea* derived from 150 year old trees grafted to 2 year old seedlings, this clone was not difficult to culture and did not display recalcitrant characteristics such as phenolic exudation and it produced new vigourous shoots. This may suggest that for *in vitro* shoots, unquantifiable but obvious characters such as shoot vigour are a poor indicator of maturation state since all seedling derived clones showed juvenile vigour. Furthermore, our observations that a 20 year old tree showed mature characteristics *in vitro* implies that some or all of the maturational events had taken place within this potentially

short period. This may open the possibility of defining the time frames(s) of the main maturation process within a period of approximately 20 years. Furthermore, it provides the challenge of identifying a set of criteria to characterise intermediate morphotypes.

Using leaf morphology Rushton (1983) identified *Q. robur* and *Q. petraea* species in a population sample of oak trees from Northern Ireland. Kleinschmit *et al.* (in press) distinguished adult and juvenile trees growing in the field based on leaf morphology. The analysis however did not differentiate between material of different species. In this study on *in vitro* grown material, leaf morphological data obtained was highly variable and did not provide significant differences between juvenile and mature material of each species. The discriminant scores for juvenile and mature shoots based on leaf characteristics were not very useful due to the large overlap between data. A larger population of clones may need to be examined coupled with examining more samples and characteristics such as those used by Rushton (1983) may be needed to obtain differentiating traits between species and states of maturity. Although *in vitro* culture of clones eliminates problems associated with ecological effects on morphological examinations the maintenance of mature cultures *in vitro* is difficult due to low vigour and propagation rates and may lead to difficulties in attaining reasonable quantities of cultures for examination. The use of image analysis and the advent of more advanced software offers a powerful tool for the morphological assessment of leaf material. Being a machine based method it leads to a much greater reproducibility between operators and laboratories and a more rapid analysis (Adams and Thomas 1987).

It is clear that the developmental age of donor trees affects the vigour and morphological traits of oak *Quercus robur* and *Quercus petraea in vitro*. For both species there was a tendency to thicker stems and plagiotropic growth in mature shoots and to increased multiplication rate and shoot proliferation in juvenile shoots. These results are consistent with those found for other species of woody plants (Brand and Lineburger, 1992; Arnauld *et al.,* 1993). Despite the inherent variability in these characteristics there were significant differences between juvenile and mature shoots which allow us to define the juvenility status of the shoot in terms of a single number which may be easily quantified. The above

methods are therefore potentially useful in monitoring growth and development *in vitro* in terms of developmental status and of quantifying the effects of treatments on the rate of shoot rejuvenation.

Inter clonal variations observed in the previous studies maty be due to the heterogeneity of material. The use of epicormic buds sourced from the same branch offered a means to examine if such an influence could be bypased, It also provided a means to further study maturation in oak material and examine the influence of zonal position on phase status. The *in vitro* cultures of clones isolated from epicormic shoots displayed mature traits in culture. Micropropagation rates when compared with findings of previous micropropagation studies were low. In this study variation can be seen in the rates based on source. Those shoots sourced closest to the trunk have higher propagation rates. Rooting was obtained for only one clone that being sourced most furthest from the trunk. Evers (1996) found that rooting of epicormic shoots from branch sections of a mature *Q. robur* tree was possible and that the average effect of age showed no difference between 35 year old, 65 year old and 100 year old trees. He concluded that this indicated towards a rejuvenation of mature material. As previously suggested micropropagation rates and ability to form roots are not a suitable indicator for the identification of phase state in oak cultures. Given that these cultures have been obtained from branch sections of the same mother tree, then the differences between micropropagation rates and rooting may be attributed to zonal aging in the trees architecture. These results suggest that the most basal part of the branch can be considered to be the most juvenile zone of the branch. This concurs with the theories of Franclet (1983) and the findings of Evers (1996). Although the rates of multiplication tend to be higher for clones sourced from section closest to trunk clonal variability is evident between section close to one another. This variability enforces the belief that micropropagation and rooting are unsuitable indicators to discriminate between clones of mature and juvenile origin. The discriminant scores obtained indicate that these clones are clearly mature in phase. This result leads one to question the actual time frame when the epicormic buds were layed down. It may be possible that the branch itself developed at a time when the tree had begun its mature phase or that the epicormic buds were themselves laid down at a time when the branch was

mature in phase. This could be as early as 20 years, given that the clones sourced from an adolescent tree (20 - 25 years) in the morphological study gave discriminant scores indicative of a mature state. It is also possible that the clones from sections closest to the trunk are more juvenile but still mature and amenable to further 'rejuvenating' treatments. An improvement in the rooting and micropropagation rates of the clones following such a treatment could lead one to conclude towards a 'rejuvenation' *in vitro* of this material.

Examining isolated protein from juvenile and mature tissue by SDS PAGE analysis proved to be difficult and problematic with streaking of gels, negative staining of bands and poor staining of gels. Inconsistent banding on gels for most isolated proteins led to difficulties in summarising findings. However for clones of seedling and stump sprout origin, bands were visualised to allow comparisons to be made. Differences occurred in the protein patterns for both membrane and soluble associated proteins. Seedling material contained soluble proteins of 39 kDa. and 20 kDa. absent in stump sprout material. Membrane associated proteins isolated from stump sprouts displayed bands at 46 kDa. which was similar to that in seedling. Remaining bands in both seedling and stump sprouts were not common. Although stump sprouts scored negatively in the discriminant analysis, indicative of a juvenile status, apparent differences in banding patterns do occur between stump sprouts and seedling clones. Considering that these two clones have been isolated from separate sources and that stump sprout are considered to be a source of mature material displaying juvenile traits (Hackett 1985; Evers *et al.*, 1996), protein banding pattern differences are to be expected. Bonn (1988) detected a 16 KDa. membrane associated protein in juvenile tissue of *Sequoiadendron giganteum*. Material rejuvenated through micrografting also showed this protein, as did a clone isolated from a 30 year old clone. This would suggest the loss or inhibition of this protein in mature material. Considering that the stump sprouts displayed some proteins associated with true juvenile material then this observation may indicate towards differences in protein content between mature and juvenile oak material. Research to date has demonstrated that there are biochemical differences between material of juvenile and mature origin (Poethig, 1995) and it is probable that the same is true for *Quercus* and other forest species. From the first investigations of proteins by

disc electrophoresis ( Fukasawa, 1966), results have demonstrated clear differences between adult and seedling material. Following rejuvenation treatments of *Sequoia sempervirens* (Huang *et al.*, 1992), patterns of proteins separated by two-dimensional PAGE were distinguishable between juvenile or rejuvenated and adult *Sequoia* shoots. Apparent differences in protein phosphorylation patterns in *S. sempervirens* were distinguishable specifically the accumulation of phosphoprotein at 32 kDa. in adult tissue. A 31 kDa. phosphoprotein originally detected in juvenile tissue failed to show up in adult tissue. Protein banding in mature material could not be compared to juvenile material due to poor results in staining. Isolation of protein from plant material is fraught with difficulties (Jervis and Pierpoint, 1989) and is confounded by many differing factors. The extraction procedure used in this study, based on that of Bon (1988), isolated membrane and soluble associated proteins. Bon (1988) encountered purification difficulties caused by the presence of lignin, cutin and polyphenols in tissue homogenates. It is accepted that phenolics affect protein purification ( Mayer *et al.*, 1987), their exact effects being poorly understood. Further problems occur through the action of proteolytic degradation by proteases. Streaking of gels was a severe problem in this study particularly of mature material. Phenolic oxidation is considered to be a causative agent of such streaking in gels. The incorporation of phenolic complexing agents such as PVP and the use of protease inhibitors in extraction buffers may provide a means to alleviate such problems. However, Granier (1988) found that inhibitors of phenol oxidases failed to prevent such action. In the analysis of meristem tissue from several forest tree species by 2-D PAGE, Bon (1989) employed the use of varying extraction buffers but still encountered problems in protein detection, background staining and resolution. Mayer *et al.* (1987) also encountered similar problems. Even closely related samples from several cell suspension cultures of *Nicotiana tabacum* and *Dactylis glomerata* yielded grossly different patterns. The technique of silver staining whilst being highly sensitive is also problematic .The question about the relationship between staining sensitivity and protein composition remains (Dunn, 1993), as do practical problems such as ease of handling, background staining and most importantly reproducibility of results (Blum *et al.*, 1987). Silver staining has been shown to be generally less sensitive to basic proteins than neutral proteins and

when applied to basic proteins, unreliable and variable staining reactions have been observed. Employing a double staining technique of Coomasie Blue and Silver Staining (Irie *et al.,* 1982) may overcome such problems. Although this study proved to be problematic, electrophoresis is still a useful tool to examine fundamental difference in proteins between juvenile and mature tissue (Bon and Monteuuis, 1991). The use of methods such as high performance liquid chromatography (Jay-Allemand, 1988) or capillary electrophoresis provide a more accurate and precise analytical tool to examine protein differences. The findings of Woo *et al.* (1994) of two mRNAs that were differentially expressed in juvenile and mature tissue of *Hedera helix* indicate that studies at a genetic level are unfolding distinct differences in material in differing phases of growth. Control of development within a plant is at a genetic level and the techniques of molecular biology are providing tools that allow researchers to investigate the events that underline differentiation and development in plants (Bradley *et al.*, 1997; Moreau *et al.*, 1994; Nam, 1997; Nilsson and Weigel, 1997).

Previous research on graft formation via *in vitro* methods has resulted in the successful grafting of several woody plant species (Jonard, 1986; Pliego - Alfaro and Murashige, 1987; Navarro 190 ; Ewald and Kretzschmar, 1996). Several authors have described successful *ex vitro* grafting of *Quercus* species (Junker and Favre, 1989; Moon and Yi, 1993). Although no reports are cited in the literature, tentative studies have resulted in successful grafting of *Q. robur in vitro* (Gerry Douglas, Personal Communication). The study in this thesis was concerned with the *in vitro* grafting of *Quercus robur* and *Quercus petraea.* Numerous problems needed to be addressed such as contamination problems, a support system for grafted partners and isolating grafting partners with similar diameters. The contamination problem was alleviated somewhat through the application of stringent aseptic techniques and isolation of material free from visible contaminants prior to grafting. It is possible that stresses placed on grafted material during the grafting process resulted in the release of microbes that were endogenous to plant material. However contamination did result in the loss of several grafts in this study.

The proper attachment of scion to stock, and the protection of the union following initial assembly and during healing presented a major obstacle in the

successful grafting of *Quercus*. Several authors have examined differing methods to enable good graft assembly and adherence ranging from paper bridges (Obeidy and Smith, 1991; Huang and Millikan, 1980), to silicon tubing (Parkinson and Yeoman, 1982; Jonard *et al.*, 1983; Gelbhardt and Goldbach 1988; Richardson *et al.*, 1996).

A suitable support system for the *in vitro* grafting of *Quercus* was obtained by using an adopted method of grafting as developed by Parkinson (1983). Silicon tubing provided a sleeve that was easy to manipulate, suitable to sterilisation via autoclaving and allowed the grafting of material of differing diameters due to tubing elasticity. Using the adopted method of Parkinson (1983) *in vitro* grafting of *Quercus* was shown to be possible. Eighteen separate media combinations were examined. Graft success ranged from 80 to 100%. Basal media combinations comprising 0.02 $mgl^{-1}$ Benzyladenine gave rise to grafts displaying best graft take (seven out of nine tested preparations giving 100% graft success). The measurements of the parameters of bud length, tip and base diameter, bud and leaf number for both scion and stock were variable for all the varying combinations. Statistical analysis of the parameters indicated that scion bud number , bud length, leaf number, base diameter and tip diameter were all significantly effected by varying auxin and cytokinin levels in the treatments. As this study was concerned with the examination of the developmental potential through serial grafting the combination yielding the best scion and stock bud development coupled with graft development would be that most desirable. Hence the effect of apical and basal media on scion and stock bud development is important.

Apical media significantly affected scion bud number. This influence, of auxin and cytokinin, appears to be variable with no specific trends. The benzyladenine concentration in apical media interacting with the benzyladenine in the basal media led to an increase in apparent bud number on the scion. Scion bud length was significantly influenced by benzyladenine level in apical media being greatest at 0.2 $mgl^{-1}$. The interaction between the apical and basal media significantly affected the stock bud length with the greatest effect being exerted at a concentration of 0.2 $mgl^{-1}$ BA, 10 $mgl^{-1}$ IAA in the apical media with 0.02 $mgl^{-1}$ in the basal media. Cytokinins are believed to cause bud break (Devlin, 1975) whilst auxins are involved in cell elongation (Leopold, 1975; Skoog and Miller, 1957) and

hence it is expected that higher levels of IAA and BA would cause shoot elongation and bud break. Micropropagation studies of oak and woody species have shown that a BA level of 0.02 $mgl^{-1}$ is ideal for the growth of oak in culture (Ballester and Meir-Dinkel, 1992). BA in basal media is not shown to be significant in the development of scion buds. However this does not provide evidence of a non transmission of BA from the basal media to the scion as BA in the apical media interacting with BA in basal media significantly influences the scion bud number. Stock bud length is influenced by the interaction of apical and basal media. In basal media containing a lower level of BA (0.02 $mgl^{-1}$) it seems that increasing BA levels in apical media result in an increase in stock bud length at each separate IAA concentration. Of note is the observation that an IAA concentration of 1 $mgl^{-1}$ in Apical Media appears to inhibit the development of bud growth given that the lower concentration of IAA results in a higher bud length at all BA concentrations in apical media. Considering that IAA moves basipetally (Jacobs and Gilbert, 1983), it is possible that IAA is not crossing the graft union as successfully as expected. A second concept may be that at lower levels of IAA, BA is influencing the growth of stock buds and as an increase of IAA concentration gradient results in first an interaction between IAA and BA which causes an inhibition of stock bud length.

Graft success appears to be influenced by the presence or absence of buds and leaves on scion or stock. Shoot development on stock is related to the number of buds on stock. Hence for good shoot development on scion and stock bud numbers of 2 or more buds on both scion and stock are advisable. Leaf presence on scion whilst not affecting graft take appears not to encourage shoot growth. Although the original number of buds on scion and stock was not noted in this study it can be assumed that buds were present at the base of the petiole and shoot elongation could occur from these buds. It is possible therefore that leaves on the stock may play a role in the development of shoots on scions. Perhaps acting as sinks or energy sources for the transport of hormones and substances towards the apex regions. This is supported by the finding that grafts consisting of defoliated scions and stocks with two leaves attached resulted in the growth of shoots on scion. It is evident that *in vitro* grafting of *Q. robur* can be achieved with 100% success

and although leaf and bud presence do not effect graft take they may retard the growth of scion and stock buds.

The grafting of *Q. robur* to *Q. petraea* is possible. However heterografts indicate that growth of scion and stock are best where grafts consist *Q. robur* scions and *Q. petraea* stocks. The presence of incompatability factors between grafted partners and etiolation of grafted partners may be proposed as a cause for graft failure but clearly the success of one set of heterografts rules out such an occurrence. Stoddard and McCully (1979) noted that in graft studies of pea roots that above graft tissue are at a nutritional advantage over those distal to the union, such advantages being correlated with hormonal differences. It is possible that in the case of grafts consisting of *Q. robur* scions and *Q. petraea* stocks a similar event occurs where hormones produced by one of the species in situ plays a significant role in the development of graft and subsequent growth of material. Gerbhardt and Goldbach (1988) observed that cell elongation after micrografting, in cherry heterografts, was related to the vigour of the scion and stock genotypes The nature of such phenomena has not been established in this study and warrants further investigation.

The *in vitro* grafting of mature *Quercus* material was problematic but achieveable. Micropropagation of mature *Quercus* cultures was difficult and led to low yield of mature material. Due to a lack of suitable material, the study of grafting of mature to juvenile material in this thesis should be considered as tentative and exploratory. Mature *Q. petraea* could be successfully grafted to juvenile *Q. petraea* with good growth of scion and stock material. From optimisation studies on the set up of scion and stock for grafting of mature *Q. petraea* to juvenile *Q.pertaea* it can be concluded that the combinations of mature leafy scions grafted to juvenile leafy stocks and juvenile leafy scions grafted to mature leafless stocks seem to ensure good growth of shoots from mature material. As to why such combinations are successful is uncertain. It may be due to the presence of leaf material providing stored nutrients and hormones to facilitate the requirements to allow shoot development. Yet the lack of leaf material on mature stocks seems not to effect the graft take or growth. Doorenbos (1955), in studies examining varying graft combinations of *Hedera helix*, showed that a combination of mature leafless stocks

grafted to juvenile leafy scions resulted in the rejuvenation of the mature stock. He argued that the juvenile leaves stimulated the process. This may also be the case in terms of graft take and shoot development in *Quercus petraea* but Juvenile leafy stocks do not encourage the growth of mature leafless scions. Clark and Hackett (1980) researched the presence of leaves on substance tranlocation across a graft union and noted that when mature leaves are retained little or no interchange between partners occurs. They hypothesised that the mature leaves acted as competitors for assimilates thus preventing the movement of material across the union. This is an important consideration in the case of rejuvenation of studies through cascade grafting as translocation of any 'rejuvenating factor' would not occur if mature material in a graft contains leaf material. Another scenario may involve the initial manipulation of the graft in assembly of partners. Although Doorenbos displayed rejuvenation of mature material through grafting after one graft, in this study one graft would not enable any conclusion to be made. In addition the combination of a leafless mature scion grafted to a leafy juvenile stock failed to give good growth and would not provide ample material for further grafting.

The technique of cascade grafting has been shown to rejuvenate other forest species and may also be suitable for such in *Quercus*. Both the above combinations, mature leafy scions grafted to juvenile leafy stocks and juvenile leafy scions grafted to mature leafless stocks, yield shoots growing from material of mature origin and provide ample material for further grafting. A second round of grafting was carried out using shoots isolated from mature material from above study. Mature material subjected to a second graft in the combination of mature leafy scion grafted to juvenile leafy scions failed to graft successfully. Mature leafless stocks grafted to juvenile leafy scions resulted in a 60 % graft union formation. The development of the mature stock was poor with no bud development on scion or stock. This may indicate to a reversion to a more recalcitrant form for mature material. It may also suggest of the transmission of mature factors across the graft union toward the juvenile material inhibiting the growth of juvenile tissue. The latter is less feasible when one considers the previously discussed hypothesis of Clark and Hackett (1980).

Grafting of material isolated from epicormic shoots resulted in poor growth of mature scions and juvenile stocks. The poor growth displayed may be a result of an aging factor or the presence of incompatibility factors between material of juvenile and mature origin. Only two clones yielded mature shoots suitable for regrafting. Whilst shoot cultures isolated from epicormic buds of the *Q. robur* tree suffered recalcitrant tendencies on the second round of *in vitro* grafting, two scions, of epicormic bud origin, did produce three shoots with good growth characteristics. These clones were sourced from sections of branches (section 7 and 11) which were close to tree trunk. As previously discussed the most basal part of the branch can be considered to be the most juvenile zone of the branch. It is plausible that epicormic buds from such regions are more prone to rejuvenation through *in vitro* cascade grafting. The failure of these shoots to root does not detract from such a possibility as rooting is by no means a sufficient indicator of juvenile status. Assessing the morphological characteristics of these shoots after *in vitro* culture and cascade grafting may provide a useful means to determine the degree of rejuvenation these shoots may have undergone. Whether or not these cultures are amenable to further cascade grafting is open to question. What is evident is the loss of material at each stage. The potential for complete loss of material at the next graft cannot be ignored nor can the possibility of a reinvigoration of shoot development and an increase in the growth of mature sourced material.

The results of different graft combinations of mature and juvenile scions and stocks with or without leaf material show that mature *Q. robur* clones can be successfully grafted *in vitro*. However the ability to produce viable shoots suited to further grafting has not been established in this study. Furthermore the lack of mature material resulting in an insufficient numbers of grafts being used for each combination renders the formulation of a conclusion difficult. To say that any of the combinations in the study of grafting mature *Q. robur* are the best suited would be purely speculative. But when coupled with the observations for grafting of *Q. petraea*, the combinations of mature leafy scions grafted to juvenile leafy stocks and juvenile leafy scions grafted to mature leafless stocks seem to ensure good growth of shoots from mature material.. The findings presented in this thesis further emphasise the importance of manipulating assimilate sources and sinks in graft

studies and the importance on the translocation of rejuvenating factor from juvenile to mature tissues in a graft.

The formation of vascular connections across the graft union is a definitive indicator that graft take has occurred. More importantly in relation to the rejuvenation of mature material, the formation of such vascular tissue is a prerequisite to transmission of a rejuvenating factor. The clearing of successful grafts in this study showed vascular connections crossing the graft union and the formation of new xylem vessels. This would suggest that in *Quercus* the transmission of factors across the union could occur and lead to rejuvenation of mature material. Warren Wilson (1982) argued that vascular connections across graft unions were capable of transporting water, minerals and hormones whilst Clark and Hackett reported the translocation of $^{14}CO_2$ labeled assimilates across the graft union in *Hedra helix*. Parkinson ( 1983) demonstrated the transport of colloidal iron oxide particles across the graft union in *N.physaloides* and *L.esculentum* homografts. Clearly a similar occurrence is possible in *Quercus* homografts and heterografts. Displaying the ability to transport such Iron Colloidal particles in *Quercus* would further reinforce this theory. The existence of a factor that can translocate across the graft union and induce a rejuvenating effect has yet to be established but the work of Bon (1988) in finding a 16kDa shoot apex protein related to juvenile *S.giganteum* and finding the same in material rejuvenated through grafting (Bon and Monteuuis, 1991) suggests the transmissibility of a signal through cells of the graft union. Finding a similar molecule in juvenile *Quercus* material and exhibiting its ability to cross a graft union would greatly enhance this belief.

Although suggesting that physiological methods such as rooting and growth rates are not a suitable means for assessing juvenility/maturity status of oak material, the identification in this study of clear differences in morphological characteristics between juvenile and mature oak material allows a discriminant scoring system. Discriminant analysis provides the means to follow the progress of phase change in oak material and an adapted method may be developed for several other forest tree species. The observation that a 20 year old tree showed mature characteristics *in vitro* implies that some or all of the maturational events had taken

place within this potentially short period. This is highly important in that it opens the possibility of defining the time frames of the main maturation process within a period of approximately 20 years. This finding reinforces the usefulness of discriminant analysis in aiding the understanding of the complex set of events that underlie phase change. Of equal importance is the development of a successful *in vitro* grafting protocol for Quercus material. Such a technique will strongly contribute to developing a 'rejuvenation' technique that will allow for mass clonal propagation of 'Elite' Oak.

**Future experimentation**

Several areas can now be examined in further detail in any future undertakings

The examination of morphological characteristics of several other woody species and the development of a Discriminant Score.

The examination of *in vitro* growing oak cultures over a large number of successive cultures to establish if their Discriminant Scores and morphological or physiological characters alter.

Applying techniques of Molecular Biology and Capillary Electrophoresis to isolate distinct genetic and protein differences between material sourced from juvenile and mature oak clones.

Further enhancement of the cascade grafting technique to examine rejuvenation of mature oak material.

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