Early Ontogenetic Male Cone Production in *Pinus radiata*

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Abstract

The tree breeding industry is interested in early ontogenetic male cone production in *Pinus radiata* in order to maximise the rate at which successive generations can be bred. The foundation of this thesis was a study of how male cone production differed in various contrasting regions around New Zealand. A study was then carried out to assess whether various morphological and anatomical characteristics of trees were correlated with the onset of male cone production. Various treatments including stress, plant growth regulator application and grafting were examined to determine whether any of these could be used to promote early ontogenetic male cone production.

The regional study found that male cone production commenced at age 3 in Nelson, at age 4 in Northern and Southern Kinleith, and age 6 in Karioi and Northland. Findings suggest that high sunshine hours and low autumn and/or winter temperatures are of significance to the precocity of male cone production. A tentative logistic model was developed that may adequately describe cone production across all regions.

Morphological and anatomical characteristics of trees were used to develop a model which predicts the probability that a tree will *not* be producing male cones. Relative cell number was found to provide the greatest ability to predict whether or not a given tree will be producing male cones, supporting the hypothesis that a certain number of cell divisions are required before male cone production commences.

Grafting did not promote male cone production in the present study. It is recommended that future grafting experiments for the purposes of promoting male cone production should reconsider the position within the crown at which grafts are made.

Male cone production on fascicle cuttings approximately two years old was promoted by growing them under stress in small pots, with minimal watering and no fertiliser application. It is suggested that a "stress" pathway, distinct from the "normal" male cone production pathway is probably involved. Male cone promotion in fascicle cuttings appears to be enhanced through the application of ABA, but not other plant growth regulators. Additionally, male cone production in fascicle cuttings appears to be enhanced by providing relatively high levels of light.

The outcomes of this study suggest that a lack of male cone production in young trees may not be the most serious impediment to the breeding program. Instead, the onus may be on the ability to make superior selections at a younger age than occurs at present.

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Chapter 1

General Introduction and Literature Review

1.1 General Introduction

With 333 000 hectares, as of June 1998, Carter Holt Harvey Forests (CHHF) is the largest plantation owner in New Zealand (MAF, 1999). As with all commercial companies, CHHF has a vested interest in improving the efficiency of its operations, in order to maximise profits. Toward this end CHHF operate their own tree breeding program, and are among the world leaders in this field.

Although micropropagation and molecular techniques are advancing rapidly, sexual crossing of "superior" parents is still the major means of tree improvement for *P. radiata*. The process is hindered by the period of time taken for each successive generation to become reproductively competent; in the case of *Pinus radiata*, this is several years. It is desirable to reduce this time lag through the promotion of precocious cone production. In *P. radiata*, there is thought to be an "adolescent phase", prior to maturity, during which trees are capable of becoming reproductive, but for some reason do not do so. With appropriate stimuli, it is thought that adolescent trees may be coerced into carrying cones relatively early (Hackett, pers. comm.[1]).

The present thesis, funded through a GRIF fellowship in conjunction with Carter Holt Harvey Forests, investigated means of inducing precocious male cone production in *Pinus radiata*. The remainder of Chapter 1 is a thorough review of literature that is pertinent to this thesis. There is an apparent lack of information in the literature pertaining to the age at which male cone production commences in *Pinus radiata*.

This problem is addressed in Chapter 2, which covers an investigation of the age at which male cone production commences in several contrasting locations around New Zealand. The study discussed in Chapter 3 builds on the preceding chapter by attempting to correlate morphological and anatomical characteristics of trees with the onset of male cone production. Chapter 4 explores the application of plant growth regulators to root restricted fascicle cuttings, with the aim of inducing precocious male cone production. Chapter 5 deals with grafting of "adolescent" scions onto both seedling and physiologically mature rootstocks, also with the aim of inducing male cone production.

1.2 Pinus radiata Background

1.2.1 Classification

At present in New Zealand, *Pinus radiata* D. Don is commonly called radiata pine. It has in the past been known by a multitude of other names, including Monterey pine, *Pinus insignis* Dougl., *P. montereyensis* Rauch., *P. californiana* Loisel (Farjon, 1984; Millar, 1986), *P. tuberculata* D. Don, *P. insignis* var. *radiata*, *P. insignis* var. *laevigata*, *P. radiata* var. *tuberculata* (Forde, 1964d; Millar, 1986), *P. adunca*, *P. monteragensis* (Bannister, 1973; Millar, 1986), *P. Sinclairii*, *P. insignis macrocarpa* (Millar, 1986) and insignis pine (Matziris, 1995). This bewildering array of names is largely due to poor communication between 19th century pine botanists, and hence redundancy of naming. Additionally, prevailing concepts during the 19th century meant that slight phenotypic variants were considered different species (Millar, 1986); considering the large phenotypic variation displayed by *P. radiata* it is not surprising that this situation arose. All of these are now reduced to synonymy under the current legitimate name *Pinus radiata* (Forde, 1964d; Millar, 1986).

It seems likely that *P. radiata*, and the other Californian closed-cone pines evolved from the Late Miocene fossil species *P. foisyi*a (Millar, 1992), or a similar species. As

[1] Wesley Hackett, Emeritus Professor, University of California, international expert on phase change, Personal communication as cited in Carter Holt Harvey Forests GRIF application.

with many plants the phylogenetic relationships between members of the genus *Pinus* are controversial. However, from the tree breeder's perspective, Duffield's classification system based on hybridisation experiments appears noteworthy (Duffield, 1952, cited in Mirov, 1967). According to this, *P. radiata* will hybridise with, and is therefore most closely related to, *P. muricata*, *P. attenuata*, *P. greggii*, *P. pringlei*, *P. patula* and *P. oocarpa*. This group was later classified as subsection *Oocarpae* (Millar, 1986). To the tree breeder, the ability to freely hybridise *P. radiata* with these other species represents an abundance of opportunities, for overcoming problems, or mitigating potential problems. Unfortunately, the repeatability of Duffields hybridisation experiments have been questioned (Millar, 1986).

P. radiata, *P. muricata* and *P. attenuata* are all present in the extant California flora (Harlow *et al.*, 1996). However, interestingly, *P. greggii*, *P. pringlei*, *P. patula* and *P. oocarpa* are species of central and southern Mexico; a distance of 640 km separates the Californian closed-cone pines from those of central and southern Mexico (Millar, 1986). This disjunction between these related species implies ancient links between the Californian and Mexican species (Axelrod, 1977).

1.2.2 Natural Range

Pinus radiata in its extant natural range is restricted to three small, isolated, coastal central Californian populations (Ano Nuevo, Monterey and Cambria), and two small island populations (Guadalupe and Cedros) off the coast of Mexico (Figure 1.1). Trees from the different populations are sometimes referred to as different varieties: those from mainland Californian as *P. radiata* var. *radiata*, those from Guadalupe as *P. radiata* var. *binata*, those from Cedros as *P. radiata* var. *cedrosensis* (Shelbourne *et al.*, 1979). The total combined area of these natural populations is less than 8000 hectares (Bannister, 1966; Matziris, 1995; Scott, 1960).

Figure 1.1: **Right:** *Natural populations of Pinus radiata.*
1. Ano Nuevo; 2. Monterey; 3. Cambria; 4. Guadalupe; 5. Cedros.
Left: *Numbered arrows show equivalent Southern Hemisphere latitudes*
(reproduced from Bannister, 1973)

The fossil record shows that the range of *P. radiata* was once much greater (Mason, 1932, Axelrod, 1977; Forde, 1966), providing at least in part an explanation for its seemingly anomalous relationship to the Mexican pines. Reasons for the decline in the species range are uncertain (Vogl *et al.*, 1977). However, several hypotheses may be advanced. Mason (1932) implies *P. radiata* may be in the process of extinction with his statement "*Pinus radiata* belongs to the Pleistocene and apparently is fast disappearing". Axelrod (1977) and Forde (1966) ascribe the shrinkage, and fragmentation of its range to the coastward spread of a drier, hotter climate during the Xerothermic (the period after the last ice age, 8000-4000 years before present). Other factors suggested as limiting to its range are soil type (Forde, 1966; Lindsay, 1932) and the limits of summer fog (Forde, 1966). Forde (1966) summarises the situation with the statement "The critical environmental factors which have allowed

this species to persist in its present locations but not in other apparently suitable habitats are not entirely understood".

Having such a small extant natural range, it might be expected that *P. radiata* inhabits a very narrow variety of environmental conditions. However, this is not the case. The range of *Pinus radiata* covers 9° of latitude, from the mainland Ano Nuevo at 37° North, to Cedros island at 28° North (Bannister, 1966; Figure 1.1, right). To put this into perspective, New Zealand spans approximately 12 degrees of latitude, from 35° to 47° South (Figure 1.1, left).

Mean annual rainfall for most of the mainland range is approximately 400 to 900 mm. However, rainfall is unreliable and may be considerably lower in some years (Scott, 1960). On Cedros Island, mean annual rainfall is only 250 mm (Matziris, 1995), and a period of fourteen months without rain has been recorded (Bannister, 1966). Throughout the range, rainfall is complemented by high humidity (Scott, 1960) and fog drip, especially in summer (Burdon and Bannister, 1973). The summer fog is the one climatic condition which is constantly present across the range (Bannister, 1973). As mentioned earlier, the bounds of this summer fog may be a major limiting factor for the survival of *P. radiata*.

Within the range, mean maximum temperature is 21°-27° C, and mean minimum temperature approximately 4° C. Absolute minimum temperatures are considerably lower. For example, in Monterey, temperatures as low as -4° C occur, with an average of 50 nights of frost per annum (Scott, 1960).

P. radiata, in its natural range, grows in a fairly wide range of different soil types. These range from skeletal soils at Cedros Island, and infertile sandy and clay loams at Monterey, to a (presumably) fertile basaltic loam at Guadalupe (Burdon and Bannister, 1973; Matziris, 1995). However, it is apparent that the species may be intolerant of heavy clay soils (Lindsay, 1932). A good example of evidence for soil type being limiting to *P. radiata* is at Cambria. Here the limits of the *P. radiata* forest correspond strikingly with the boundaries of the Arnold sandy loam soil type (Lindsay, 1932; Forde, 1966).

Notwithstanding the presence of summer fog across its entire range, it is apparent that *P. radiata* is tolerant of a fairly wide variety of environmental conditions. Perhaps the most compelling evidence for this is the fact that the species northern limit is bounded by Douglas fir, and its southern limit by cacti (Bannister, 1973). Comparing trees from the different populations, there is an associated variability in their anatomy (Forde, 1964b; Forde, 1964c), morphology, wood properties, frost and disease tolerance, and susceptibility to boron deficiency (Burdon and Bannister, 1973). The species is also extremely variable within each population (Forde, 1964a). It is apparent that much of this variability is genetic, a situation which is most fortuitous for breeding purposes.

1.2.3 As an Exotic

In its natural range *P. radiata* is of little or no commercial value, but it is used for decorative purposes in many Pacific slope cities (Harlow *et al.*, 1996). Conversely, the species is grown commercially on a large scale in countries including Australia, Chile, New Zealand, South Africa, and Spain (Scott, 1960). In these countries it is an important source of lumber, pulp, paper, and other wood products (Forde, 1964a). *P. radiata* is also grown, or has in the past been grown, to a lesser extent in other countries including Kenya, Malawi, Tanzania, Uganda, Bolivia, Brazil, Equador, United Kingdom, and Italy (Scott, 1960).

According to Forde (1964a), the primary reason for its prevalence is "that in areas of suitable climate Monterey pine is inexpensive and easy to establish, and outstanding among softwoods in growth rate and productiveness". Other major reasons probably include its hardiness, and the utility of its general purpose wood.

1.2.4 In New Zealand

It is difficult to ascertain exactly how and when *P. radiata* arrived in New Zealand. David Douglas, the notable Scottish explorer and botanist introduced *P. radiata* into

England in 1833, and within four or five years seedlings were being sold by nurserymen in England, France, and Germany (Bannister, 1973). Bannister (1973) suggests that wealthy Canterbury settlers may have imported seedlings from England in the early 1840's. It is possible, although unsubstantiated, that a Dunedin nurseryman was selling *P. radiata* as early as 1845 (Bannister, 1973). It was reported to have been planted in Canterbury at Acland's Mount Peel Station in 1859 (Barr, 1996). A general consensus is that miners arriving from California during the gold rush would have established trees they brought with them, including *P. radiata*. The 1858 *Planting of Trees Ordinance* in Canterbury appears to be the earliest New Zealand legislation aimed at encouraging tree planting (Roche, 1987).

By around 1890 the first harvests of *P. radiata* occurred, and the useful traits of the species began to be appreciated. It was also becoming apparent that the indigenous forest resource could not meet the countries demands indefinitely (Scott, 1960).

Organised planting by the State of *P. radiata* and other exotic conifers began in about 1900, and continued on a small scale until 1923 (Scott, 1960). A planting boom then arose, extending from 1923 to 1936, during which around 240 000 hectares were established. Scott (1960) states that this was largely motivated by "exaggerated ideas of financial profits to be made from afforestation as put forward by company promoters and bond sellers". Additionally, there was strong motivation by the Forest Service to produce an exotic substitute for indigenous forest products. At the time, approximately 40% of State and 85 to 90% of private plantings were *P. radiata* (Scott, 1960).

From the late 1930s until 1960, a period of indifference to exotic tree planting occurred. During this period planting averaged only 2800 hectares per year (Sutton, n.d., cited in Bilek, 1996). The main reasons for this were a general wood surplus, and low wood value. The wood surplus was largely a result of the yield of *P. radiata* being higher than expected. This oversupply, coupled with State policy to sell wood cheaply to Tasman Forestry Ltd., in which the State was a major shareholder, accounted for the low wood prices (Bilek, 1996).

In 1959 it was forecast that New Zealand's forests would be incapable of meeting the country's demand for forest products, in the long term. Additionally, the potential to increase exports was recognised (Bilek, 1996). The government increased planting, and encouraged private planting through incentive schemes. A second planting boom resulted, starting in 1961. In 1987 the Labour Government began to phase out the incentives, and introduced a "cost of bush" tax regime which was unfavourable to forestry (Bilek, 1996). Planting rates slumped dramatically.

The National Government changed the tax regime pertaining to forestry yet again in 1991 (Bilek, 1996). This change had a positive effect, and a new planting boom occurred, lasting until the present day.

Currently, in New Zealand, *P. radiata* is by far the dominant exotic species grown in commercial plantations. As of April 1998, there were 1 520 000 hectares of the species, representing 90.5% of New Zealand's total exotic production forest area (MAF, 1999). The planting rate for most of the 1990's has been well in excess of 50 000 hectares per annum, and in the outstanding 1998 year approached 100 000 hectares (MAF, 1999).

1.3 Breeding of *Pinus radiata* in New Zealand

1.3.1 Nomenclature

In New Zealand, a system of numbers is used to designate a particular *Pinus radiata* clone, for example "268.405". The first three digits are referred to as the clone's "series number". The first digit of the clone series number refers to the region of origin, based on the old New Zealand Forest Service conservancy code (2 = Rotorua, 6 = Canterbury, 7 = Southland), or if 8 signifies other selection programs undertaken, nation-wide (Shelbourne, 1986a; Burger, pers. comm.[2]). The second and third digit of the series number refer to the year in which the selection was made (Shelbourne,

1986a). The digits following the point refer to the particular clone within the series. Thus, 268.405 is clone 405, selected in 1968 from around Rotorua.

1.3.2 Background

In this thesis, tree breeding is defined as in Thulin (1957), to be all measures directed towards the improvement of quality and yield of trees by the regulated production of desirable offspring.

Tree breeding began in New Zealand with the establishment of the Forest Research Institute in 1951 (Thulin, 1957). As previously described, by this time *P. radiata* had already been grown in New Zealand by the State for half a century, and for much longer by private growers (see Roche, 1987). It is thought that all of the early plantings were of mainland (California) provenances; the first known New Zealand plantings of Guadalupe and Cedros provenances occurred in 1962 and 1964 respectively (Libby *et al.*, 1968). According to Burdon and Bannister (1973), natural and silvicultural selection during the early days may have provided a head start with genetic improvement. Accordingly, the New Zealand *P. radiata* population can be considered to be distinct from the Californian provenances from which it originated (Burdon and Bannister, 1973).

Based on morphological evidence, it appears that there is an extremely wide genetic variation within each mainland California population; with the exception of four characteristics, tree-to-tree variation accounts for more than 70% of the total variation of the species (Forde, 1964d). More recently, these findings have been substantiated, based on allozyme variation (Millar 1988). Biochemical characteristics, such as allozyme variation are more reliable than morphological traits for studying genetic variation. Thus, even if many of New Zealand's early seedlots were from only one, or a few local areas of just one population, a reasonably wide genetic base would still ensue. As it happens, evidence reveals that the genetic composition of

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[2] Fred Burger, Manager tree breeding, Carter Holt Harvey Forests. Personal communication 24 February 2000.

New Zealand's *P. radiata* population is around 55% Ano Nuevo, and 45% Monterey provenances (Shelbourne *et al.*, 1979).

1.3.3 Breeding Programs

The initial phase of New Zealand's *P. radiata* breeding program began with an intensive program of "superior" tree selection, during the 1950s (Shelbourne, 1986a). Right from these early days, Ib Thulin appears to have had a very clear view of what he considered to be a superior tree, and states "...it is considered that the highest-quality timber is cut from trees with light multinodal branching, straight stem form, and high heartwood content. A tree which combines these attributes with above average growth rate and resistance against disease will come very close to the ideal tree type" (Thulin, 1957). Selections made in the 1950's from plantations around the country would have been to meet these requirements. This selection program was very intense, with about one tree (ortet) being selected per 100 hectares (Shelbourne, 1986a). These clones were registered as the "850" series (Shelbourne, 1986a).

Grafted ramets from the "850" series were used to establish seed orchards, at several locations. The earliest orchards established at Kaingaroa and Gwavas were based on just 14 clones, but this number was eventually increased to 36 clones. Another orchard was also established at Waimihia utilising 36 clones (Shelbourne, 1986a). For a detailed discussion of New Zealand seed orchards see Vincent (1986). In the "850" series orchards, mortality due to graft incompatibility was as high as 60% on some sites (Shelbourne, 1986a). This implied that thinning out of inferior clones could not be achieved optimally; incompatibility losses were almost as high as the desired thinning rate (Shelbourne, 1986a). Despite this, according to Shelbourne (1986a) the "850" series afforded appreciable genetic gains in stem straightness, decreased malformation, and branch habit, as well as modest gains in growth rate.
In 1968 the original strategy of using a small number of intensively selected clones (as was the case with the "850" series) was revised (Shelbourne, 1986a). By this time, experience revealed that a small number of clones did not allow very intensive re-selection in favour of superior clones (Burdon and Shelbourne, 1971). The revised

strategy resulted in the selection of 588 superior trees in northern Kaingaroa Forest, and these were the progenitors of the clones designated the "268" series (Shelbourne, 1986a).

In 1969 progeny trials were established on three north island sites to evaluate all of the "268" series clones. In 1971 a further four trials based on 220 clones were established at different locations, including three in the south island (Shelbourne, 1986a). Hedged archives were also established to provide a ready supply of clonal material (Firth, 1986). The 1969 progeny trials were assessed at age 5 and age 10, and the resultant selections were designated the "875" and "880" series clones, respectively (Shelbourne, 1986a). Selection of the "875" series was based on stem diameter and straightness, branch quality, and also height and wood density of the better clones. Selection of the "880" series was based on these criteria and, additionally forking and susceptibility to *Cyclaneusa* (Shelbourne, 1986a). The "268" series are the parents and grandparents of many of the more recent series of clones (Shelbourne 1986c).

The introduction of *P. radiata* seedlots into various parts of New Zealand has been a complex process. Within New Zealand, regional stocks have arisen due to differences in genetic origin, hybridisation of the natural provenances, and several generations of natural selection under a variety of environmental conditions. This phenomenon may be referred to as the "founder effect" (Burger, pers. comm.[3]) Subtle differences appear to exist between regional stocks, and these are putatively considered to be different "land races" (Burdon, 1986).

In 1985 it was realised that the opportunity to make new selections from the New Zealand "land races" was diminishing, because the last remnants of "land race" stock were progressively being clearfelled, and only seed orchard stock was being utilised for new plantings. It was decided to carry out a final round of "plus-tree" selections

[3] Fred Burger, Manager tree breeding, Carter Holt Harvey Forests. Personal communication 24 February 2000.

from the "land races" to broaden the genetic base of the existing breeding population (Burger, pers. comm.[4]).

Between 1985 and 1988, 979 "plus" trees were selected from unimproved "land race" stands; these series were designated "885", "887" and "888" according to the selection year. Selections were made at the central North Island plateau, Northland, Hawkes Bay, Nelson and West Otago. Selection intensity varied widely from 1:0.8 ha to 1:4.4 ha but was commonly 1:2.0 ha. (best tree in every 2 ha). The primary selection criterion was vigour, followed by stem straightness and crown health (Burger, pers. comm.[4]). More recently the emphasis of the breeding program has been on wood properties.

The information above is a brief outline of tree breeding in New Zealand, and pertains largely to the "multi-nodal" breeding population. For more detailed discussions of general interest see Shelbourne (1986b; c). Discussion on the disease resistant breeding population is provided by Carson (1986), and on the long internode breeding population by Jayawickrama *et al.* (1997).

1.4 Morphology and development in pine

1.4.1 Terminology

In *Pinus radiata*, branches generally occur in what superficially appear to be true whorls, and foresters often refer to these as "branch whorls". However, as Bannister (1962) rightly points out, whorls cannot occur in plants with a spiral phyllotaxis, such as *Pinus radiata*, and instead he refers to "branch clusters". In this thesis, the term "branch cluster" will be utilised to describe the region of attachment of branches to the stem.

[4] Fred Burger, Manager tree breeding, Carter Holt Harvey Forests. Personal communication 24 February 2000.

The region where a branch cluster occurs is often, erroneously, referred to as a "node", and the region between branch clusters as an "internode". In strict botanical terms, a "node" may be defined as the part of the stem where one or more leaves (cataphylls, or short shoots in the case of *Pinus*) arise (Thain and Hickman, 1996). Doak (1935) clearly accepted this definition of a node in *Pinus*. Furthermore, he describes the shoot as being comprised of repeating "stem units", which he defines as "an internode, together with the node and nodal appendages (leaf fascicles on a mature tree) at its distal extremity". This terminology will be employed in this thesis.

1.4.2 Terminal Bud Structure

The terminal bud of *Pinus* is a compound structure, with unextended internodes that contain all of the primordia for the following cycle's growth (Sacher, 1954). The bud is formed by periodic activity in the peripheral zone of the shoot apex. Firstly a series of spirally arranged cataphylls, that do not subtend buds are produced, i.e. sterile cataphylls. Next a series of cataphylls that will ultimately bear axillary structures are produced. The majority of axillary structures borne will be short shoots, however the final few may be long shoots. Finally a series of cataphylls are produced that will be the terminal bud scales of the bud to be formed during the following season (Sacher, 1954).

In a *P. radiata* bud, there are typically about 50 sterile cataphylls, 150 cataphylls subtending short shoots, and 10 to 15 cataphylls subtending long shoots (Taylor *et al.*, 1984).

1.4.3 Tree growth, development and branch arrangement

Pinus radiata is generally polycyclic, producing several cycles of growth in each year (Bollmann and Sweet, 1976). Each cycle of growth is laid down in the terminal bud of the previous cycle. Figure 1.2 provides a diagrammatic representation of shoot development in *Pinus radiata.*

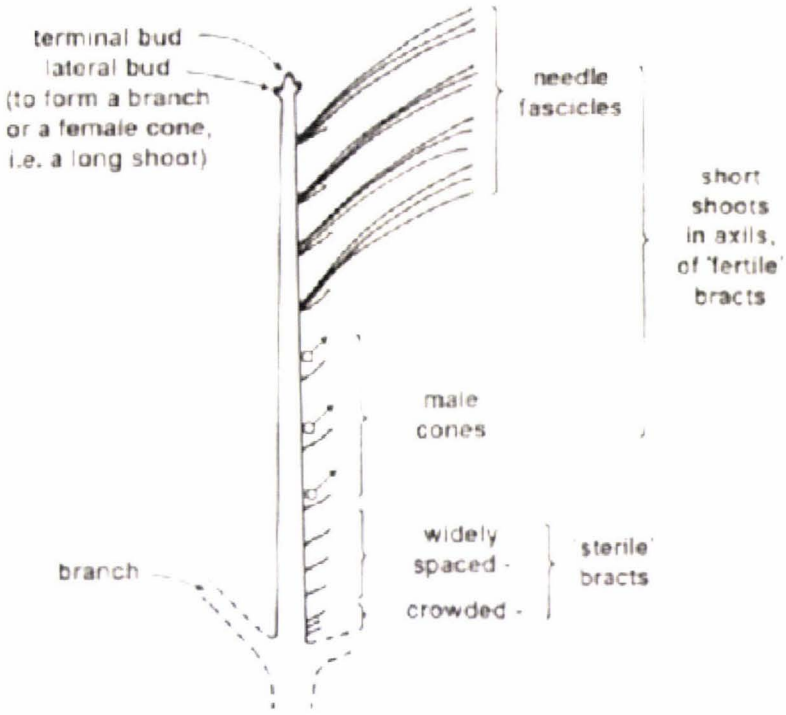

Figure 1.2: *Diagrammatic representation of pine shoot (solid lines). Each cycle of shoot growth in Pinus radiata is characterized by the following distinct regions, which develop in the following ontogenetic order:*
i) a set of sterile bracts, that were originally the scales protecting the bud formed in the previous cycle.
ii) a region of bracts that accommodate male cones.
iii) a region of needle fascicles.
iv) lateral buds which will form long shoots - either branches or female cones.
v) the terminal bud.
(reproduced from Cremer, 1992)

The "overwintering" bud of monocyclic species growing in relatively extreme climates typically undergoes a relatively long, well defined "resting", or "dormant" period. In some regions of New Zealand, such as the extreme south, *P. radiata* may

also undergo a distinct period of dormancy with an associated resting bud (Jackson *et al.* 1975). In contrast, *Pinus radiata* growing in most regions of New Zealand does not have a well defined period of bud rest (Jackson *et al.* 1975).

From samples of trees ranging from 2-44 years of age, Bannister (1962) established that the number of branch clusters of the annual shoot generally varies from 1 to 5, but is occasionally 0 or 6. Additionally, it was ascertained that the number of clusters produced annually is relatively low at the beginning of the tree's lifespan, steadily increases until about the 20th year, and thereafter remains fairly constant.

1.4.4 Recognition of the first cycle of the annual shoot

In order to understand the pattern of shoot development in a species which may produce five, or more, cycles of growth in a year, it is necessary to determine which cycle is the "starting cycle" of the annual shoot i.e. which cycle corresponds to the annual shoot of a monocyclic species. In some cases, where there is a well defined cool period, a resting bud may occur, defining the "starting cycle" (Bollmann and Sweet, 1976). Its position may be detectable for several years by the presence of a large band of sterile cataphylls, which are bunched in comparison with those produced in later cycles, of that particular annual shoot (Bannister, 1962; Bollmann and Sweet, 1976; Jacobs, 1937).

In cases where a well defined resting bud does not occur (which is the likely scenario in most parts of New Zealand), the first cycle of the annual shoot may be retrospectively recognised by the distance between the stem, and where the first lateral buds of the secondary branches occurred. According to Jacobs (1937), the (secondary) branches of the first cluster of the annual shoot are almost invariably larger than those of subsequent clusters. Thus, in progressing from the first cluster, to subsequent clusters, there is a gradual reduction in the distance from the main stem, to the position along the secondary branch, where tertiary branches occur.

1.4.5 Sexuality

According to Fielding (1953; 1960), most *Pinus radiata* trees produce both female and male cones, but trees do vary between those that are completely female, and those that are completely male. However, Fielding (1960) also states that "Monterey pines (*P. radiata*) undoubtedly vary in their requirements for flowering (cone production), and it is probable that individuals which are phenotypically unisexual or asexual might have flowered (produced cones) normally if they had been grown in a different environment".

1.4.6 Cone Position

In *Pinus radiata*, male cones are generally produced on subordinate shoots of the tree (Cremer, 1992). A typical subordinate shoot bearing male cones is shown in Figure 1.3. Male cones originate as short shoot primordia, which would otherwise have formed needle fascicles. As previously mentioned, the growth pattern is predetermined, with all components of the current cycle having been initiated in the terminal bud of the previous cycle (Cremer, 1992).

Figure 1.3 *A typical Pinus radiata subordinate shoot, bearing male cones.*

Cremer (1992) states that "the subordinate shoots of *P. radiata* are unicyclic". However, observations at Amberley seed orchard revealed bicyclic growth of subordinate shoots in some genotypes (Welsh, 1997).

Leading shoots (i.e. shoots at the tip of the tree, and vigorous upper branches) of *P. radiata* are generally polycyclic. Female cones are usually formed on these shoots, originating as long-shoot initials, which would otherwise have formed branches (Cremer, 1992). The pattern of ontogenetic development of leading shoots is considerably more variable than that of subordinate shoots. A typical vigorous upper branch of *Pinus radiata* is shown in Figure 1.4.

h: terminal bud & lateral buds [late 1999]

g: lateral buds [spring 1999]

f: female cone [early 1999]

e: cluster of branches [1998]

d: cluster of branches [1998]

c: 2 year old female cones & branches in same cluster [1997]

b: cluster of branches [1997]

a: 3 year old cone & branches in same cluster [1996]

Figure 1.4: *Female branch. Early January 2000.*

1.4.7 Characteristics of female cone buds

Female cone buds can usually be distinguished from vegetative buds, by the following characteristics (from Fielding, 1960):-

1. When cone buds and branch buds occur in the same cluster, as is generally the case, the cone buds are attached to the stem slightly below the branch buds (Figure 1.5, bud labelled "d").

2. The cone bud is typically sub-conic and tapers to a sharp point (Figure 1.5, bud labelled "d"), whereas the branch bud is sub-cylindrical and lacks such a sharp point (Figure 1.5, buds labelled "c").

3. The scales of cone buds are typically broader, and less numerous than those of branch buds.

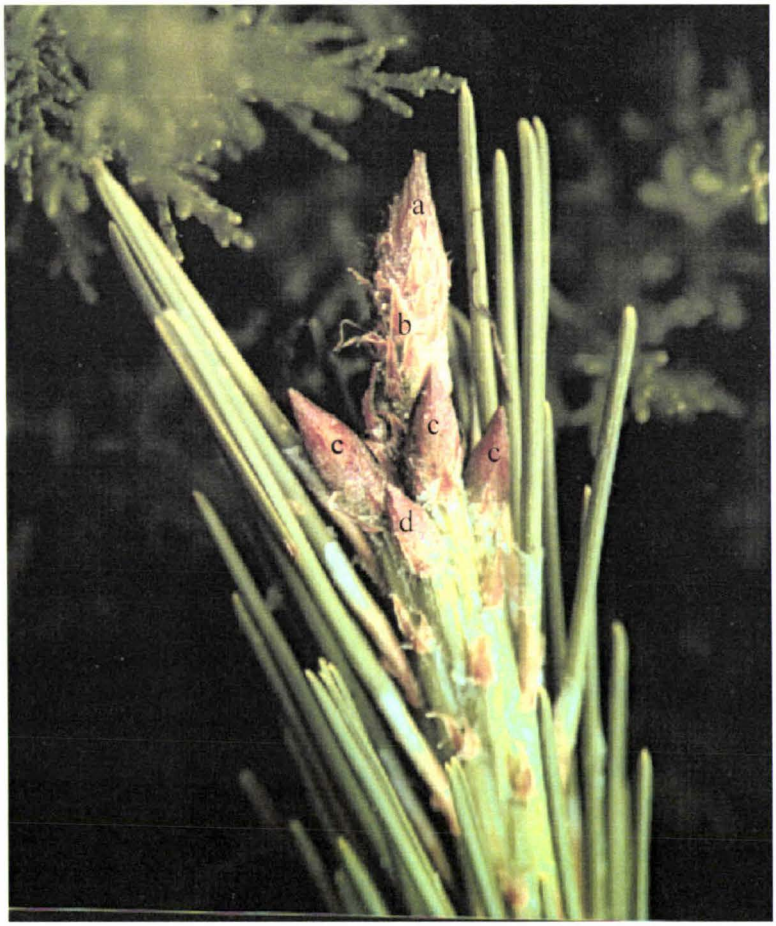

Figure 1.5: *Branch apex.(a) terminal bud, enclosed by scales (b). (c) & (d) long shoot lateral buds. The upper three will probably develop into branches. The lower one (d) will probably develop into a female cone.*

1.4.8 Female cones

Bannister (1962) established that the number of female cone bearing clusters, in the annual shoot, is in most cases either two less than the total number of branch clusters, or zero if the total number of clusters is only one or two. This is because female cones are almost invariably absent from the first (earliest formed) branch cluster, scarce in the second cluster, and generally present in all other branch clusters of the annual shoot. Hence, polycyclism is a necessary prerequisite for female cone production, and the number of cones produced is positively correlated with the number of branch clusters. Fielding (1960) reinforces this in his statement "Truly uninodal (i.e. trees producing only one *branch cluster* each year) Monterey pine do not normally produce any female flowers (cones) at all: at least, the writer has never found a cone on a truly uninodal tree".

According to Bannister (1962), appearance of the first (female) stem cones is dependent on attainment of a certain height, irrespective of variations in age or environment. However there is still a wide degree of variation in the value of this "certain height".

In studies carried out in the Australian Capital Territory, *Pinus radiata* begins to produce female cones at about four years of age, and the majority of trees are producing female cones at ten years of age (Fielding, 1960).

1.4.9 Male cones

In contrast to some *Pinus radiata* trees which never produce any female cones, some trees appear to never produce any male cones (Fielding, 1960). However, according to Fielding (1960), trees completely lacking male cones are far rarer than those completely lacking female cones.

In the Australian Capital Territory, *Pinus radiata* begins to produce male cones at three years of age, and most trees are producing male cones by seven years of age

(Fielding, 1960). Burdon and Bannister (1973) provide results from a trial carried out in Kaingaroa Forest, comparing the performance of *P. radiata* originating from the five natural populations, and two New Zealand populations (Kaingaroa and Nelson). For the New Zealand and Ano Nuevo provenances, approximately 25% of trees were found to be producing male cones at age five (from seed). Figures for the other provenances were much lower. Unfortunately no details for other ages were provided.

1.4.10 Short Shoot and Male Cone Development

1.4.10.1. Early Short Shoots

In *Pinus*, following germination, the juvenile leaves which develop after the spiralled cotyledons are single and needle-like. They are borne on the main axis (long shoot) of the seedling. The earliest short shoots arise as buds in the axils of some of these juvenile leaves (Foster and Gifford, 1974). As ontogenetic development progresses, subsequently formed short shoots arise as buds subtended by non-photosynthetic cataphylls, referred to by Doak (1935) as bracts.

1.4.10.2. Short Shoot Development

Sacher (1955a) provides an excellent account of short shoot development in *Pinus lambertiana*. Observations of bud sections reveal that processes involved appear to be very similar in *P. radiata*. Initiation of short shoot primordia occurs in the median axillary region of the subtending cataphylls, a few internodes from the apical meristem of the long shoot. The first evidence of short shoot initiation is the occurrence of frequent divisions in various planes of the hypodermal cells in the axillary region of the cataphylls. Following this, there is a series of mostly periclinal divisions in the overlying surface layer, which result in evagination of the short shoot primordium. Following emergence of the primordium the surface cells divide and enlarge in an anticlinal orientation to contribute to surface growth, while underlying divisions in various planes add to volume growth (Sacher, 1955a).

In *P. radiata*, during December, well developed short shoot primordia are clearly visible in the axils of cataphylls (Figure 1.6). The figure shows a typical median longitudinal bud section from a subordinate lateral branch of clone 268.405, collected at Amberley seed orchard in December 1996.

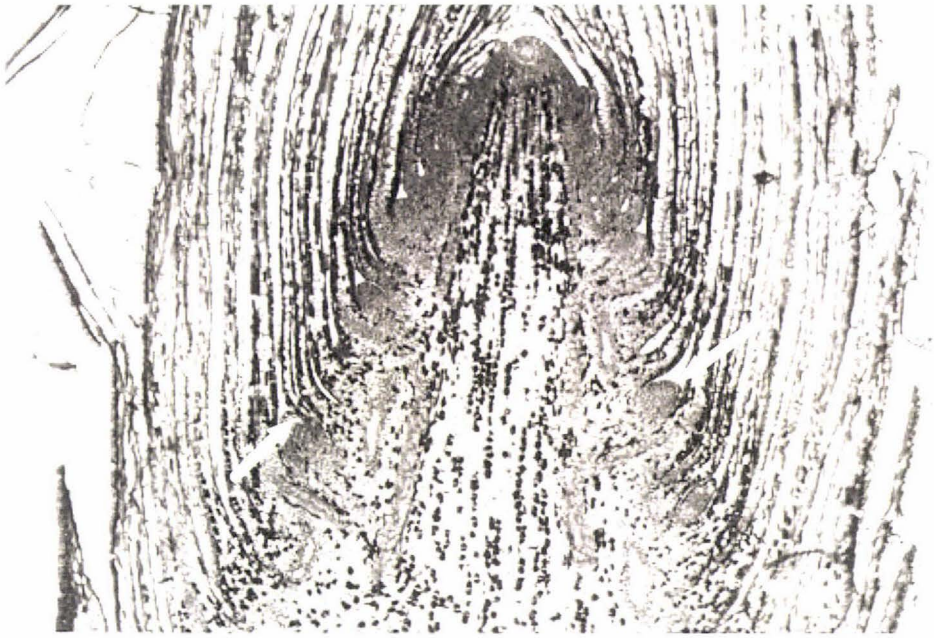

Figure 1.6: *Median longitudinal bud section. Arrows indicate clear examples of short shoot primordia.*
P. radiata *Clone 268.405, 23rd December 1996. Bud section x32. (Welsh, 1997)*

Short shoot development in *P. radiata* is known to vary, depending upon clone, location and year (Riding, unpublished). Buds collected on 22 November 1996 near Rotorua also had well developed short shoot primordia, as did buds from Seddon collected in early December of the same year. Based on the stages of development present in these samples, under New Zealand conditions, it is estimated that short shoot initiation in *P. radiata* occurs in late October (Welsh, 1997).

1.4.11 Cataphyll numbers

Bollmann and Sweet (1976) investigated the morphogenesis of the terminal bud of the leading shoot of one clone of *Pinus radiata*, growing at two sites. The morphogenesis of the terminal bud is important, since it establishes the pattern of

primordial initiation, and consequent development of the shoot. Additionally, the number of cataphylls produced during the ontogenetic development of the tree may be correlated (indirectly) with the attainment of reproductive competence (Hackett pers. Comm.[5]). Based on data from the two sites studied, it appears that *Pinus radiata* produces in the order of 1000 cataphylls, in each annual shoot (Bollmann and Sweet, 1976).

1.5 Maturation or Phase Change

1.5.1 Characteristics of Maturation

During the ontogenetic development of plants established from seed, changes in the characteristics of structures produced by the shoot apical meristem occur. As a result, there are progressive morphological, anatomical, physiological and developmental changes in vegetative characteristics. Depending on the species involved, these may include leaf shape, thickness and epidermal characteristics, phyllotaxis, thorniness, shoot orientation, shoot growth vigour, anthocyanin pigmentation, photosynthetic characteristics, disease and insect resistance, and competence to form adventitious buds and roots, and somatic embryos (Hackett and Murray, 1997). Moreover, reproductive competence is achieved and, with suitable environmental and endogenous stimuli, flowering or the production of cones can occur.

Characteristics that occur early in development are referred to as juvenile, while those that appear later are referred to as mature. As maturity approaches, changes in all or some of the characteristics mentioned above are to be expected. However, according to Hackett (1985) "attainment and maintenance of the ability or potential to flower is the only consistent criterion available to assess the termination of the juvenile period". The change from juvenile to mature characteristics is known as phase change, or maturation (Hackett and Murray, 1997). Poethig (1990) provides a further distinction within the mature phase, referring to a mature vegetative phase,

[5] Wesley Hackett, Emeritus Professor, University of California, international expert on phase change, Personal communication 11 February 1999.

and a mature reproductive phase. Hence, a minimum of three phases are recognisable:

- a juvenile phase
- a mature vegetative phase
- a mature reproductive phase

These distinctions prove invaluable for further consideration, and discussion of phase change.

Hackett (1985) is careful to distinguish between definitions of *maturation*, and *aging*. While both occur during plant ontogenetic development, *maturation* refers to the transition from the juvenile to mature phase, while *aging* refers to the loss of vegetative vigour associated with increasing complexity of the plant. Once reproductive maturity is reached, it is relatively stable, and reversion to the juvenile state does not *generally* occur through techniques such as the taking of cuttings, or grafting. In contrast, the loss of vegetative vigour, as a consequence of *aging* can be reversed if the aged shoot is grafted onto a seedling rootstock, or a cutting rooted.

Normally (without intervention), once the mature reproductive phase is reached, flowering or the production of cones will occur, providing the required inductive stimuli are present. Additionally, certain cultural treatments, and application of some plant growth regulators can induce precocious flowering or cone production in *apparently* otherwise juvenile individuals of some species (Pharis and Ross, 1986). This situation appears to contradict the commonly accepted usage of the word "juvenile". However, flowering (or the production of cones) in such cases is temporary, and plants return to the usual non-flowering juvenile condition, unless treatments are reapplied (Hackett, 1985).

1.5.2 Mechanisms of Maturation

Relatively little is known about the actual mechanisms of maturation, but molecular techniques are beginning to provide insights into possible mechanisms. Hackett and Murray (1997) have outlined a range of possible maturation models (Figure 1.7).

With the onset of maturation, individual maturation related characteristics tend to change at different rates. Therefore, it does not seem feasible that a single master switch is the mechanism involved in phase change. Evidence suggests an array of switches or processes are involved (Figure 1.7, from Hackett and Murray, 1997).

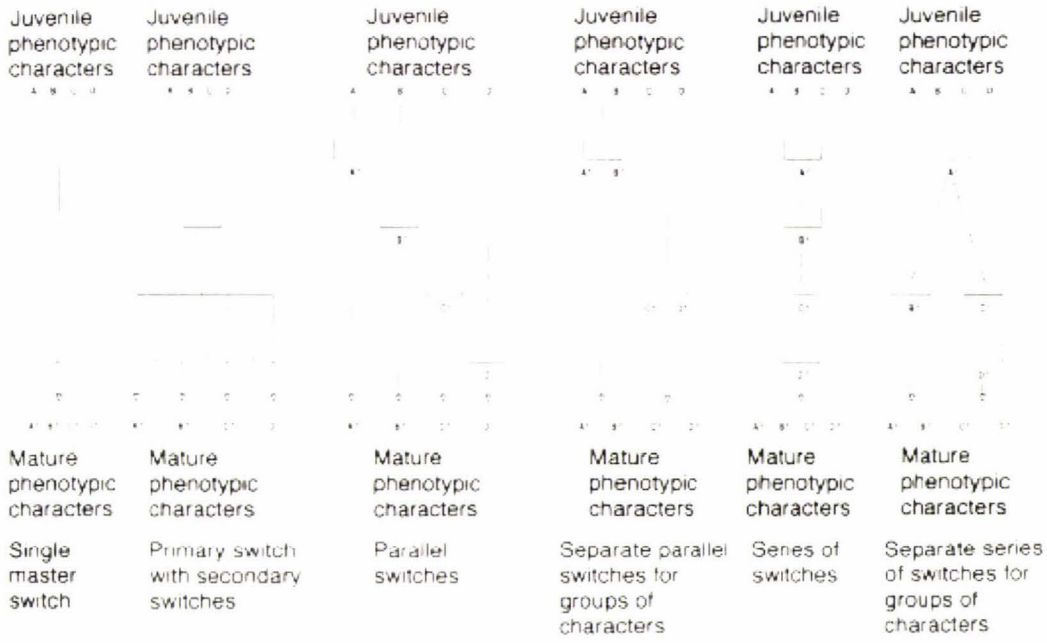

Figure 1.7: *Alternative models of phase change or maturation. (reproduced from Hackett and Murray, 1997)*

Poethig (1990) states "development of the shoot is specified by a series of independently regulated, overlapping programs that modify the expression of a common set of processes for shoot growth". Poethig and others provide the following evidence in support of this statement.

In maize, leaves produced during the transition from the juvenile to mature phase possess a combination of juvenile and mature cell characteristics (Bongard-Pierce and Poethig, unpublished observations, cited in Poethig, 1990). Similarly, in conifers, the transition from the juvenile to the mature phase is typified by shoot development that combines cellular (Hutchison *et al.*, 1990), and morphological traits from each phase (Steele *et al.*, 1989). New Zealand's indigenous woody flora possesses an abundance of heteroblastic species, in which the differences between juvenile and mature forms are great, and occur abruptly (Godley, 1985). Nevertheless, in at least some of these

species, transitional stages of development, with traits intermediate between juvenile and mature can be observed (Day *et al.*, 1995).

The transition from vegetative mature to reproductive mature phases is also characterised by the production of intermediate traits by the shoot (Poethig, 1990). For example, in *Arabidopsis thaliana* the transition to fully reproductive development is immediately preceded by the production of rudimentary leaves, and elongated lateral branches similar to those produced by rosette nodes (Poethig, 1990), characteristic of the mature reproductive phase.

In larch (*Larix laricina*), shoots that possess an aberrant combination of juvenile and mature traits can be produced experimentally by grafting scions in one phase of development onto rootstocks in a different phase of development (Greenwood *et al.*, *1989*). Similar aberrant behaviour can also be induced experimentally in *Hedera helix* by hormonal treatment (Frydman and Wareing, 1974; Rogler and Hackett, 1975).

Mutants with prolonged expression of juvenile characteristics or accelerated appearance of mature characteristics provide further insight into the mechanisms involved in maturation (Poethig, 1990; Lawson and Poethig, 1995). Evidence suggests that a complex, interacting array of pathways operate to control the multitude of changes that are associated with maturation. Interactions may occur between genes that act early in the regulatory pathways, or between genes that operate downstream. These genes may interact with each other in a combinatorial, or an antagonistic manner. Lawson and Poethig (1995) provide a functional model of how these pathways might operate (Figure 1.8).

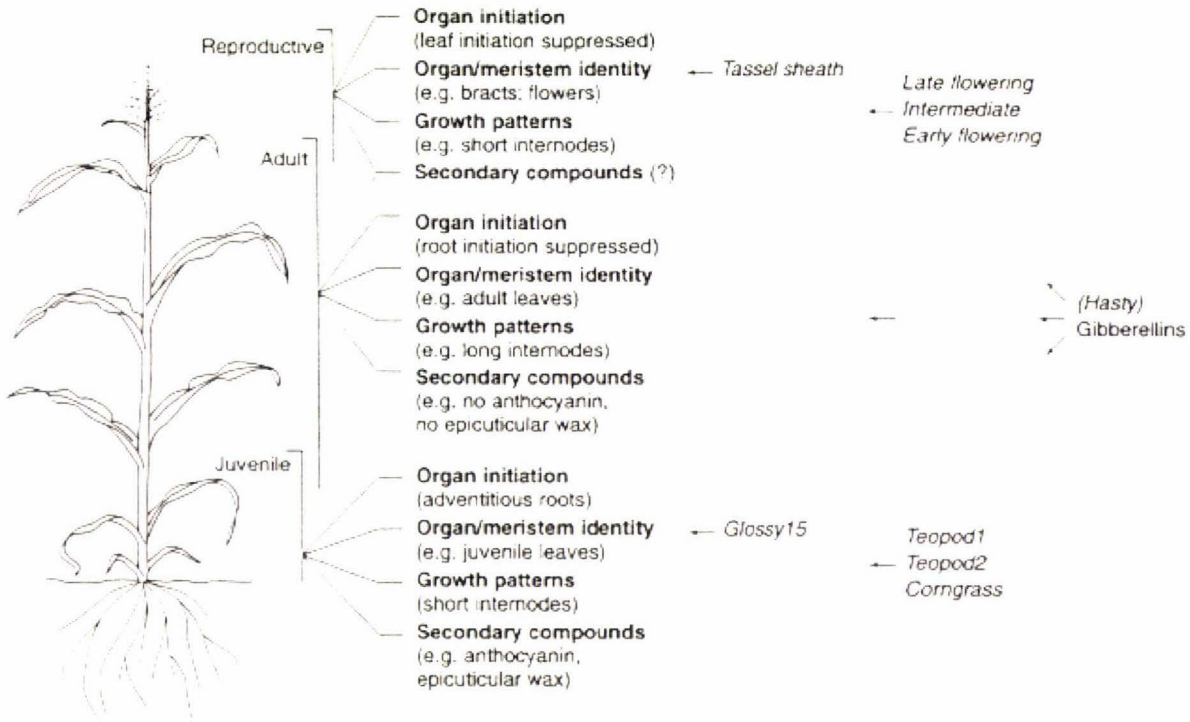

Figure 1.8: *Simplified model for phase change in maize showing juvenile, mature vegetative, and reproductive phases. Because traits characteristic of a particular phase can vary independently of one another, it is likely that maturation is regulated by a highly branched genetic pathway. Some genes of these pathways have been defined by mutations in maize and Arabidopsis, and are shown in italics. (adapted from Lawson and Poethig (1995))*

1.5.3 Factors Controlling Maturation and/or flowering

1.5.3.1. Classical theories accounting for the inability to flower

i) Insufficient leaf area

The hypothesis that a certain leaf area is required before flowering can occur is discussed by Bernier *et al.* (1981). This hypothesis is often regarded with indifference, since experiments have shown that in many species *very little* leaf area is required for flowering to occur. However, in *Pharbitis* and other species insufficient leaf area has been shown to reduce flower initiation (see Bernier *et al.*, 1981).

In olive (*Olea europaea* L.), removal of 90% of each leaf blade has no significant effect on subsequent inflorescence formation (Hackett and Hartmann, 1964). However, 100% defoliation completely inhibits inflorescence formation, but buds

may still develop vegetatively. Evidence suggests that the inhibition of flowering in defoliated olive plants is probably not due to a lack of carbohydrates (Hackett and Hartmann, 1964). Hackett and Hartmann (1964) suggest that the leaves may be involved in producing a flowering hormone or other substance, which in small quantities, might promote inflorescence formation. The fact that at least a small leaf area is required for flowering to occur in olive complies with this hypothesis; a very small leaf area may be sufficient to synthesise the required level of the "flowering hormone or other substance".

Bernier *et al.* (1981) suggest that the photosynthetic contribution of leaves is important in attaining "ripeness-to-flower", which Bernier *et al.* (1981) define as "attainment of sufficient carbohydrate and nutrient reserves for flowering to occur". In tomato, Hussey (1963) provides evidence that flower initiation is promoted by increased availability of carbon substrates. The olive trees used by Hackett and Hartmann (1964) were three years old, and therefore, presumably would have already built up considerable carbohydrate and nutrient reserves prior to experimentation.

ii) Ratio of juvenile to mature leaves

Some evidence suggests that a certain ratio of mature to juvenile foliage must be reached before floral initiation can occur (Bernier *et al.*, 1981).

In soybean, the ratio of mature to juvenile leaves is more highly correlated with the onset of flowering, than plant age. A ratio (mature:juvenile leaves) in the order of 10:1, varying slightly depending on growing conditions and soybean variety, is required for flowering to commence (Fisher, 1955). Additionally, removal of mature leaves impedes flowering, while removal of juvenile leaves hastens flowering (Fisher, 1955). In tomato, Hussey (1963) found that removal of the first two leaves hastened flowering by eight days, and fewer mature leaves formed before flowering, relative to controls.

It is hypothesised that juvenile leaves may produce floral inhibitors, or interfere with the transport of floral stimuli from mature leaves to the meristem (Bernier *et al.*, 1981).

iii) Leaf insensitivity to daylength

In some photoperiodic species, insensitivity of juvenile leaves to daylength conditions favourable for flowering is considered to be a primary reason for the inability of juvenile plants to flower (Bernier *et al.*, 1981).

In the short day plant cocklebur (*Xanthium pensylvanicum* Wallr.), the cotyledons are relatively large, and photosynthetic (Jennings and Zuck, 1955). However, in grafting experiments Jennings and Zuck (1955) showed that cotyledons exposed to short photoperiods did not elicit a flowering response, as would a similar, or even a smaller area of mature leaves.

Experimental evidence, from the short day plant *Perilla crispa* also supports this hypothesis (Zeevartt, 1958). Two groups of *Perilla* plants were sown four weeks apart under long daylength, so that the second leaf pair of the late sown group, and the fifth leaf pair of the early sown group were fully expanded at the same time. Leaves of similar area from the two groups were then grafted onto rootstocks, which had been maintained under long day conditions. After graft unions were established, the donor leaves were then exposed to several cycles of short day conditions. For successful flower induction, it was found that leaves from the second node required about twice as many short day cycles compared to those from the fifth node. It was concluded that, in *Perilla*, the sensitivity of leaves to inductive conditions increased with ontogenetic rank, at least up until the fifth node (Zeevaart, 1958). Anatomical observations, and translocation experiments with labelled sucrose indicated that a phloem connection between donor and receptor is required for transmission of the "floral stimulus" (Zeevartt, 1958).

However, in the above work, no indication of the relative photosynthetic efficiency of leaves from the second node, compared to the fifth node was provided. It is possible that older leaves, from the fifth node, were more efficient and therefore these plants may have attained "ripeness to flower" earlier (Bernier *et al.*, 1981). This may also be the case for the findings of Jennings and Zuck (1955) in *Xanthium*.

The results obtained in *Xanthium* and *Perilla* are not universal, and there have been different findings for other species. For example, in the long day plant spring turnip rape (*Brassica campestris* L. cv. Ceres) cotyledons are fully sensitive to a single inductive photoperiod four days after germination, and will flower shortly after such treatment (Friend, 1968).

iv) Influence of the root system

In some species, flowering appears to occur when the shoot reaches a certain species-dependent size. For example, in the short day plant black currant (*Ribes nigrum*), rooted shoots with 14 nodes, taken from mature bushes, cannot be induced to initiate flowers by short day treatment (Schwabe and Al-Doori, 1973). Shoots with a greater number of nodes, treated in the same manner, do initiate flowers. In order to elucidate the reasons for this, Schwabe and Al-Doori (1973) undertook an elaborate set a experiments involving shortening of stems, defoliation, bud removal, grafting, and the formation of adventitious roots on the stem. It was concluded that the inability of plants with fewer nodes to flower is associated with the proximity of the shoot tips to the root system. The nature of this effect was tentatively traced by Schwabe and Al-Doori (1973) to gibberellin-like activity emanating from the root and travelling upwards.

The exact nature of the reputed inhibitory effects of the root system remains unknown (Poethig, 1990).

v) Number of meristematic cell divisions

Results obtained by Robinson and Wareing (1969) suggest that size *per se* is not the factor that limits flowering. With prior knowledge of the height at which flowering usually occurs, black currant (*Ribes nigrum*) seedlings were grown to a height at which flowering would not occur. At this stage seedlings were then decapitated, and the tips grown as cuttings. These plants were treated as for the original seedlings, and the process repeated twice. Cuttings from the third and fourth decapitations began to flower, although they had not reached the height required for flowering to occur in intact plants. Robinson and Wareing (1969) suggest that phase change occurs after a

certain number of cell divisions of the meristem and hence phase change is correlated with, but not determined by, attainment of a certain plant size.

vi) Meristem insensitivity to floral promoters

"Fully juvenile" scions from trees such as citrus and larch (*Larix*) do not flower, or initiate cones, precociously when grafted onto mature reproductive plants (Bernier *et al.*, 1981). This suggests that the meristems of juvenile scions are not capable of responding to floral stimuli from mature leaves. The term "fully juvenile" is used here because Robinson and Wareing (1969) found that in some cases, supposedly juvenile, and "near mature" scions of European larch (*Larix decidua*) did initiate cones when grafted onto mature rootstocks. Their "juvenile" and "near mature" plants were all nine years old, although individuals from the "juvenile" population were much smaller than those from the "near mature" population. Consideration of normal population distributions suggests that a few members from the upper tail of the "juvenile" population could be reaching "near mature" or adolescent status.

This situation emphasises the requirement to develop reliable criteria for assessing maturity of plants. It seems likely that reliable biochemical markers exist to distinguish juvenile, adolescent, mature vegetative, and mature reproductive phases. Such markers for some characteristics of maturation are already known (Hackett and Murray, 1997).

The literature involving the "classical hypotheses" discussed above provides a wealth of interesting ideas and concepts attempting to explain why juvenile plants are not able to flower. However, there appears to be considerable disparity between findings and conclusions of many of these studies, and the overall picture presented is rather confused. Some of these differences may emanate from inherent variations between species used in different studies, and the very different approaches used in these studies. It is also apparent that in many of these studies, possible confounding elements have been overlooked in reaching conclusions. This seems especially so in studies involving experimental manipulation of plants, such as stem modifications and defoliation. These manipulations would undoubted invoke a multitude of confounding plant responses.

1.5.3.2. Epigenetic Studies

A useful experimental approach for studying maturation is the epigenetic approach. This involves comparison of differences in gene expression between juvenile and mature tissues of the same genotype. Epigenetic studies are particularly useful in perennial species with a prolonged juvenile phase, since genetic studies are hindered by the associated long generation times. As mentioned above, such studies have led to the identification of markers in a variety of species, that can be used to assess the progression of maturation related events (Hackett and Murray, 1997). Study of the underlying basis of juvenile versus mature characteristics, individually, will eventually provide elucidation of maturation mechanisms (Figure 1.2).

i) Physiological and biochemical

In English ivy (*Hedera helix*), hypodermal collenchyma cells of juvenile stems and leaf petioles accumulate anthocyanin pigment, while genetically identical mature tissues do not (Murray and Hackett, 1991). Murray and Hackett (1991) undertook a study to assess which enzyme(s) involved in the biosynthesis of anthocyanin might limit its accumulation in the mature phase tissue. Tissue from both phases accumulated similar levels of the flavonols, kaempferol and quercetin (both precursors to anthocyanin), suggesting that enzymes involved in their synthesis were not limiting in the mature tissue. In contrast to juvenile tissue, mature tissue did not accumulate either leucocyanidin or anthocyanin, suggesting that mature tissue lacked dihydroflavonol reductase (DFR) activity. This hypothesis was supported by enzyme assays, which could not detect DFR activity in mature tissue. Hence, in ivy, DFR activity is a reliable marker for this characteristic of maturation.

ii) Genetic

Further to their biochemical studies, Murray *et al.* (1994) compared gene expression in juvenile and mature tissues of English ivy. It was found that mature tissue lacked transcription of the DFR gene, resulting in lack of accumulation of DFR mRNA. Accordingly, DFR and ultimately anthocyanin pigmentation are absent in the mature tissue.

1.5.3.3. Genetic Studies

The genetic approach is another useful experimental method for studying maturation. This involves comparing the usual ontogenetic development of wild type plants, to that of mutants with altered progression of maturation related characteristics (Hackett and Murray, 1997).

i) How do Plants Measure Developmental Time?

In order for plants to correctly gauge when maturation events should occur, they must have some means of measuring developmental time. Developmental time could be measured reliably by either temporal or spatial signals (Lawson and Poethig, 1995). The concept discussed above, of the proximity of the root system to the shoot apex, is the basis for the spatial signalling hypothesis. The root system might produce a factor which promotes juvenility or inhibits maturation. Shoot maturation might then occur when this factor falls below a certain threshold level, due to the distance between the apex and the root system. The alternative hypothesis is that the plant can measure real time by a steady increase in factors that promote mature characteristics, or by a decline in factors that promote juvenile characteristics.

Based on these hypotheses, predictions about the phenotypes generated by mutations in maturation pathways can be devised (Lawson and Poethig, 1995). If maturation timing is controlled spatially, then mutations that affect the size of the shoot would also affect the characteristics of leaves at particular positions on the shoot, independently of when the leaves were produced. Alternatively, if maturation is controlled temporally, then such mutations would affect leaves depending on when they were produced, regardless of where they were on the plant.

Evidence exists in support of both of these hypotheses. Birch (*Betula verrucosa*) grown under conditions that hasten growth, tend to flower relatively early (Longman and Wareing, 1959), lending support to the spatial hypothesis. However, in this work no mention is made of the other facets of maturation, and furthermore, the precocious flowering obtained may have been due to some other confounding cause. Shoot restriction experiments in *Metrosideros excelsa* suggest that vegetative phase change cannot be fully accounted for by the spatial hypothesis (Clemens *et al.*, 1999).

Evidence from the *Arabidopsis paused (psd)* mutant favors the hypothesis for temporal regulation of maturation (Lawson and Poethig, 1995). Seedlings homozygous for this gene fail to produce the first true leaf primordium, until several days after germination. The first true leaf that is eventually produced in the *psd1* mutant morphologically resembles later leaves that are being produced at the same time by wild type seedlings of the same age, rather than having the usual morphology of the first leaf. Additionally, despite having fewer leaves, *psd1* plants flower at the same time as wild type plants (Lawson and Poethig, 1995).

ii) Genes Controlling Maturation Characteristics

Maturation traits maybe regulated independently of each other, or they may be interrelated, with the expression of one trait being dependent on the manifestation of another (Lawson and Poethig, 1995). Independent regulation of maturation-related traits would allow greater plasticity in development, and therefore possibly provide a selective advantage (from an evolutionary viewpoint), compared to connected regulation of traits. Experiments with woody species suggest that each maturation-related trait may be regulated independently. Experiments show that vegetative, and reproductive maturation can be dissociated (Lawson and Poethig, 1995), and that the onset of individual mature vegetative characteristics are not always well correlated (Steele *et al.*, 1989).

Maize mutants have been identified that have pleiotropic effects on shoot development. *Teopod 1, 2* and *3* (*Tp1, Tp2* and *Tp3*), and *Corngrass* (*Cg*) mutations all prolong juvenile traits, but have little effect on mature vegetative characteristics, or the timing of tassel initiation (Poethig, 1988; Lawson and Poethig, 1995; Hackett and Murray, 1997). These mutations affect *all* traits associated with juvenility, and juvenile traits are observed together with normal mature features, at the same node (Moose and Sisco, 1994). Clonal analyses demonstrate that *Tp1* and *Tp2* are involved in the production, or control of a factor that promotes juvenility in cells that are normally destined to contribute to mature structures (Dudley and Poethig, 1991; Dudley and Poethig, 1993). Since these genes (*Tp1, Tp2, Tp3* and *Cg*) are pleiotropic, acting on several juvenile traits, it appears that there is some degree of

linking of pathways involved in maturation. However, it appears that other pathways, such as those involved in regulating mature characteristics, are not associated directly with these pathways.

The maize *glossy15* (*gl15*) mutant causes precocious mature traits, but affects only the epidermis. Moose and Sisco (1994) demonstrated that the *GL15* gene product operates in a cell-autonomous way, to direct juvenile epidermal differentiation, but does not affect factors that regulate the entire maturation process. Moose and Sisco (1994) refer to the assemblage of genes promoting juvenility (including *Tp1, Tp2, Tp3* and *Cg*) as "juvenility program" genes. It appears that maturation may be regulated in a hierarchical fashion, with the juvenility program genes operating upstream of *GL15*. This scenario is intuitively appealing, since the juvenility program affects the entire raft of juvenile traits, while the subordinate *GL15* is only involved in regulating epidermal traits.

The viviparous8 (vp8) mutation of maize increases the number of juvenile leaves produced by increasing the rate of leaf initiation early during shoot development, and delaying vegetative phase change (Evans and Poethig, 1997). Additionally, it reduces the number of adult leaves produced, but does not delay flowering. Hence, it appears that Vp8 normally functions both to restrain the rate of early shoot development, and to promote vegetative phase change, but has no significant role in flowering (Evans and Poethig, 1997).

iii) Source Strength Regulation of Maturation

Transgenic plants with modifications in carbohydrate sink or source strength are an ideal system for studying the effects of the altered sink/source ratio on shoot development. In tobacco, Tsai *et al.* (1997) compared shoot development in Rubisco antisense plants, with that in the wild type. Rubisco is a key regulatory enzyme in the assimilation of photosynthetic carbon resources (Tsai *et al.*, 1997). The Rubisco antisense plants used in this study had a 20% reduction in Rubisco, and therefore reduced carbohydrate production (source strength), compared to the wild type. It was found that antisense plants remained in the juvenile phase for longer, and produced more leaves with greater longevity, than the wild type. These results suggest that the antisense plants were compensating for the lower carbohydrate assimilation rate brought on by the reduction in Rubisco, by delaying developmental events. In conclusion, it was suggested that in tobacco, carbohydrate source strength regulates the transition from the juvenile to mature phase (Tsai *et al.*, 1997).

The findings of Clemens *et al.* (1999) suggest that source strength may also be involved in vegetative phase change in *Metrosideros excelsa*. These findings pertain to the attainment of vegetative maturity. Nevertheless, the overriding importance of source strength could also help to explain some of the disparities noted at the end of section 1.5.3.1, regarding the classical theories accounting for the inability to flower. Many of the classical experiments would have been affecting source strength, but this was not regarded in consideration of results.

1.5.4 Practical consequences and manipulation in Pinus

An appreciation of phase change is an inherent component of any study involving reproductive activities in plants, especially woody perennials, which tend to have a relatively long juvenile phase. This point is especially important in tree breeding, since the duration of the juvenile phase is inversely related to breeding efficiency; a long juvenile phase implies many years may transpire before reproductive activity occurs, and improved breeds can be produced.

It was stated previously "once reproductive maturity is reached, it is relatively stable, and reversion to the juvenile state does not *generally* occur through techniques such as the taking of cuttings, or grafting" (Hackett, 1985). This may not *appear* to be strictly true for *Pinus*, as *apparent* rejuvenation, or at least partial rejuvenation can occur through the taking of cuttings (Jacobs, 1939) or grafting (Menzies *et al.* 1985; Hackett 1985). However, great care needs to be exercised in defining the cause of these phenomena associated with the rooting of cuttings and grafting. On this subject Hackett (1985) states "... it is difficult to distinguish between rejuvenation and reversal of physiological ageing (invigoration) ... some reports of rejuvenation may in reality be temporary invigoration related to reversal of physiological ageing".

In *Pinus radiata*, there is thought to be an "adolescent phase", at some stage prior to vegetative maturity (Hackett, pers. comm.[6]). This "adolescent phase" is probably analogous to that which Robinson and Wareing (1969) refer to as "near mature", in reference to their experiments with scions of larch. With the progression of phase change events, it appears that a critical point in time is reached, at which reproductive competence is attained. Presumably at this point in time, shoot primordia have developed sufficient sensitivity to perceive the requisite signals, and reproductive activity may commence, without attainment of the full raft of characteristics associated with maturity (Figure 1.9). Alternatively, the source strength (or the strength of some other factor) may have reached a threshold level, switching on the particular pathways involved in the reproductive facet of maturation.

[6] Wesley Hackett, Emeritus Professor, University of California, international expert on phase change, Personal communication as cited in Carter Holt Harvey Forests GRIF application.

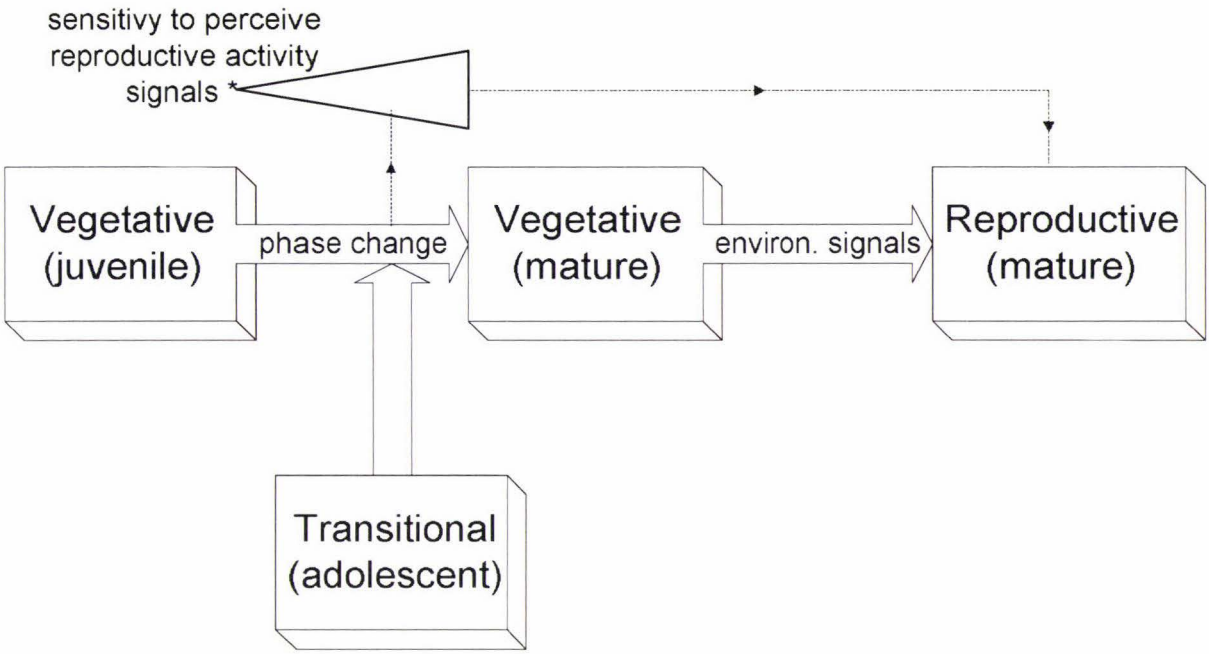

Figure 1.9: *Characteristics of phase change during plant ontogenetic development.*
** or reproductive activity factor has reached a threshold level*

The ability of the plant to produce cones, without having attained the full complement of mature traits, may be a consequence of there being a complex array of programs that act to regulate maturation. It is appears that the pathway(s) regulating cone production, may be detached from the array of pathways regulating other facets of maturation, and still remain operational. This point is illustrated by the occasional presence of male cones on *P. radiata* cuttings, which appear juvenile in many other respects (Figure 1.10).

Regulation of cone production is further complicated by the fact that male and female cones develop from different primordia - short shoot and long shoot primordia respectively (Cremer, 1992). Furthermore, the "requisite signals" required for the commencement of male and female development may differ, since different genes are involved in male and female development (Mouradov and Teasdale, 1999).

Figure 1.10: *Young Pinus radiata cutting, with male cones.*

1.6 Genes Involved in Flowering and Cone Production

Flowering and cone production involves the activity of two main groups of genes. Firstly, there are the floral meristem identity genes, that switch the meristem from vegetative to reproductive activity. Secondly, there are the floral organ identity genes that direct the development of the various organs of the flower.

1.6.1 Floral Meristem Identity Genes

As discussed previously, the production of flowers or cones, instead of leaves or branches, is a result of changes in the characteristics of structures produced by the shoot apical meristem. In *Arabidopsis* the development of flowers, instead of other structures, is mediated by the action of the floral meristem identity genes, *LEAFY,*

APETALA1 and *CAULIFLOWER* (Weigel and Nilsson, 1995). Floral meristem identity genes appear to have overlapping and partially redundant functions, since plants with mutations in one of these genes typically develop some flower-like structures. Weigel and Nilsson (1995) proposed a model for floral induction, in which the floral meristem identity genes interact, and positively regulate each other. While *APETALA1* and *CAULIFLOWER* also appear to be involved, *LEAFY* alone has been demonstrated to encode a developmental switch, the operation of which is sufficient to induce flowering (Weigel and Nilsson, 1995).

Weigel and Nilsson (1995) introduced *LEAFY* into aspen (*Populus tremula* x *tremuloides*) under control of the 35S cauliflower mosaic virus promoter. The *35S::LEAFY* aspen plants were obtained by *Agrobacterium*-mediated transformation of stem segments, and subsequent regeneration of transgenic shoots in tissue culture. Aspen naturally flowers after 8-20 years, whereas the transgenic line flowered within five months.

LEAFY homologues have been isolated in several tree species, including *MEL* in *Metrosideros excelsa* (McKenzie *et al.*, 1997), *ELF* in *Eucalyptus globulus* (Southerton *et al.*, 1998), and *NEEDLY* in *Pinus radiata* (Mouradov *et al.*, 1998). A different *LEAFY* homologue, *PrFLL* has also been isolated in *P. radiata* (Mellerowicz, *et al.*, 1998). *NEEDLY* and *PrFLL* are both expressed in juvenile and mature vegetative buds. Their expression pattern differs between male and female cone buds. *NEEDLY* expression occurs in developing male and female cones, but to a higher degree in female cones. In contrast, *PrFLL* expression occurs in developing male cones, but not in developing female cones (Mouradov and Teasdale, 1999).

Although *P. radiata NEEDLY* and *PrFLL* share extensive homology with *LEAFY*, they lack the proline-rich and acidic motifs thought to function as transcriptional activation domains. Expression of *NEEDLY* in transgenic *Arabidopsis* promotes flowering, demonstrating that, despite its sequence divergence, *NEEDLY* encodes a functional orthologue of the *LEAFY* gene (Mouradov *et al.*, 1998). The evidence suggests that *PrFLL* is involved in determination of male cone primordium identity (Mellerowicz, *et al.*, 1998).

It is of particular interest that two *LEAFY* homologues exist in *Pinus radiata*, and that their expression patterns differ in developing male and female cones. It is speculated that in ancestral plants, prior to the divergence of the angiosperms, there were separate male and female floral meristem identity genes. Furthermore, in angiosperms, with the evolution of the perfect flower, one of these then became redundant (Mellerowicz, *et al.*, 1998).

1.6.2 Floral Organ Identity Genes

Generally, angiosperm flowers are assemblages of four distinct types of organs, which are arranged as follows: outermost are the sepals, the leaf-like structures which protect the other flower parts while in the bud stage. Within the sepals are the petals, the familiar, usually conspicuous and brightly coloured structures, which often act to attract insects. Within the petals are the stamens (microsporophylls) which are the male parts, on which pollen is produced. The carpels (megasporophylls) occupy the centre of the flower, and these are the female parts, where ultimately seeds are produced (Thain and Hickman, 1996).

The relative position of a cell in the apical meristem (or its flanks) instructs that cell, and its derivatives, as to what type of organ they should become (Meyerowitz, 1994). Genetic studies of flowers from normal and mutant *Arabidopsis* plants have produced a simple model that explains this pattern of organ development (Figure 1.11). In *Arabidopsis*, activity of the *AGAMOUS (AG)* gene alone instructs specific cells to develop into carpels (Figure 1.11, region C alone). Similarly, activity of the *APETALA2 (AP2)* gene alone instructs specific cells to develop into sepals (Figure 1.11, region A alone). *APETALA3 (AP3)* and *PISTILLATA (PI)* activity, combined with *AG* activity instructs cells to develop into stamens (Figure 1.11, region B combined with C). *AP3* and *PI* activity, combined with *AP2* activity instructs cells to develop into petals (Figure 1.11, region B combined with A) (Coen and Meyerowitz, 1991; Meyerowitz, 1994).

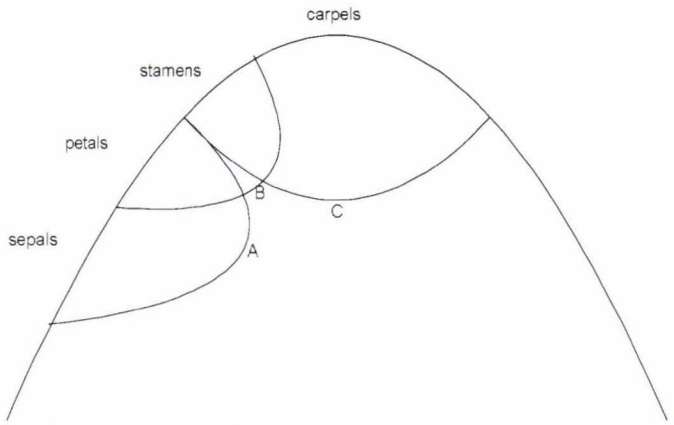

Figure 1.11: *Simple schematic diagram of floral primordium in tangential view, showing the organs that are specified by activities of genes, or gene combinations. Region enclosed by arc A - AP2 activity Region enclosed by arc B - AP3 & PI activity Region enclosed by arc C- AG activity (Concepts derived from Coen and Meyerowitz, 1991; Meyerowitz, 1994)*

The "ABC" model outlined above provides a fundamental overview of the genetics of flower development; in reality a multitude of other genes are involved. The organ identity genes represent an essential stage in the intracellular interpretation of position within the meristem. Clearly, other genes are involved, upstream of the organ identity genes, which establish the positional differences within the meristem (Meyerowitz, 1994). Additionally, numerous other genes are known to be involved in controlling flowering time, floral repression pathways, promoting autonomous flowering, and promoting flowering under different environmental conditions (Levy and Dean, 1998). Furthermore, new genes involved in flowering are frequently being discovered.

In *P. radiata*, MADS-box genes with homology to *Arabidopsis (AG)* and *(AP3)* have been isolated (Wang *et al.*, 1997). As discussed above, in *Arabidopsis* the activity of *AP3, PI* and *AG* together specifies stamens, while activity of *AG* alone specifies carpels. In reference to the *P. radiata AP3* and *AG* homologues, Wang *et al.* (1997) states "these are possibly genes involved in the developmental process leading to male cone formation".

Mouradov and Teasdale (1999) have isolated a gene, *PrDGL*, which is exclusively expressed in developing male cones of *P. radiata*. *PrDGL* was first detected in emergent male cone primordia, and its expression persisted throughout the early stages of male cone bud differentiation. *PrDGL* is significantly homologous to the *Arabidopsis AP3/PI* (B activity, in ABC model outlined above) floral organ identity genes, which when combined with *AG* specify stamens. It appears that *PrDGL* may play a role in distinguishing between male cone development when switched on, and female cone development when switched off (Mouradov and Teasdale, 1999).

A family of genes, *PrMALE 1, 2, 3*, has been isolated in *P. radiata* which are expressed only in male cone buds (Mouradov and Teasdale, 1999). These have been found to be homologous to *FIL1*, a stamen specific gene in *Antirrhinum*. Another male-specific gene, *PrMALE4*, has also been isolated, and Mouradov and Teasdale (1999) suggest that this may be involved in controlling pollen development.

Understanding of the genetic mechanisms involved in cone production in *P. radiata* is at present very limited. Further research into molecular aspects of *P. radiata* will undoubtedly lead to a better understanding, and possibly establishment of useful tools for the manipulation of cone production.

1.7 Plant Hormones

Bonnet-Masimbert (1987) suggests that the key to successful promotion of male cone production lies, as with female cone production, in application of hormonal treatments at the correct time i.e. in the period between short shoot initiation, and the commitment to either male cone or fascicle development. In many experiments, treatments have been applied at times which would have been more suited to promotion of female cone production, rather than male.

1.7.1 Gibberellins

i) Endogenous levels

In *Pinus radiata*, the forms of endogenous gibberellins present in terminal buds are known to vary during development. Taylor *et al.* (1984) examined levels of gibberellin-like compounds in first order branch buds of *P. radiata*, harvested at three dates encompassing both short shoot and long shoot initiation. Early on, during short shoot initiation, the less polar gibberellins, putatively GA_9, GA_4 and GA_7 were found to be predominant. Later, at the time long shoots were beginning to initiate, there was a shift towards more polar forms of gibberellin, such as (putatively) GA_1 and GA_3 (Taylor *et al.*, 1984).

GA_1 and GA_3 are considered to be the most biologically active forms of gibberellin, and most other forms are thought to be intermediates in their biosynthesis (Mohr and Schopfer, 1995). As mentioned, in *P. radiata* the onset of long shoot initiation appears to be correlated with an increase in GA_1 and GA_3 biosynthesis (Taylor *et al.*, 1984). Whether there is a causal link involved between the increase in GA_1 and GA_3, and the transition to long shoot initiation is unknown. This increase in GA_1 and GA_3 levels, may be related to other developmental activities, such as branch extension, which is also occurring at about this time.

ii) Exogenous application in Pinaceae

In many Pinaceae species, several studies have displayed successful promotion of female cone production, using a mixture of gibberellin A_4 and A_7 ($GA_{4/7}$). Pharis and Ross (1986) provide a list of 19 tree species (including *Pinus radiata*), from five genera of the family Pinaceae, that respond positively to the application of $GA_{4/7}$. In some *P. radiata* clones, increases in female cone production of over 200% are achievable by stem injection of $GA_{4/7}$ (Siregar and Sweet, 1996).

Many of the studies undertaken have concentrated on the promotion of female cone production. Considerably less work has been carried out regarding the promotion of male cone production. However, in *Picea* species, *Pinus sylvestris* (Bonnet-

Masimbert, 1987), *Pseudotsuga menziesii* (Pharis *et al.*, 1987), and *Larix leptolepis* (Anon, n.d.), male cone production has been enhanced by the application of $GA_{4/7}$.

Until recently, it was unclear whether exogenous gibberellins acted in a pharmacological, or morphogenic manner in Pinaceae cone induction. Pharis *et al.* (1987), in relation to Pinaceae, state "The GA effect on flowering is of undetermined physiological significance. The amount of GA that must be applied to a single shoot to have any effect on flower bud initiation is many thousand times higher than the levels of endogenous GA-like activity that can be detected by bioassay...Furthermore, the effect of applied GA is not unique. Other chemicals and cultural treatments produce comparable effects." Thus, it was considered that exogenous GA may be acting in a pharmacological manner to promote cone production.

However, it is now generally accepted that both endogenous, and exogenously applied gibberellins regulate bud growth and development (Oden *et al.*, 1994). One hypothesis for gibberellin-controlled cone production in Pinaceae is that imposed stress decreases shoot elongation, which is a possible strong sink for gibberellins. As a result, there is a build up in concentration of gibberellins which may then be utilised for cone induction (Oden *et al.*, 1994). Similarly, exogenously applied gibberellins might complement endogenous levels, which could be limiting to cone induction. However, Oden *et al.* (1994) state that "It... is more likely that the availability of active GAs is more delicately regulated in specific cells and cell compartments and that the metabolism is directly influenced by factors like root activity, stomatal turgor and temperature."

In Pinaceae, little progress has been made towards elucidating the precise mechanisms linking gibberellin with cone induction. However, in *Arabidopsis* Blázquez *et al.* (1998) have linked the effect of GA on flowering to *LEAFY* transcription and activity. In mutants unable to flower due to defective GA biosynthesis, *LEAFY* promoter activity was shown to be reduced compared to the wild type. Flowering ability in this mutant was restored by a constitutively expressed *LEAFY* transgene.

It is possible that GA is associated with *LEAFY* homologue activity in a similar fashion in *P. radiata*. Ross and Pharis (1987) suggest that "GAs function as a triggering agent to induce initial changes in the apical meristem which lead to reproductive as opposed to vegetative development." It is possible that the "triggering agent" alluded to is GA, and one of the "initial changes in the apical meristem" is the upregulation of the *P. radiata LEAFY* homologue(s) *NEOLY* and/or *PrFLL*.

1.7.2 Auxins

Auxins are a group of plant growth regulators that are involved in many aspects of plant development, and are implicated in flower initiation (Thain and Hickman, 1996). Naturally occurring auxins include indole-3-acetic acid (IAA) and indole-3-acetonitrile (IAN) (Thain and Hickman, 1996). The synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA), which have plant growth regulatory activity, have also been developed (Thain and Hickman, 1996).

i) Endogenous levels

Pilate *et al.* (1990) induced Douglas fir trees to produce cones using a combination of GA_{4+7} and NAA. Subsequently, IAA levels in vegetative and cone-producing shoots were quantified immunologically with anti-IAA antibody after separation of extracts by HPLC. Comparisons indicated that IAA levels differed only slightly between vegetative and cone-producing shoots (Pilate *et al.*, 1990).

In *Pinus sibirica*, Goroshkevitch and Menyailo (1995) compared levels of IAA present in buds from potentially vegetative and potentially reproductive parts of the crown, at the time of male cone initiation, and female cone initiation. In general, IAA levels were found to be fairly similar across all samples, except in the case of potentially male buds at the time of male cone initiation. In this case the IAA level

was approximately double that of other samples (Goroshkevitch and Menyailo, 1995).

ii) Exogenous application

Pharis and Ross (1986) mention examples of species in which cone production has been enhanced using the synthetic auxin, naphthaleneacetic acid (NAA), but generally only in concert with $GA_{4,7}$. For example, Sweet (1979) used a combination of $GA_{4,7}$ with NAA to promote female cone production in *Pinus radiata*. Similarly, Marquard and Hanover (1985), and Pharis *et al* (1986) increased cone production in white spruce (*Picea glauca*) using a combination of $GA_{4,7}$ and NAA.

In some species of the Pinaceae family, there is some evidence that NAA applied in conjunction with $GA_{4,7}$ may enhance male cone production, at the expense of female cone production (Ross, 1989; Ross and Pharis, 1987).

In general, application of NAA alone does not appear to enhance cone production. Sheng and Wang (1990) discuss a few examples where NAA alone may enhance cone production, but stipulate that these cases have not been readily repeatable.

1.7.3 ABA

i) Endogenous levels

In *Pinus sibirica*, there is a marked reduction in the level of ABA in buds from the male generative storey at the time of male cone initiation (Goroshkevitch and Menyailo, 1995). It is possible that the reduction in ABA has some bearing on male cone initiation. However, according to Goroshkevitch and Menyailo (1995), the reduction in ABA may be due to the preferential synthesis of gibberellins at the same time, from the common precursor mevalonic acid.

Increases in endogenous levels of ABA are known to occur in plants, in response to various stresses, particularly water stress (Bray, 1993; Kermode, 1997). A variety of hormonal and cultural treatments are known to be associated with an increase in cone

production in Pinaceae family species (Pharis and Ross, 1986; Bonnet-Masimbert, 1987). It seems likely that many of these treatments impose considerable stress when applied to trees, thus mediating an increase in endogenous ABA levels. There is no evidence that causally links an increase in ABA with increased cone production, but there is sufficient evidence to suggest these may be associated.

Pilate *et al.* (1990) treated Douglas fir trees with a combination of $GA_{4/7}$ and NAA, in order to induce cone production. Both vegetative and cone-producing shoots subjected to this treatment had levels of endogenous ABA approximately twice that of control shoots, which did not receive exogenous hormone application. Endogenous ABA levels in shoots were quantified immunologically with anti-ABA antibody, after separation of extracts by HPLC. Pilate *et al.* (1990) suggested that the increase in endogenous ABA levels in tissues from hormonally treated trees may indicate that a mild stress has occurred.

ii) Exogenous application

ABA is widely considered to be a growth inhibitor, and slowing of vegetative growth may be associated with the promotion of cone production (Pharis *et al.*, 1987). Timely application of ABA, between short shoot initiation and the commitment to either male cone or needle fate, may slow development at a critical stage thereby promoting male cone production (R. Riding, pers. comm.[7]). In *Pinus radiata*, Welsh (1997) demonstrated that stem injection of ABA approximately four to six weeks after short shoot initiation increased male cone production and realisable pollen yield.

1.7.4 Cytokinins

Cytokinins are a group of phytohormones that can be divided into two broad structural classes, adenines and ureas. Although the adenines and ureas are structurally different, both groups are able to interact with the cytokinin receptor (Iwamura, 1992). The cytokinins are thought to be synthesised mainly in root apices, and transported via the xylem to the shoot (Mohr and Schopfer, 1995). Cytokinins

[7] Richard Riding, University of New Brunswick, Canada. Personal communication 14 June 1997.

are implicated in a range of physiological and morphological processes in plants, including phase change (Zhang, 1998).

In the endemic New Zealand tree *Elaeocarpus hookerianus*, Day *et al.* (1995) compared endogenous cytokinin levels in juvenile and mature leaves. Active cytokinin levels were found to be higher in the juvenile phase leaves, compared to the mature phase leaves. While a reduction in cytokinin levels was found to be correlated with maturation, it was not determined that there was a causal link between the two.

The *in vitro* rejuvenation of *Pinus pinaster* meristems, in buds grown on cytokinin containing medium, has been reported (David *et al.*, 1978, cited in Hackett, 1985). In enclosed buds of mature *P. radiata* cultured *in vitro*, the cytokinin 6-benzylaminopurine (BAP) causes an apparent rejuvenation of the plantlets; primary leaves characteristic of the juvenile phase are produced, instead of three leafed fascicles characteristic of the mature phase (Horgan, 1987). Further to these findings, Zhang (1998) analysed endogenous levels of various cytokinins in buds from juvenile and mature trees. The highest concentrations of cytokinin free bases and ribosides were found in seedling buds. In progressively older buds the concentrations were found to be progressively lower. This, and other findings of Zhang (1998), suggest that cytokinins play an important role in controlling maturation in *P. radiata*.

Doumas *et al.* (1989), and Zaerr and Bonnet-Masimbert (1987) discuss the possibility that endogenous cytokinins have a role in the induction of strobili in Douglas fir (*Pseudotsuga menziesii*). Treatments that reduce root area, such as root pruning, are known to induce cone production in forest trees (Bonnet-Masimbert, 1987). Zaerr and Bonnet-Masimbert (1987) working with Douglas fir found that a relatively low concentration of endogenous cytokinins in the shoot was associated with the production of cones, rather than vegetative development. Since cytokinins are largely produced in the roots, Doumas *et al.* (1989) suggest that reducing root area may lead to a reduction in the flow of endogenous cytokinins to the shoot. The reduction in cytokinin levels in the shoot might, therefore, be involved in cone induction. This hypothesis ties in very well with the findings of Zhang (1998) and others, that a reduction in endogenous cytokinin levels is associated with the

maturation. Interestingly, Zaerr and Bonnet-Masimbert (1987) allude to the possible use of cytokinin antagonists to promote cone production. Such compounds are now successfully used to promote flowering in asparagus (see below).

As mentioned previously, levels of various forms of endogenous cytokinins in *Pinus radiata* are known to vary during development. Sweet (1979) found levels of endogenous cytokinins (zeatin and zeatin riboside) to be relatively lower in buds he considered to be potentially female cone bearing, compared to those he considered to be potentially vegetative.

Taylor *et al.* (1984) examined levels of cytokinins in lateral buds of *P. radiata*, harvested at three dates encompassing both short shoot and long shoot initiation. Early on, during short shoot initiation, the less active glucoside conjugate of zeatin riboside dominated. Later, at the onset of long shoot initiation, the more biologically active zeatin riboside appeared. Male cones and leaves are homologous structures, both being derived from short shoots. Similarly, female strobili and branches are homologous, both being long shoots (Doak, 1935). Hence, it is possible that the cytokinin forms present in the bud at a given time influences the developmental pathway taken by the primordia; either male cones/leaves, or female cones/branches are produced, depending on the cytokinin form present. However, as Zhang (1998) points out, the range of cytokinins studied by Taylor *et al.* (1984) is incomplete (the nucleotide cytokinins were ignored). Hence, the situation is undoubtedly more complex than that presented by Taylor *et al.* (1984).

1.7.4.1. Anticytokinins

Anticytokinins are a synthetic group of compounds allied in structure to the cytokinins. When applied exogenously to a plant they function by binding to receptor sites and blocking the action of endogenous cytokinins (Iwamura, 1994). Anticytokinins, such as anilide, benzamide, and phenylcarbamate derivatives are able to induce flowering in one month old asparagus (*Asparagus officinalis* L.) seedlings, when seeds are germinated in the presence of these compounds (Yanosaka, 1989; Hara *et al.*, 1992). Asparagus does not normally flower until two or three years old.

In the case of some carbamate derivatives, successful flowering in up to 100% of seedlings was attained (Yanosaka, 1989).

Asparagus seedlings induced to flower by treatment with either carbamates or thiocarbamates have been shown to accumulate an acidic 17-kDa polypeptide (Yeo *et al.*, 1996). This protein appears early during the transition from vegetative to floral activity, and has been named early flowering protein (EFP). Carbamates and thiocarbamates that induced flowering in more than 80% of seedlings also induced high levels of EFP, while weak flower-inducing compounds induced only low levels of EFP.

1.8 Cultural treatments to enhance cone production

1.8.1 Grafting

1.8.1.1. Background

As early as 1820, *Pinus nigra* Arnold var. corsicana was being successfully grafted onto *P. sylvestris* to facilitate seed production (Jayawickrama *et al.*, 1991).

Grafting is important for the vegetative propagation of conifers, since in many species there are difficulties in rooting cuttings, especially when these are taken from mature trees. One such example is that of *Pinus taeda* (loblolly pine) which, according to Buijtenen and Shaw (1985), is "notoriously difficult to propagate by rooted cuttings". The problem is far more marked for cuttings taken from six year old trees, compared to cuttings from one year old seedlings. Other United States southern pines are also problematic in this regard (Dorman, 1976). On the other hand, straight forward, reliable grafting methods for these species have been developed, and are routinely used for the establishment of seed orchards in the southern United States (Dorman, 1976).

In *P. radiata*, the rooting of mature cuttings is also challenging. However, methods have been developed to overcome many of the associated difficulties (Van Dorsser and Faulds, 1991). Nevertheless, grafting is still relied upon as an intermediate step in the methodology. Grafted *P. radiata* ramets are extensively used in New Zealand commercial seed orchards (Sweet and Thulin, 1973; Menzies *et al.*, 1985; Vincent, 1986; Vincent, 1997), and for clonal archives (Menzies *et al.*, 1985; Firth, 1986).

These are the only widespread uses for grafting in *P. radiata*, yet in horticulture grafting is utilised far more extensively. Fruit tree rootstocks have been developed for a multitude of uses including: control of ramet size, precocious flowering, fruit yield and quality, cold tolerance, disease resistance, and adaptation to site (Jayawickrama *et al.*, 1991). Work has been carried out attempting to identify rootstocks that may provide similar benefits in some Pinaceae family species. However, the results of the various studies are often conflicting, and consequently our knowledge of the effects of grafting in Pinaceae is sketchy. The remainder of this section attempts to clarify some of what is known regarding this topic.

1.8.1.2. Terminology

According to Jayawickrama *et al.* (1991), the terms graft survival, graft success, and graft (in)compatibility are sometimes used interchangeably, and undoubtedly this may lead to confusion. Jayawickrama *et al.* (1991) defines *graft success* to be the formation of a graft union between the scion and rootstock. A successful graft will still be alive a few weeks after grafting. If the vascular tissues of the scion and rootstock do not properly unite, the scion will die within a short period of time.

Jayawickrama *et al.* (1991) defines *graft incompatibility* to be when a successful union forms but the grafted part of the plant later develops physiological and developmental malfunctions. Graft incompatibility may be expressed soon, or several years, after grafting. In conifers, symptoms of graft incompatibility include yellowing of leaves, and relatively short leaves (Vincent, 1997). These are typical early symptoms of graft incompatibility in *P. radiata*. In more extreme cases the symptoms

may ultimately include death of the scion. These definitions will be adhered to in this thesis.

1.8.1.3. Graft success

As might be expected, grafts between closely related individuals generally tend to be more successful than those between distantly related individuals (Jayawickrama *et al.*, 1991). In a study involving 5580 grafts using scions from six families of conifers, the success rates were: 97% for intraspecific grafts, 68% for interspecific grafts, 37% for intergeneric grafts, and 0% for interfamily grafts (Yakovleva, 1974).

Schmidtling (1973) grafted scions from three different *Pinus taeda* clones onto *P. taeda, P. echinata, P. elliottii, P. glabra,* and *P. serotina* seedling rootstocks. In this work, graft survival averaged above 90%, and differed little between clones or rootstocks.

P. radiata may be successfully grafted intraspecifically. Additionally, *P. radiata* scions may be successfully grafted onto rootstocks of several other *Pinus* species, including *P. attenuata, P. contorta, P. elliottii, P. muricata, P. patula, P. ponderosa* and *P. taeda* (Faulds, pers. comm.[8]).

1.8.1.4. Graft Compatibility

Graft compatibility tends to follow similar patterns to graft success; grafts made between more closely related scions and rootstocks tend to be more compatible than between those more distantly related (Jayawickrama *et al.*, 1991). Graft compatibility figures provided by Yakovleva (1974) are: 96% for intraspecific grafts, 61% for interspecific grafts, 17% for intergeneric grafts, and 0% for interfamily grafts. The time after grafting when these assessments were made is not given in the English abstract.

[8] Trevor Faulds, Forest Research nursery, New Zealand. Personal communication 30 November 1999.

Based on evidence from Ahlgren (1972), the graft compatibility of some rootstock/scion species combinations appears to be quite poor, while for other similar combinations compatibility is relatively high. For example, *P. griffithii* (also known as *P. excelsa* and *P. wallichiana*) scions appear to be completely incompatible with most rootstocks tried in this study. On the other hand, compatibility of *P. peuce* and *P. strobus* scions on the same rootstock species was much higher. These three species are closely related, all belonging to subsection *Strobi* (Farjon 1984).

In Schmidtling's (1973) study, involving scions from three different *Pinus taeda* clones grafted onto *P. taeda, P. echinata, P. elliottii, P. glabra,* and *P. serotina* rootstocks, the findings were similar. As time progressed considerable scion mortality occurred, due to incompatibility. The mortality varied depending on scion clone, and rootstock species. One scion clone suffered much higher mortality than the other two, which were similar. The worst rootstock species was *P. glabra*; after four years there was 49% mortality. According to Schmidtling (1973), *P. glabra* is less closely related to the scion species, than the other species of rootstock. However, this claim may be disputed based on other classification systems (Mirov, 1967; Farjon, 1984). Interestingly, grafts of *P. taeda* scions on *P. elliottii* rootstocks were the most compatible (82% survival after four years), and those on *P. taeda* rootstocks were second to worst (63% survival after four years).

In intraspecific grafts of *P. elliottii*, Bower and McKinley (1987) tested the effect of scion/rootstock relatedness. They concluded that no significant gain in compatibility was to be realised by grafting scions onto rootstocks to which they are closely related. Conversely, for intraspecific grafts of *P. taeda*, Schmidtling (1983a) found that scion and rootstock genotype had a significant effect on graft compatibility. In an extreme case, for one particular scion/rootstock combination, mortality due to incompatibility was 100%.

Incompatibly is particularly problematic in New Zealand *P. radiata* seed orchards, where it causes the death of many ramets (Sweet and Thulin, 1973; Copes, 1980). The economic significance of this problem is substantial, since death of ramets results in a reduction in the availability of genetically "superior" seed. The severity of this

incompatibility problem varies markedly, depending on genotype (Copes, 1980). In view of the very wide genetic variability that *P. radiata* possesses (Forde, 1964d), it is not particularly surprising that intraspecific graft incompatibility exists in this species (Copes, 1980).

1.8.1.5. Cone Production

Schmidtling (1969) examined the influence of grafting seedling-derived *Pinus echinata* scions onto three species of seedling rootstock. Seven years after planting out in the field, cone production (female?) was assessed. Grafts on *P. taeda* rootstocks appeared to favour cone production, compared to those on *P. echinata* and *P. elliottii* rootstocks.

For three different clones of *P. taeda* scion, grafted onto different rootstock species, Schmidtling (1973) found that rootstock species significantly affected the number of female cones produced. For the first three years the number of cones produced per tree was consistently highest on *P. glabra* rootstocks, which were the least compatible (see discussion above under compatibility). Additionally, the scion clone which was least compatible produced the most female cones. From year four the situation changed dramatically, and ramets on *P. glabra* rootstocks began to produce the lowest number of female cones. At this time female cone numbers became highest on *P. elliottii*, followed by *P. taeda* rootstocks. Later reassessments indicated that this general trend continued, at least until year ten (Schmidtling, 1983a). Details of male cone numbers in this trial are also provided by Schmidtling (1983a). Male cone numbers were also highest on ramets grafted onto *P. elliottii* rootstocks, followed by *P. taeda* rootstocks, with *P. glabra* rootstocks being the worst.

Schmidtling (1983a) furthered the earlier studies by grafting *P. taeda* scions onto two additional rootstock species, namely *P. densiflora* and *P. virginiana*. Up until age five, female cone production was greater on ramets grafted onto dwarfing *P. densiflora* rootstocks, than on other rootstock species tried. As for *P. glabra* rootstocks in the earlier study, this advantage was short lived, and other rootstock species provided better cone production in later years. Evidence from Schmidtling

(1983a) also suggests that in intraspecific grafts of *P. taeda*, the genotype of the rootstock also influences both female and male cone production.

Ahlgren (1972) undertook a trial involving grafting of *Pinus strobus, P. peuce, P. koraiensis, P. cembra,* and *P. griffithii* scions onto rootstocks from five species of *Pinus* and *Abies balsamea*. Based on the results of this study, it is suggested that scion/rootstock combinations with "slight" incompatibility may stimulate the production of male cones. Melchior (1984) grafted *Picea abies* scions onto various rootstocks. It was concluded that dwarf rootstocks, *Picea abies* 'Conica' and *Picea abies* 'Clanbrasiliana', may stimulate precocious cone production. Unfortunately, in this paper it is not clear whether male or female cones were being discussed; the author mentions "fruit setting", so presumably it is female cones (if these are considered to be "fruit").

In the East African tree breeding program in 1966, *Pinus patula* scions were grafted onto *P. radiata* rootstocks, instead of the usual *P. patula* rootstocks. While the grafts on *P. radiata* rootstocks were successful, it was subsequently found that they had a slower growth rate than intraspecific grafts. Five years after planting a large proportion of the ramets on *P. radiata* rootstocks had a dwarfed, bushy form but appeared to bear more female cones than grafts made on *P. patula* (Dyson, 1975). Undoubtedly the phenomenon observed by Dyson (1975) was associated with mild graft incompatibility.

For *P. taeda* seed orchards Burris *et al.* (1991) provide a method which they claim reduces the period required for female cones to be produced by one year. Three year old scions, grafted onto 2 or 3 year old rootstocks, and subjected to a regime of drought stress and several $GA_{4/7}$ applications, may produce female cones 14 months after grafting. Ten month old scions treated similarly may produce both male and female cones 26 months after grafting. Thus the minimum total time taken, from germination to cone production, was 36 months (Burris *et al.*, 1991). This method *might* provide a worthwhile saving in time for breeding purposes, but would probably be very expensive.

In *P. taeda* grafts, Jayawickrama *et al.* (1992) studied variations in scion physiology associated with the effects of different rootstocks, scion clones, sites, and interactions of these factors. Rootstocks significantly affected levels of hexose, total sugars, P, K, Ca, and Mg in the leaves of scions. Both male and female cone numbers correlated significantly with concentrations of some nutrient levels. Levels of some sugars (especially hexose during spring), and N and K appear to be significantly positively correlated with male cone production. Conversely, levels of Mg and Ca appear to be negatively correlated with male cone production. It is suggested that rootstocks that appear to increase cone numbers may do so by altering the levels of sugars and some mineral nutrients.

Evidence from the studies of Schmidtling and others suggest that certain rootstocks can provide worthwhile gains in cone production, in the years immediately following grafting. It should be noted, however, that not all studies showed a significant effect of rootstock on cone production (Jayawickrama *et al.*, 1991). Nevertheless, a predominant theme in the literature is that incompatibility of the scion and rootstock tends to favour early cone production. Obviously, the degree of incompatibility needs to be mild so that mortality is not too great.

Increased cone production in these formative years is of key interest to the New Zealand tree breeding industry. It seems likely that judicious selection of rootstocks could provide worthwhile gains in early ontogenetic cone production in *P. radiata* too. Supporting evidence for this hypothesis comes from observations of grafted *P. radiata* ramets. Relatively heavy cone production is known to be associated with a high degree of graft incompatibility (Faulds, pers. comm.[9]). Furthermore, *P. radiata* scions have been successfully grafted onto several different species of rootstock, and found to be very incompatible on some (*P. attenuata, P. contorta, P. elliottii, P. muricata, P. taeda*), and not so incompatible on others (*P. patula, P. ponderosa*) (Faulds, pers. comm.[9]). Unfortunately, cone production was never assessed on these

[9] Trevor Faulds, Forest Research nursery, New Zealand. Personal communication 30 November 1999.

grafts; in any case, it is likely that for some rootstock species, mortality would have occurred before cone production could be expected.

Since *P. radiata* possess a very wide degree of genetic variability (Forde, 1964d) it can be hypothesised that the choice of rootstock may vary greatly, depending on the genotype of scion used. Scions of each superior genotype would most likely require a specific type of rootstock for optimum cone production; the rootstock would most likely provide a balance between sufficient incompatibility to provide gains in cone production, while not causing excessive ramet mortality. With the multitude of rootstocks available (different genotypes of *P. radiata*, and different species), it seems likely that suitable rootstocks could be identified.

1.8.2 Root restriction

Root restriction is known to stimulate cone production in some trees of the Pinaceae family (Pharis *et al.*, 1987). In seedlings of *Pinus caribaea* var. *hondurensis*, Bacon (1985) found that root wrenching induced a marked reduction in endogenous cytokinin, and gibberellin levels, and an increase in levels of ABA. As previously mentioned, Doumas *et al.* (1989) suggest that reducing root area may lead to a reduction in the flow of endogenous cytokinins to the shoot, which may enhance cone production. This is possibly associated with the role of cytokinins in maturation; a reduction in endogenous cytokinin levels is associated with maturation (Zhang, 1998).

The increase in ABA levels noted by Bacon (1985), due to root wrenching, is probably a stress response. ABA is now known to be involved in the upregulation of several genes, in response to various stresses (Bray, 1993; Kermode, 1997).

1.8.3 Drought Stress

Drought stress is commonly used to enhance cone production in conifers, both alone or in addition to other treatments (Pharis and Ross, 1986; Bonnet-Masimbert, 1987).

As for root wrenching, Bacon (1985) found that drought stress induced a marked reduction in endogenous cytokinin, and gibberellin levels, and an increase in ABA levels, in *Pinus caribaea* var. *hondurensis* seedlings.

In this thesis, hormone treatments, grafting, root restriction and drought stress were investigated as methods of inducing precocious male cone production in *Pinus radiata*.

Chapter 2

Regional study of male cone production

2.1 Introduction

In the New Zealand tree breeding industry, there is considerable interest in increasing the rate at which successive generations are bred. A significant bottleneck in the breeding process is the period of time, from planting of seedlings or cuttings, until male cone production commences.

There is an apparent lack of detailed information in the literature regarding the age at which male cone production commences in *Pinus radiata*. Anon. (1990, in What's New in Forest Research No. 182) states "...radiata pine grafts or cuttings of physiologically mature trees rarely produce pollen until around four years after planting". However, Vincent (1997) states "...radiata pine grafts or cuttings from physiologically mature trees...(on most sites in New Zealand) rarely produce pollen until around five years after planting".

Burdon and Bannister (1973) provide results from a trial carried out in Kaingaroa Forest, comparing the performance of *P. radiata* originating from the five natural populations, and two New Zealand populations (Kaingaroa and Nelson). For the New Zealand and Ano Nuevo provenances, approximately 25% of trees were found to be producing male cones at age five (from seed). Figures for the other provenances were much lower. Unfortunately no details for other ages are provided. Fielding (1960) assessed male cone production at two Australian Capital Territory forests in 1956. At age three years, 4 % and 1 % of trees were producing male cones at Kowen and Uriarra respectively. At age five years, the respective figures were 82 % and 46 %.

Comparing these results, there are obvious differences in the incidence of male cone production depending on the provenance from which seed is derived and the location of the trial. The reasons for this phenomenon are unclear. However, a combination of genetic and environmental factors probably play a part. In order to elucidate this situation (at least to some degree) an appraisal of male cone production at some contrasting locations around New Zealand was carried out to determine whether certain regions with their unique climatic conditions appeared to favour precocious male cone production compared to others. It was also considered necessary to obtain an appreciation of male cone production before the major morphological study presented in Chapter 3 was carried out.

2.2 Materials and Methods

Five regions were selected in which to undertake this study, namely Northern Kinleith, Southern Kinleith, Northland, Karioi, and Nelson. The proportion of trees producing male cones was assessed in forests from each region. In Northern Kinleith, Southern Kinleith and Karioi regions age classes 3 to 10 years (1996-1989 plantings) were assessed. In the Nelson region age classes 3 to 8 years (1996-1991 plantings) were assessed. In the Northland region age classes 3 to 7 years (1996-1992 plantings) were assessed.

In Northern and Southern Kinleith, for plantings prior to 1993 (1989 to 1992) information on genotype was not recorded. However, these were most likely all seedlings with a broad mixture of genotypes. More recent plantings also encompass a broad range of genotypes in most age classes. In the Nelson region most plantings were from "268" series orchards. No information on genotypes at Northland and Karioi was available; most likely these were open pollinated seedlings with a wide genetic background. This study was carried out in late autumn to winter of 1999.

For forests other than Kinleith, compartment maps were assembled detailing areas stocked with unpruned trees of the required age classes. For Northern and Southern Kinleith, compartment maps were no longer available and maps containing the

required details were generated by ARCINFO from GIS data (see Kinleith section of discussion for further details).

For each age class, in each region, three repetitions of 40 x 5 m transects were utilised. Transect start points were randomly assigned on maps detailing age classes of interest. Transects were laid northward from these start points, with the aid of a compass and hip-chain. When necessary a corridor was cut through weeds with a slasher. Trees within 2.5 m either side of the hip-chain line were numbered using aerosol spray paint. On completion of each 40 m transect, numbered trees were carefully scrutinised for the presence or absence of male cones, during the return to the start point. The number of trees carrying male cones, and the total number of trees in each transect plot were recorded.

Data was analysed using the SAS procedure LOGISTIC (SAS, 1995).

Climate data for each region was obtained from the New Zealand Meteorological Service, and Climate Research and Information Services, National Institute of Water and Atmospheric Research Ltd. Climate stations closest to the forest regions studied were selected. In some instances these stations did not measure sunshine hours and this was derived from a second nearby station. Nevertheless, this data is still very pertinent to the regions studied. Table 2.1 relates the regions studied to the climate stations used to source data for them. Data are averages from readings taken over many years, as detailed in Table 2.1. More recent data is available from some of these climate stations, whereas others have ceased operating. Thus, the data provided is the latest available that provides data for all climate stations of interest.

Table 2.1: *New Zealand climate stations used to source data for the regions employed in this study. The period data for each parameter pertains to is in parentheses below the climate station name. Locations of climate stations are provided in New Zealand Meteorological Service (1983).*

	Climate Station used as parameter source, and period data covers		
Study region	**Sunshine hours**	**Temperature**	**Rainfall**
Northland	Kaitaia Airport (1951-1980)	Aupori Forest (1967-1980)	Aupori Forest (1967-1980)
Northern Kinleith	Whakarewarewa (1935-1977)	Kinleith (1967-1980)	Kinleith (1967-1980)
Southern Kinleith	Taupo (1949-1980)	Atiamuri Power Stn (1967-1980)	Atiamuri Power Stn (1966-1980)
Karioi	Ohakune (1962-1974)	Karioi (1930-1980)	Karioi (1927-1980)
Nelson	Nelson Airport (1935-1980)	Golden Downs Forest (1930-1980)	Golden Downs Forest (1929-1980)

2.3 Results

2.3.1 Incidence of Male Cones

Assessment results for the proportion of trees bearing male cones in each region are summarised in Figure 2.1.

In the Northland forests assessed in this study there were no plantings aged 8 to 10 years, and this is representing by * in Figure 2.1. For Nelson, the * denotes that no assessment of 9 and 10 year old trees was undertaken because 100% cone production had already clearly been attained by younger age classes. Cuttings were only present in Northern and Southern Kinleith, and planting of these commenced in 1993 (age 6). In the Northern Kinleith region, no seedlings aged 5 and 6 were available which were suitable for this study; all areas of these age classes at the correct altitude had been pruned.

In the Nelson region, male cone production commenced in a small proportion (2%) of trees at age 3 and by age 4 90% of trees were producing male cones. In Northern and Southern Kinleith, male cone production commenced at age 4. In Northland and Karioi, male cone production did not begin until age 6 (Figure 2.1)

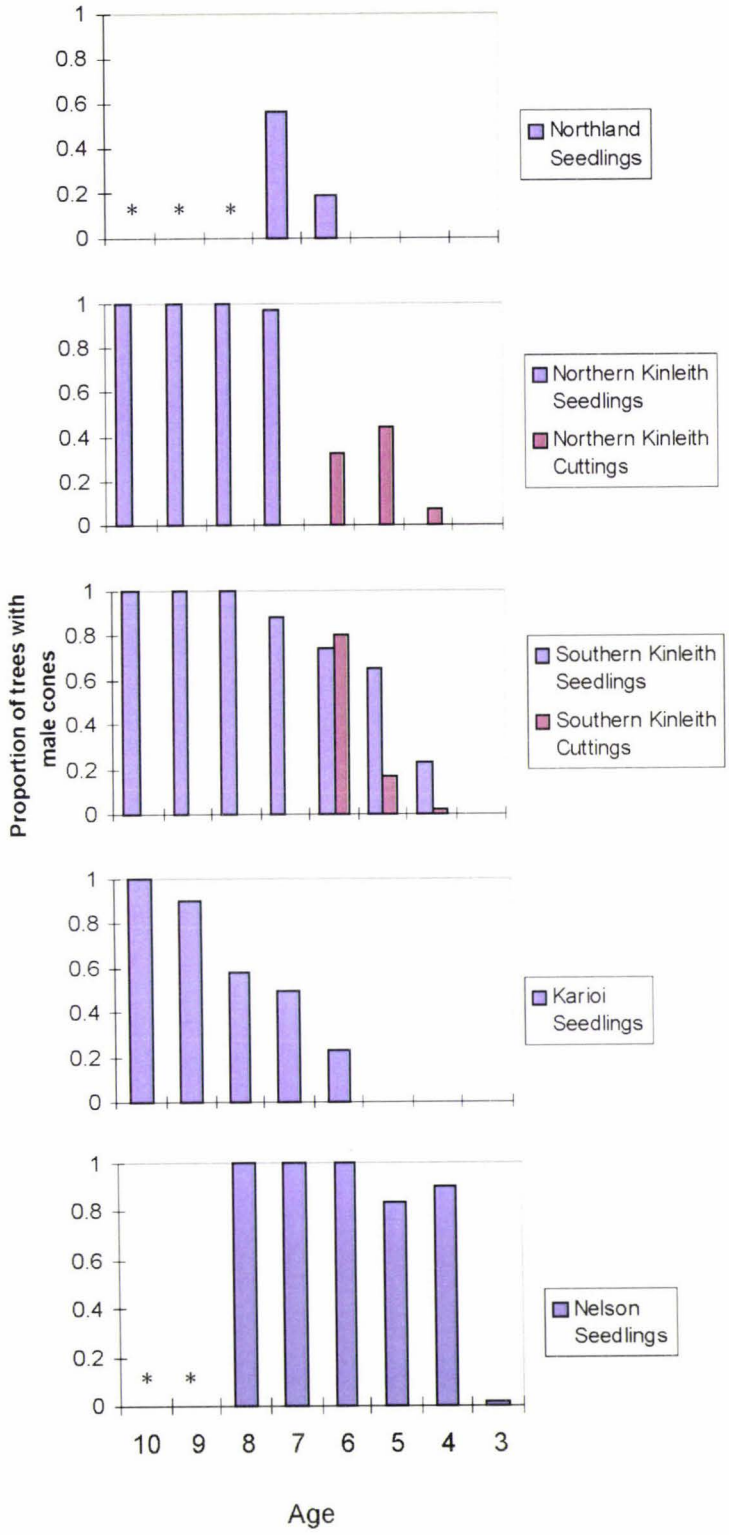

Figure 2.1: *The proportion of trees bearing male cones in Northland, Northern Kinleith, Southern Kinleith, Karioi, and Nelson regions. Each bar represents the mean of data from three 40 x 5 m transects.*
** denotes no data (see text).*

2.3.2 *Climate Data*

Mean sunshine hours, average daily minimum temperatures, and mean rainfall experienced each month are provided in Figures 2.2, 2.3, and 2.4 respectively. Figure 2.2 reveals that the Nelson region experiences considerably greater sunshine hours throughout the year, compared to the other regions studied. Of these other regions, all experience similar levels of sunshine hours during summer and autumn. During winter and spring, Northland experiences greater and Karioi experiences lesser sunshine hours than Northern and Southern Kinleith.

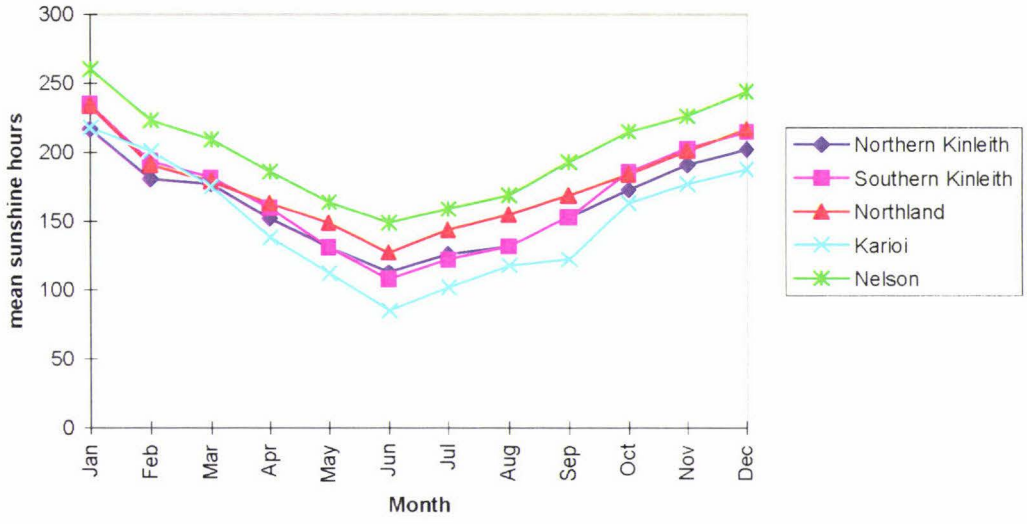

Figure 2.2: *Mean sunshine hours per month in Northland, Northern Kinleith, Southern Kinleith, Karioi, and Nelson regions. (Data source: New Zealand Meteorological Service, 1983)*

Average minimum daily temperature experienced each month is considerably higher (~4° C in summer to ~7° C in winter) throughout the year in Northland, compared to any of the other regions studied. The other regions fall within an envelope of approximately 4° C of each other throughout the year, with Nelson experiencing the coolest winter and Karioi the coolest summer temperatures (Figure 2.3).

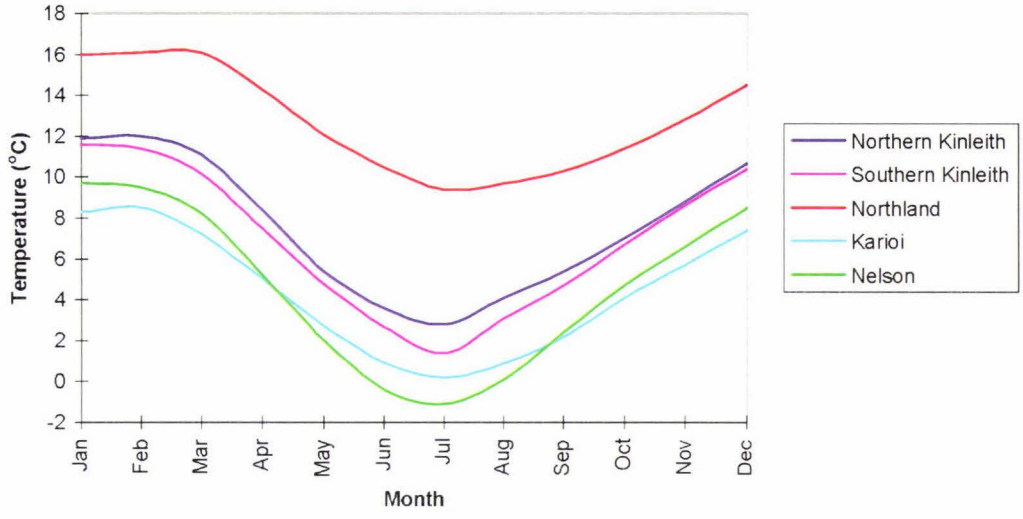

Figure 2.3: *Average daily minimum temperature experienced each month in Northland, Northern Kinleith,Southern Kinleith, Karioi, and Nelson regions.*
(Data source: New Zealand Meteorological Service, 1983)

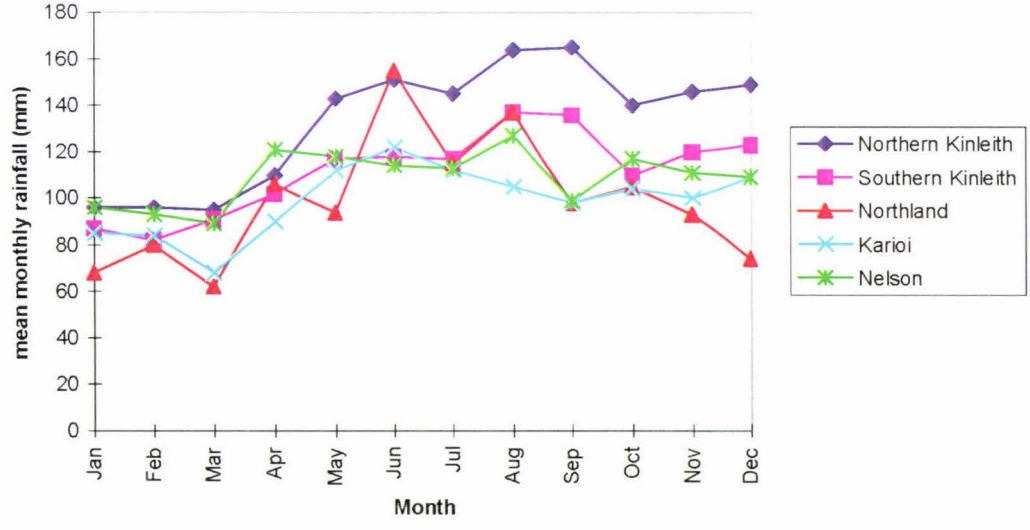

Figure 2.4: *Mean rainfall per month in Northland, Northern Kinleith, Southern Kinleith, Karioi, and Nelson regions.*
(Data source: New Zealand Meteorological Service, 1983)

Figure 2.4 reveals that rainfall patterns are more complex than those for sunshine hours and temperatures. In general, Northern Kinleith experiences the highest and Karioi the lowest rainfall. All regions in this study experience what may be regarded as "moderate" rainfall, when compared to extremes experienced in some other parts of New Zealand (New Zealand Meteorological Service, 1983).

Table 2.2 provides data from climate stations in locations experiencing greater than 2200 hours of sunshine each year.

Table 2.2: *New Zealand climate stations recording sunshine hours greater than 2200 hours per year, and associated lowest average daily minimum temperatures.*
(Data source: New Zealand Meteorological Service, 1983)

Climate Station / Region	Mean Yearly Sunshine Hours	Lowest Average Daily Minimum Temperature per Month (°C)
North Island		
Gisborne Airport	2204	4.4
Napier	2245	4.6
Tauranga Airport	2277	4.5
Whakatane	2329	4.1
South Island		
Blenheim	2447	1.5
Mt John	2399	-1.7
Nelson	2397 (Nelson Airport)	-1.1 (Golden Downs Forest)
Riwaka, Motueka	2418	1.4
Takaka	2369	not recorded

2.3.3 Statistical Analysis

The statistical analysis pertains only to seedlings, since insufficient data pertaining to cuttings was available. Statistical analysis by ANOVA reveals that the male cone production differs significantly between regions ($p < 0.05$).

Due to the prevalence of missing data for the Northern Kinleith Region, this region was excluded from the model development process. The increase with age in the proportion of trees bearing cones is not a linear function, and differs markedly

between regions. Nevertheless, a tentative logistic model was developed that adequately describes cone production across all regions (except Northern Kinleith) in this study. An iterative approach was used to assess the value of various combinations of climate variables in describing the proportion of trees producing male cones at a given age. The "best" model arrived at is based on the independent variables age, age^2, mean yearly sunshine hours and average minimum July temperature, and is as follows:

$$P_{cones} = 1 / 1 + e^{-(-31.2343 + 3.3363age - 0.1506age\hat{}2 + 0.00874sun - 0.3225temp)}$$

Where: P_{cones} = Proportion of trees producing male cones

e = the base of the natural logarithm (2.7183).

age = age of trees in years

sun = mean yearly sunshine hours in region of interest

temp = average daily minimum July temperature in region of interest

These climate variables are available for most New Zealand regions from New Zealand Meteorological Service (1983).

It must be stressed that this model is based on only four regions, and as such the two climate variables (temperature and sunshine hours) are represented by only four observations each. This implies that there are insufficient degrees of freedom for the model to be very reliable. A follow-up study is required to develop a more reliable model. This would necessitate obtaining data for a minimum of ten, but preferably twenty regions (Hedderley, pers. comm.[10]). Additionally, the limitations of the logistic function imply that the model does not perform well for young age classes (3 years and younger); under these circumstances the model predicts a very small proportion of trees will be producing male cones, when in reality the proportion is probably zero. Despite these limitations, for older age classes the model provides a

[10] Duncan Hedderley, Statistics Research & Consulting Centre, Massey University. Personal Communication, 27 march 2000.

very good indication of the proportion of trees producing male cones, at least for the regions studied.

Because of the shortcomings of the above model, separate statistically robust models based solely on age were developed for each region to describe cone production, and these are as follows:

Northland

$$P_{cones} = 1 / 1 + e^{-(-14.8863 + 2.1837\ age)}$$

Southern Kinleith

$$P_{cones} = 1 / 1 + e^{-(-6.7261 + 1.3203\ age)}$$

Karioi

$$P_{cones} = 1 / 1 + e^{-(-9.7949 + 1.3431\ age)}$$

Nelson

$$P_{cones} = 1 / 1 + e^{-(-16.4627 + 5.7606\ age - 0.3539\ age^2)}$$

Where: P_{cones} = Proportion of trees producing male cones
e = the base of the natural logarithm (2.7183)

2.4 Discussion

2.4.1 Kinleith

Kinleith, located in the central North Island of New Zealand (Figure 2.5), is Carter Holt Harvey Forests' major holding of production forest, and is therefore of central interest to the company. For this reason Kinleith was used as a benchmark for this study.

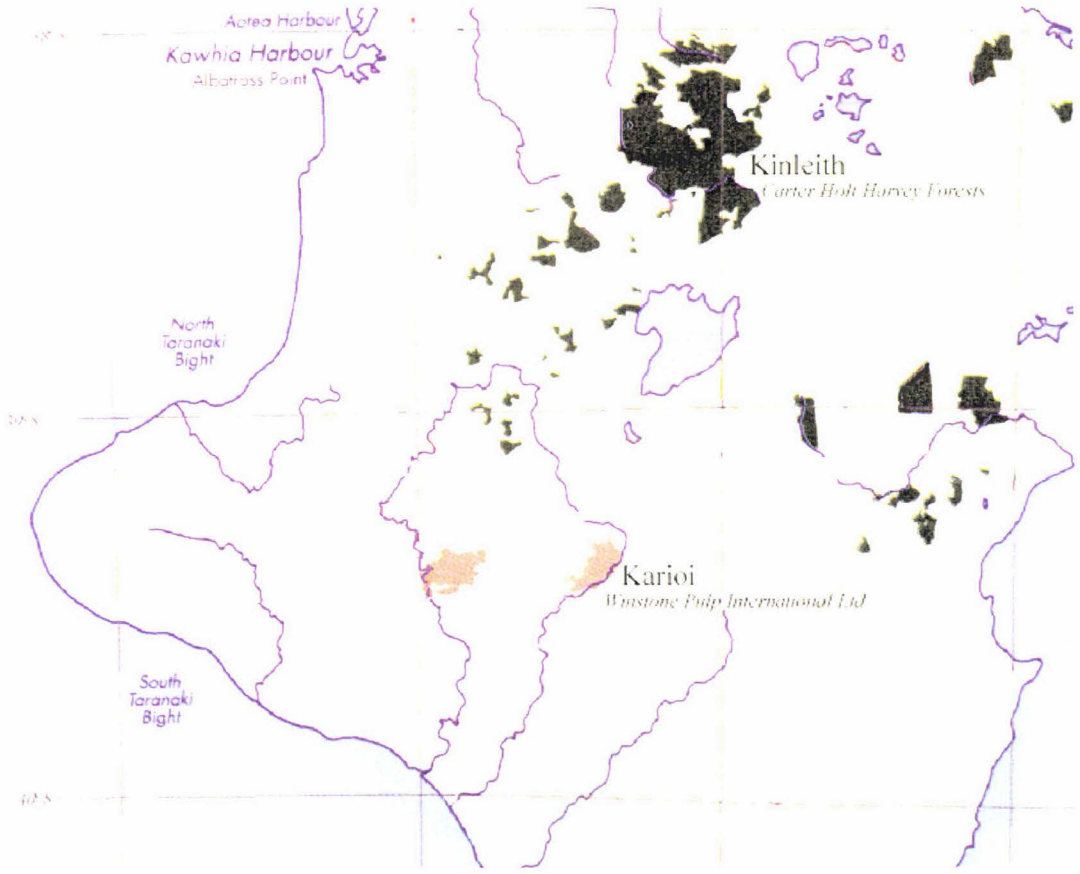

Figure 2.5: *Map showing general location of Kinleith and Karioi Forests (Adapted from Kirkpatrick, 1999)*

In order to provide relatively in-depth information, this key region was divided into Northern and Southern sub-regions. This division was based on the broadly similar conditions within each sub-region. The Northern sub-region encompasses CHHF map sheets 90425, 90426, 90427, 90514, 90517, 90518, 90521, 90522, 90523, and

90524. The Southern sub-region encompasses map sheets 90721, 90722, 90723, 90724, 90728, 90731, 90732, 90733, 90728, 90735, 90736, and 90737.
The map sheets listed above were produced from GIS data by the computer program ARCINFO. These provided the following details:

- Areas of unpruned *Pinus radiata* planted from 1989 to 1993.
- Stock codes associated with these plantings.
- Altitude information (contour lines and spot heights).
- Rivers and streams.
- Roads and harvesting skid sights.
- Areas restricted for safety reasons, or because other trials are present.

Utilising information from these maps, areas stocked with unpruned 1989 to 1996 plantings were selected. Unpruned trees were considered necessary for the present study because male cones are often produced on lower branches. Incorporating pruned trees (with the lower branches removed) into the study would have introduced an unnecessary confounding element. When information was available, stock codes were used to determine whether plantings in each area were seedlings or cuttings. Prior to 1993 (1989 to 1992) such information was not recorded. However, at that time almost all plantings were seedlings as cuttings were not commonly available.

In order to further minimise confounding elements, areas were narrowed down to provide the greatest possible environmental consistency, both within each age class and across all age classes. This involved selecting areas with flat or nearly flat topography, and which were at a similar altitude. An iterative approach was used to determine how tightly these constraints could be imposed in practice; if the constraints were too rigid, complete age classes were eliminated, which was obviously not acceptable. Altitude ranges found to be a reasonable compromise were 170-250 meters in the northern sub-region, and 350-400 meters in the southern sub-region.

Mean sunshine hours and average daily minimum temperatures experienced each month in Northern and Southern Kinleith are moderate compared to other regions employed in this study (Figures 2.2 and 2.3). Interestingly, commencement of male cone production is also intermediate in these regions. Results indicate that male cone production commences in both Northern and Southern Kinleith at age 4, compared to age 3 at Nelson, and age 6 at both Northland and Karioi (Figure 2.1). These findings lend support to the hypothesis that climate variables may influence male cone production.

2.4.2 Northland

The Northland region affords a climate quite different from other parts of New Zealand. The climate of the region is characterised by warm humid summers and mild winters (New Zealand Meteorological Service, 1983; Kirkpatrick, 1999). The primary motive for incorporating Northland into this study was the very mild winters. Aupori forest, located along Ninety Mile Beach was considered ideal for this study. Its close proximity to the sea and low altitude provide a very equitable winter climate. Tepaki forest occupies a more exposed, slightly higher altitude site than Aupori. For this reason, Aupori forest was employed as much as possible in this study. However, since one required age class was not present in Aupori, it was necessary to sample it at Tepaki. The general location of Aupori and Tepaki Forests is shown in Figure 2.6. Both of these forests are managed by Juken Nissho Ltd.

A list of all compartments stocked with unpruned 1992-1996 plantings, in Juken Nissho's Northland forests was generated. Compartments located in low lying, coastal forests (Aupori and Tepaki) were retained, while those located inland were abandoned. As outlined above, the objective of this exercise was to provide study areas with the warmest winter climate possible. Data from Aupori Forest and Tepaki climate stations indicate that these areas do in fact experience warmer winter temperatures than stations located inland (data not shown; New Zealand Meteorological Service, 1983).

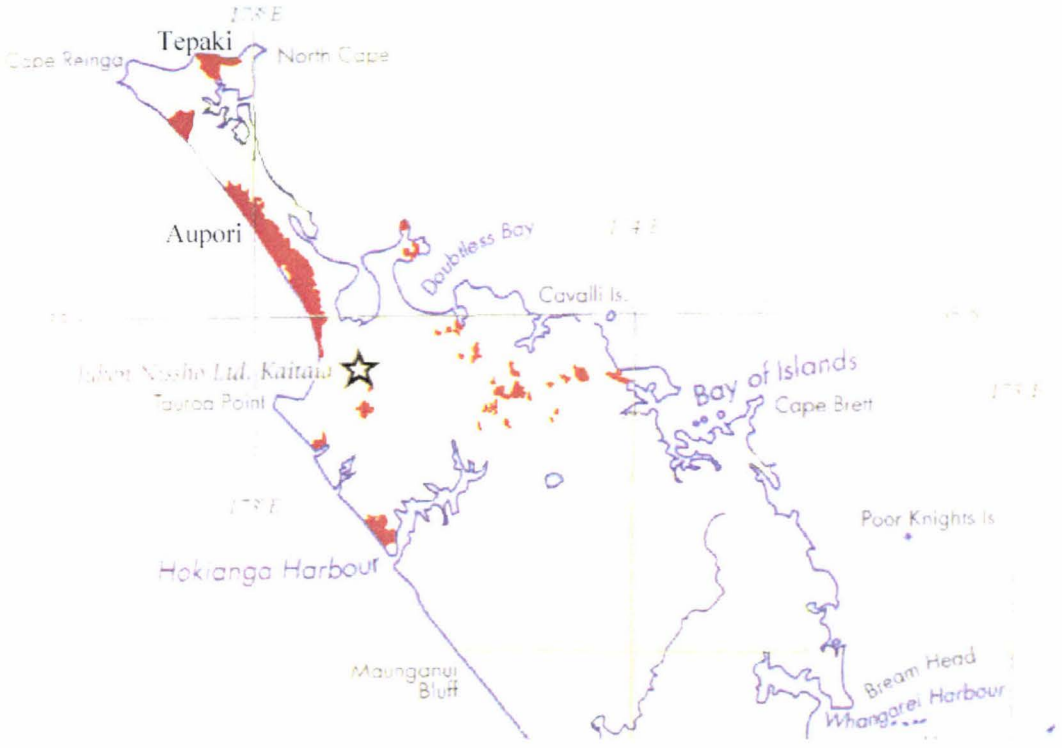

Figure 2.6: *Map showing general location of Aupori and Tepaki Forests, Northland.*
(Adapted from Kirkpatrick, 1999)

Anecdotal evidence suggests that a cold winter period may favour cone production. Northland was incorporated into the present study to test this hypothesis; in order to support this hypothesis, cone production should commence relatively late during ontogenetic development in Northland. Results of this study indicate that male cone production in Northland is relatively late, commencing at age 6, compared to age 4 in both Northern and Southern Kinleith (Figure 2.1). Sunshine hours experienced in Northland are quite similar to those in Northern and Southern Kinleith. Consideration of these findings indicate that cooler temperatures may favour earlier ontogenetic male cone production.

2.4.3 Nelson

Nelson (Figure 2.7) was selected because this region is anecdotally purported to provide exceptionally profuse cone production. The results of the present study provide strong evidence in support of this claim. Male cone production in the Nelson

region was both more profuse (subjective observations) and more precocious than in other regions assessed in this study (Figure 2.1). A small proportion of trees assessed in the Nelson region were producing male cones by age 3, and by age 4 most trees were.

It is apparent from the climate data that Nelson experiences far greater sunshine hours, and cooler autumn and winter temperatures than other regions assessed in this study (Figures 2.2 and 2.3). From this, it may be hypothesised that high sunshine hours, and low autumn and winter temperatures are conducive to precocious male cone production.

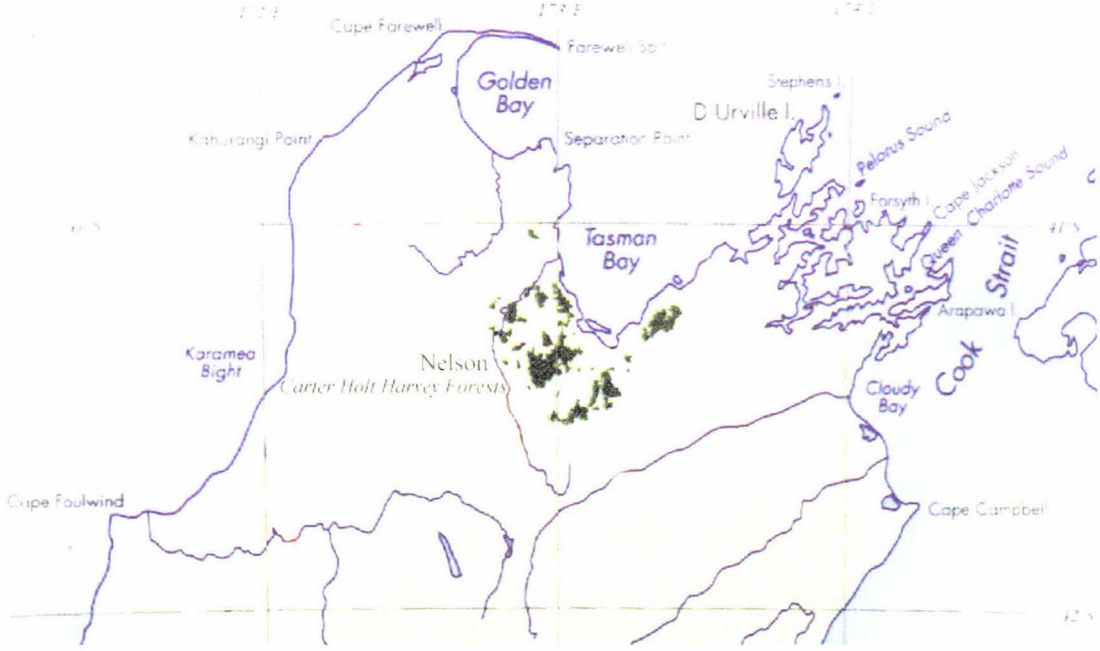

Figure 2.7: *Map showing general location of Carter Holt Harvey Forests, Nelson.*
(Adapted from Kirkpatrick, 1999)

2.4.4 Karioi

Karioi forest is managed by Winstone Pulp International Ltd. Its general location is provided in figure 2.5.

The climate at Karioi is characterised by low sunshine hours, and relatively cool temperatures throughout the year (Figure 2.2 and 2.3). This combination of climate

variables is of considerable interest to this study. As mentioned above, the Nelson region experiences high sunshine hours, and this may be associated with relatively high levels of male cone production. Also, anecdotal evidence suggests that low winter temperatures, as experienced by both Karioi and Nelson, may also influence cone production. Thus, information from Karioi was considered to be valuable in elucidating whether high sunshine hours, or low winter temperatures were of the most significance to male cone production.

Results indicate that male cone production is relatively poor at Karioi, commencing at age 6, compared to age 4 at Northern and Southern Kinleith, and age 3 at Nelson. This information, combined with climatic considerations suggests that high sunshine hours may be of greater importance to early male cone production than low autumn and winter temperatures.

2.4.5 General Discussion and Conclusions

The findings described in this chapter suggest that high sunshine hours, followed by low autumn and/or winter temperatures are of importance to precocity of male cone production. High sunshine hours and low autumn/winter temperatures are experienced in Nelson, and this appears to be associated with precocity of male cone production relative to other areas. Removal of either of these conditions appears to be associated with a loss of precocity, as evidenced by findings for Karioi and Northland.

Other regions with similar climates to Nelson do exist in New Zealand, and it seems likely that these regions may also favour precocious male cone production. Table 2.2 was developed from a comprehensive search for New Zealand climate stations that experience relatively high sunshine hours.

The North Island stations do not experience sunshine hours as high, or minimum temperatures as low, as any of the South Island stations listed. However, the most

precocious male cone production in the North Island could be expected in the vicinities of Gisborne, Napier, Tauranga and Whakatane.

As might be expected, the South Island climate stations listed in Table 2.2 experience sunshine hours and minimum temperatures most comparable to Nelson. Possibly of the most interest are the Motueka and Blenheim regions, which experience even higher sunshine hours than Nelson, albeit with higher minimum temperatures. The climatic conditions at Mt John (Tekapo) appear to be very comparable to those at Nelson. These conditions are experienced throughout the Mackenzie Basin (Stuart Burgess, pers. comm.[11]). Future studies in these regions would help to elucidate how critical each of these climatic factors are in providing precocious male cone production.

Because of the limitations of this study, the hypothesis that high sunshine hours and low autumn/winter temperatures are required for precocious male cone production must be regarded as preliminary in nature. Future studies in the regions mentioned above, especially Blenheim and the Mackenzie Basin, would substantiate this hypothesis. Follow-up studies are also necessary to validate and fine tune the cone production model.

It is possible that factors other than sunshine hours and temperature, such as soil moisture status, are important. Water stress, associated with low soil moisture may be important for the initiation of male cones, as evidenced by experiments with water stressed potted plants (see Chapter 4). However, initiation of male cones begins in October (Cremer, 1992; Welsh, 1997) and based on evaporation and rainfall data (Riwaka, Motueka climate station, New Zealand Meteorological Service, 1983) it is not likely that water stress would be occurring around this time of the year in the Nelson region. However, at Wither Hills, Blenheim, evaporation exceeds rainfall at this time of the year (New Zealand Meteorological Service, 1983), and water stress would probably occur. Future studies in the Blenheim region would elucidate the

[11] Stuart Burgess, Climate Research and Information Services, National Institute of Water and Atmospheric Research Ltd. Personal communication, 17 March 2000.

possible importance of water stress in precocious male cone production in field grown trees.

It is possible that high sunshine hours may favour precocious male cone production through relatively early accumulation of carbohydrate reserves, sufficient for cone initiation to occur. Bernier *et al.* (1981) suggest that the photosynthetic contribution of leaves is important in attaining "ripeness-to-flower". Presumably, the photosynthetic contribution of leaves would be higher with greater sunshine hours, assuming other factors were equal.

Exposure of plants to low temperatures for long periods is known to lead to an accumulation of water-soluble carbohydrates (Howarth and Ougham, 1993). It is hypothesised that this may be a side-effect resulting from a shift in the metabolic balance of the plant; at low temperatures there is a reduction in growth rate without an equivalent reduction in photosynthesis (Howarth and Ougham, 1993). Viewed another way, there is a reduction in sink strength (due to reduced growth), while the source strength (the photosynthetic contribution of the leaves) remains largely unchanged.

Cold temperature is also one of the factors that may elicit morphogenetic switches in plants, such as the transition from vegetative to floral development (Howarth and Ougham, 1993). Low temperatures may favour precocious male cone production through relatively early accumulation of carbohydrate reserves, sufficient for cone initiation to occur, and the triggering of the morphogenetic switch(es) necessary for the transition to reproductive development.

Chapter 3

Characterisation of *Pinus radiata* development patterns in relation to cone production

3.1 Introduction

The aim of this study was to characterise the crown architecture and vegetative development of *Pinus radiata*, and relate this to the onset of reproductive competence. In particular this study focused on the onset of male cone production. Such information is of general interest to those who study phase change, or maturation. Furthermore, it is useful to the tree breeding industry since it may provide valuable clues as to how male cone production can be manipulated.

It is hypothesised that reproductive competence in (at least) some plants is reached after the apical meristem of the leading shoot has undergone a certain number of cell divisions (Robinson and Wareing, 1969). Accordingly, reproductive phase change would be correlated with, but not determined by, attainment of a certain plant size. Extension of this hypothesis leads to the hypothesis that commencement of male cone production in *Pinus radiata* may occur after a certain number of cell divisions of the apical meristem has occurred (Hackett, pers. comm.[12]).

In *Pinus radiata* this hypothesis may be tested by comparing the relative number of cell divisions between male cone producing and non-male cone producing trees. One would expect a greater number of cell divisions to have occurred in a cone producing tree, compared to a non-cone producing tree if this hypothesis were to be true.

[12] Wesley Hackett, Emeritus Professor, University of California, international expert on phase change. Personal Communication 11/2/99.

Since it would be extremely difficult to count meristematic cell divisions directly, this study utilises a surrogate parameter to provide an indication of this. It was decided to utilise an estimate of the relative number of cells present in a given tree based on tracheid length and height as the surrogate parameter. Additionally other parameters, tree height, diameter and the number of branch clusters produced were measured. The presence, or absence of male cones on each tree was also noted. These data were then used to establish whether reproductive competence is correlated with the other developmental characteristics measured.

3.2 Materials & Methods

3.2.1 Sample plots

This trial was located in the stand identified as planting operation 27448, off Juniper road, Kinleith Forest. This stand is comprised of a wide mixture of seedling genotypes (stock code R95BS), and is situated in an area of flat topography, thus providing a high degree of environmental uniformity. The trial was carried out in the winter of 1999, and at this time trees were four years old. Sampling was by way of three 200 m transects which were established in randomly selected rows within the stand. The first 60 trees *not* producing male cones encountered on the transects were numbered 1 to 60 with green spray paint. The first 60 male cone producing trees encountered on the transects were numbered 1 to 60 with blue spray paint. Since only about 20% of the trees were producing male cones, a far greater distance was covered to provide the male cone producing sample, compared to the sample not producing male cones.

3.2.2 Data collection

For each sample tree, height was measured to the nearest 0.1 m using a telescopic height pole, and diameter at breast height (d.b.h.) (1.4 m) was measured to the nearest cm using a diameter (pi) tape. A count was made of the total number of

branch clusters present on each tree. A small block of wood (approximately 10 mm wide, 10 mm deep, and 15 mm tall) was cut from each tree at a height of two meters using a sharp chisel and hammer. Each block of wood was immediately covered with FAA (Appendix A) in an appropriately labelled specimen container.

3.2.3 Tracheid measurement

Wood samples were prepared by firstly removing the bark when this was still intact. Using a razor blade a section of wood approximately 2 mm thick was cut from the outer (tangential) face of the block and discarded. In cases where the bark was no longer intact the outer face of the block was clearly recognisable by its smooth, slippery texture relative to the other faces. Taking care to maintain the orientation of the block a second section approximately 2 mm thick was cut from the freshly formed outer tangential face. Two 2 mm wide pieces were then cut from this section in a radial longitudinal orientation. Thus, the resulting wood pieces resembled a short match-stick.

For each sample, the two wood pieces were immediately transferred to labelled 20 x 150 mm test tubes and triple rinsed using water to remove excess FAA. Maceration was carried out using a modified version of Franklin's Method (Berlyn and Miksche, 1976). Five ml 50% (v/v) hydrogen peroxide and 5 ml glacial acetic acid were added to each test tube. Test tubes were then placed in a water bath at 90° C for 4 h in a fume hood. The maceration fluid was then drained off and the samples carefully triple rinsed with water. After rinsing, approximately 5 ml of water was added to each test tube. Maceration was completed by lightly shaking each test tube.

Two 25 x 75 mm microscope slides were prepared for each sample. Two drops of the maceration were placed onto each slide and covered with a 22 x 60 mm cover slip. Projected tracheid images were observed on an ampliscope and measured with a plan measuring wheel (Scalex Planwheel™, Geosystems Ltd., Christchurch). The setup was calibrated and the appropriate conversion factor calculated with a stage micrometer. Calibration was checked before each slide was assessed. The lengths of

25 randomly selected intact tracheids were measured on each slide, and a mean tracheid length calculated. A modification of the methods of Harris (1966) was used to check the measurements. The mean tracheid length results of the two slides from the sample were compared. If these agreed within 7% then the results were pooled and the mean tracheid length calculated from the 50 observations. If the two results did not fall within this range a third slide was prepared and a mean tracheid length obtained from this. Invariably this third sample agreed within 7% of one of the first two slides, and the results from the slide in disagreement were discarded. The two favourable results were then pooled as before and the mean tracheid length for the sample tree calculated from the 50 observations.

3.2.4 Estimate of relative cell number

An indicator of the relative number of cell divisions undergone by a given tree was calculated by:

Relative cell number = tree height (m) / the mean tracheid length (mm)

The resulting "relative cell number" figure for each sample tree is arbitrary, and is not intended to indicate the total number of cell divisions undergone by that tree. Instead "relative cell number" provides a means of comparing the relative number of cell divisions undergone by each sample tree.

2.2.5 Statistical analysis

Data was analysed using SAS version 6.12. Correlation data was produced using the procedure CORR (SAS, 1990). Logistic regression was performed using the procedure LOGISTIC (SAS, 1995).

3.3 Results

3.3.1 Tracheid measurement

Tracheids were easily discernible from the other major cell type, ray tracheids, by their much larger size and the very different shape of the cells (Figure 3.1).

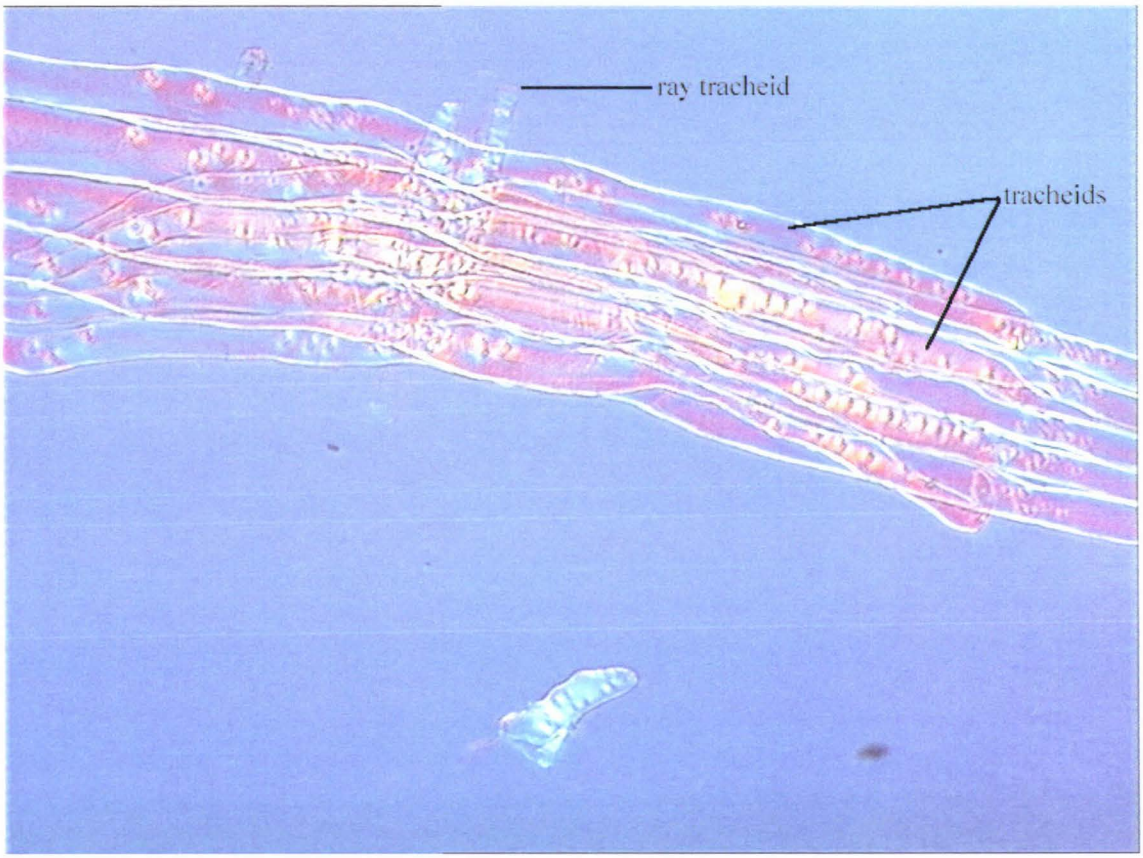

Figure 3.1: *Phase contrast image showing tracheids and ray tracheids, the two main cell types present in* Pinus radiata. *Note the different size and shape of the two cell types, which makes them easily distinguishable. Ray tracheids have obtuse end walls, while tracheids have oblique or forked end walls.*

3.3.2 Comparison of cone-producing and non-cone-producing parameters

Mean tracheid length did not vary significantly ($p > 0.1$) between cone-bearing and non-cone-bearing samples (Figure 3.2). However, cone bearing trees were highly significantly taller ($p < 0.0001$), had a significantly greater d.b.h. ($p < 0.001$),

significantly more branch clusters ($p < 0.001$) and had undergone a highly significantly greater number of cell divisions ($p < 0.0001$) compared to non-cone-bearing trees (Figure 3.2).

3.3.3 Correlation between morphological and anatomical parameters

Due to the nature of tree growth and development some of the morphological and anatomical parameters of this study are correlated to varying degrees; generally, as a tree increases in height it also increases in diameter, and both of these occur by the addition of cells by the cambium (see discussion). Table 3.1 summarises the data for correlation between parameters for the entire 120 tree sample.

Tree height is most strongly correlated with cell number ($R = 0.824$), and to a slightly lesser extent d.b.h. ($R = 0.7724$). Tree height is moderately correlated with the number of branch clusters ($R = 0.824$) but only weakly correlated with tracheid length ($R = 0.3725$). Tracheid length is also only weakly correlated with the other morphological parameters: $R = 0.3015$ with d.b.h., $R = 0.1732$ with the number of branch clusters.

Table 3.1: Correlation Coefficients (R) for tree height, diameter at breast height, number of branch clusters, relative cell number, and tracheid length.

	Height	d.b.h.	# branch clusters	Relative Cell #	Tracheid length
Height	1.0000	0.7724	0.4807	0.8240	0.3715
d.b.h.	0.7724	1.0000	0.4107	0.6290	0.3015
# branch clusters	0.4807	0.4107	1.0000	0.3999	0.1732
Relative Cell #	0.8240	0.6290	0.3999	1.0000	-.2155
Tracheid length	0.3715	0.3015	0.1732	-.2155	1.0000

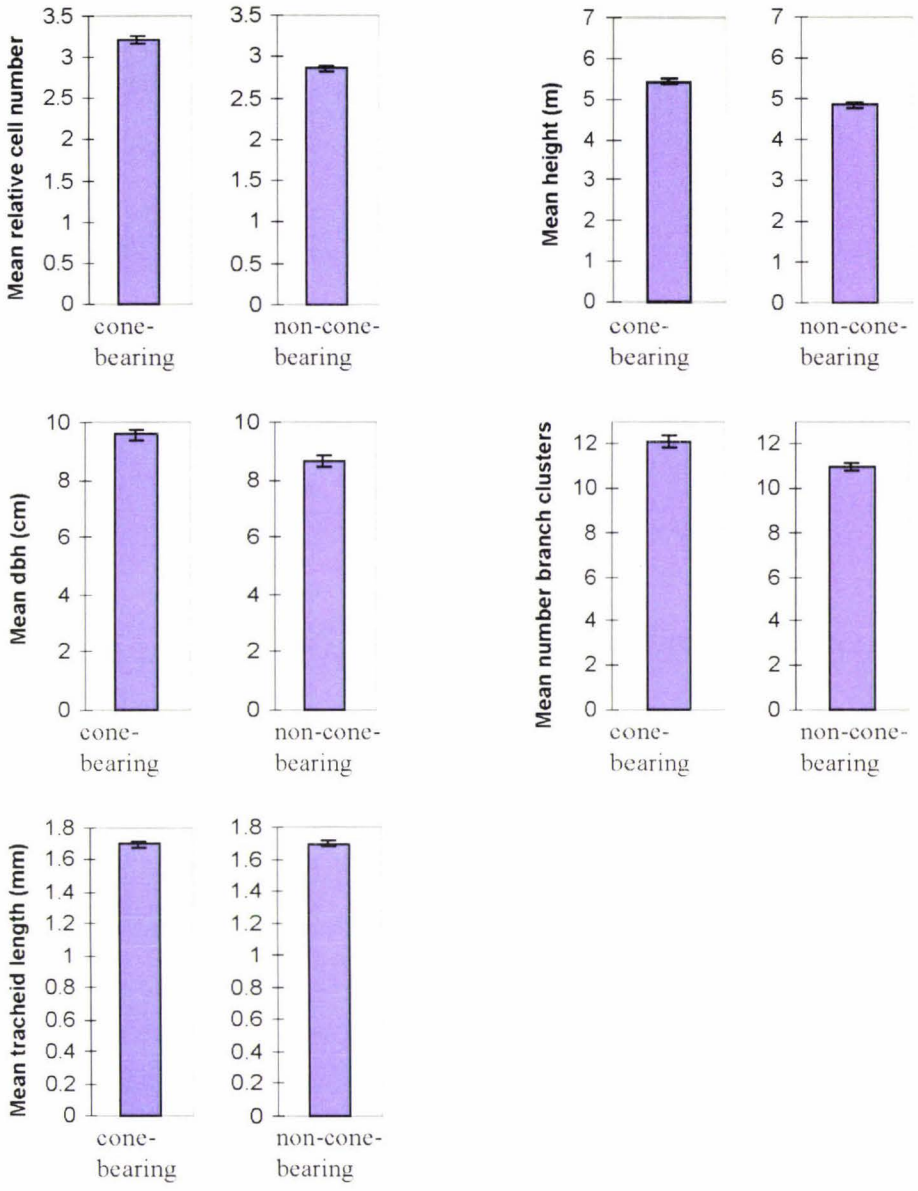

Figure 3.2: *Comparison of cone-bearing and non-cone-bearing tree parameters. Each main bar represents the mean of 60 sample trees. Error bars indicate ± s.e.* ***Top Left:*** *mean relative number of cells of cone-bearing and non-cone-bearing samples, means highly significantly different ($p < 0.0001$).* ***Top right:*** *mean height of cone-bearing and non-cone-bearing samples, means highly significantly different ($p < 0.0001$).* ***Middle left:*** *mean d.b.h. of cone-bearing and non-cone-bearing samples, means significantly different ($p < 0.001$).* ***Middle right:*** *mean number of branch clusters of cone-bearing and non-cone-bearing samples, means significantly different ($p < 0.001$).* ***Bottom left:*** *tracheid length of cone-bearing and non-cone-bearing samples, means do not differ significantly ($p > 0.01$).*

3.3.4 Prediction of cone production based on morphological and anatomical parameters

An iterative approach was used to construct a model which predicts the probability that a tree will *not* be producing male cones based on its morphological and anatomical characteristics. The best single independent variable model attained is based on relative cell number; height also provides a good single independent variable model, although not as good as relative cell number. Using the relative cell number model as a foundation, addition of height and/or d.b.h. did not improve the model significantly, but addition of number of branch clusters did. It transpired that no significant improvement over the model utilising these two independent variables (relative cell number and number of branch clusters) could be attained. This model is as follows:

$$P_{\text{without cones}} = 1 / 1 + e^{-(10.487 - 0.2605\ \#\text{clusters} - 2.4712\ \text{cell}\#)}$$

$$P_{\text{with cones}} = 1 - P_{\text{without cones}}$$

where: #clusters = the number of branch clusters
cell# = the relative number of cells
e = the base of the natural logarithm (2.7183)

Figure 3.3 provides a three dimensional surface chart which represents an approximation of the above model. This can be used to simply and quickly determine an estimate of the proportion of trees not producing cones, from relative cell number and the number of branch clusters.

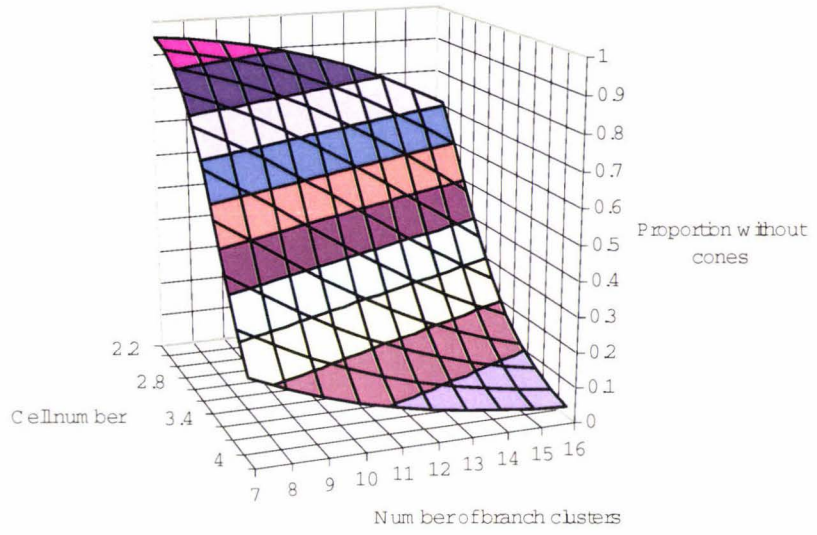

Figure 3.3: *Three dimensional surface chart which utilises relative cell number and number of branch clusters to estimate the proportion of trees that will not be producing cones.*

3.4 Discussion

3.4.1 Justification of methodology

At the onset of this study two surrogate parameters were considered in order to estimate the number of cell divisions undergone by the apical meristem.

Firstly, an estimate of the relative number of cataphylls present in cone-producing and non-cone-producing trees could be used as an estimate of the relative number of cell divisions undergone by the apical meristem. This hypothesis can be justified by considering the fate of cells recruited from the flanks of the apical meristem in *Pinus*. Cataphylls are initiated in a fairly regular pattern, both spatially, and temporally (Sacher, 1955b; Bollman & Sweet, 1976). Thus, a count of the number of cataphylls present would be reasonably well correlated with the number of divisions having occurred at the flanks of the apical meristem.

Secondly, an estimate of the relative number of cells present in cone-producing and non-cone-producing trees could be used as an estimate of the relative number of cell divisions undergone by the apical meristem. This hypothesis can be justified by consideration of the following: it is obvious that the majority of a tree is comprised of wood. Furthermore, from examination of wood anatomy, it is apparent that in *Pinus* most of this wood is comprised of tracheids (Esau, 1953). In order to understand this justification it is necessary to appreciate the ontogenetic development of tracheids. Briefly, tracheids arise from the fusiform cambial initials, which develop from the procambium, which in turn originated from differentiation of derivatives at the flanks of the apical meristem (Esau, 1953; Steeves and Sussex, 1989). It is apparent that tracheids do not arise directly from divisions at the flanks of the apical meristem, but the cambial initials do. While tracheids elongate slightly with respect to the cambial initials from which they arise (Esau, 1953), this is necessarily so for *all* trees. Thus the *relative* number of tracheids present between trees may provide a reasonable indication of the *relative number of divisions* that have occurred. The relative number of tracheids present may be estimated by sampling mean tracheid length and tree height.

In the present study it was decided to utilise an estimate of the relative number of cells present in a given tree based on tracheid length and height as the surrogate parameter. A major reason for this decision was that it would be extremely difficult to perform cataphyll counts while trees were standing, and felling of 120 trees was not considered justifiable in view of the alternative method available.

3.4.2 General discussion

The hypothesis that a certain number of cell divisions are required before male cone production commences in *Pinus radiata* is strongly supported by this study. Modelling of single independent variables (either height, d.b.h., number of branch clusters, or relative cell number) indicates that relative cell number provides the greatest ability to predict whether or not a given tree will be producing male cones. Thus, the number of cell divisions undergone by the apical meristem as indicated by

the relative cell number is more strongly correlated with male cone production than the other variables studied.

It is apparent that relative cell number and height are strongly correlated (Table 3.1); indeed cell number is derived in part from tree height (relative cell number = height/tracheid length). Nevertheless, when the height for a particular tree is divided by mean tracheid length for that tree, the relative cell number for that particular tree results. Modelling reveals that cell number has a greater ability than height to predict whether or not a particular tree will be producing cones.

Of the other independent variables, the number of branch clusters is the only one that significantly improves the single variable model based on cell number. This point also provides some evidence in support of the hypothesis that a certain number of cell divisions are required before male cone production commences in *Pinus radiata*. Each "appendage" on a tree is the result of primordium activity at the flanks of the apical meristem, which in turn is derived from cell divisions of the apical meristem itself. Thus, from this reasoning a relatively high number of branch clusters is likely to be strongly (positively) correlated with a relatively high number of divisions of the apical meristem. Inspection of Table 3.1 suggests that this may not be the case; cell number and the number of branch clusters have a correlation coefficient of only 0.4.

An alternative explanation for the number of branch clusters being of significant secondary importance to the model is as follows: it is likely that an array of interacting pathways are involved in the transition to reproductive development (Poethig, 1990; Hackett and Murray, 1997). It is possible that a certain number of divisions of the apical meristem (as reflected in the number of cells present) represents one of the pathways required to be activated in order for male cone production to occur. Additionally, it is likely that other pathway(s) would also need to be activated either concomitantly or sequentially for this to occur. It is possible that the requirement for other pathway(s) to be activated is reflected in the number of branch clusters present. A high number of branch clusters presumably provides for a relatively high leaf area. This might impart a relatively high photosynthetic ability and in turn a relatively high contribution to carbohydrate reserves; this state may be a

requirement for attaining "ripeness to flower" (Bernier *et al.*, 1981), which allows male cone production to occur. These hypotheses may be a reflection of another of the pathways mentioned above.

Chapter 4

Hormonal treatments of stressed fascicle cuttings

4.1 Introduction

Successful promotion of cone production in Pinaceae species has been reported following the application of a mixture of gibberellin A_4 and A_7 ($GA_{4/7}$) (Pharis & Ross, 1986). Many of the studies undertaken have concentrated on the promotion of female cone production. Considerably less work has been carried out regarding the promotion of male cones. However, male cone production has been promoted in some species through the application of $GA_{4/7}$, for example in *Picea* species, *Pinus sylvestris* (Bonnet-Masimbert, 1987), *Pseudotsuga menziesii* (Pharis *et al,* 1987), and *Larix leptolepis* (Anon., n.d).

Bonnet-Masimbert (1987) suggests that the key to successful promotion of male cone production lies in the application of hormonal treatments at the correct time. However, treatments in most experiments have been applied at times which have been more suited to the promotion of female cone production rather than male.

ABA is widely considered to be a growth inhibitor, and slowing of vegetative growth may be associated with the promotion of cone production (Pharis *et al,* 1987). Timely application of ABA may slow development at a critical stage thereby having a positive effect on male cone production (R. Riding, pers. comm.[13]). This has been shown to be the case in *Pinus radiata*, in which Welsh (1997) demonstrated that stem injection of ABA approximately four to six weeks after short shoot initiation increased male cone production.

[13] Richard Riding, University of New Brunswick, Canada. Personal communication 14 June 1997.

Male cones have been observed on young stressed *Pinus radiata* cuttings growing in very small pots (Figure 1.10). The cuttings in Figure 1.10 were surplus to requirements and had been abandoned. Hence, they had received no attention such as fertiliser application or watering and accordingly would have been very stressed.

In this chapter, attempts to stimulate fascicle cuttings to produce male cones are reported. A range of hormonal treatments which were considered to have potential to stimulate cone production were applied to fascicle cuttings growing in small pots, with minimal watering and no fertiliser application.

4.2 Materials & Methods

4.2.1 Cuttings

Fascicle cuttings were obtained from the CHH nursery at Rotorua, in trays of forty 80 ml pots. For this trial each tray was cut into four, providing an experimental unit of ten cuttings. Cuttings were set in the winter of 1997, so at the commencement of this experiment the cuttings were approximately eighteen months old.

4.2.2 Hormone stock solutions

The following hormone stock solutions were prepared:

$GA_{4/7}$: 10 µg/µl in propylene glycol (Regulex™)

ABA: 10 µg/µl 96 % ethanol

NAA: 1 µg/µl 96 % ethanol

4-Fluorophenyl N-(2-chloro-4-pyridyl)carbamate: 0.5 µg/µl DMSO

At the time of each application the required hormone concentrations were made up by diluting the stock solutions. $GA_{4/7}$, ABA and NAA were diluted with 96% ethanol.

The anti-cytokinin, 4-Fluorophenyl N-(2-chloro-4-pyridyl)carbamate was dissolved in DMSO (dimethylsulfoxide), but dilutions were made using water.

4.2.3 Hormone application and plant growth

Hormonal treatments were applied on: 16 October 1998
27 October 1998
11 November 1998

Experiment 3 only, ABA application on: 24 November 1998

With reference to the experimental design, appropriate hormones/concentrations were applied to 2 blocks of 10 cuttings per treatment. Application to each shoot apex was carried out using a Hamilton PB600 dispenser. Treatments were applied at a rate of 2 µl per plant for Experiments 1-3. Controls received 2 µl of 96 % ethanol. For Experiment 4, 8 µl per plant was applied, with the control receiving DMSO.

Cuttings were situated outside on a raised mesh table to facilitate air root pruning, at the CHHF Rotorua greenhouse complex. Trays of treatment cuttings were randomly arranged contiguously in a square pattern. Trays of unused cuttings were positioned around the perimeter of the treatment trays to act as a buffer zone. Watering was carried out at a minimum as required, and no fertiliser was applied.

4.2.4 Experimental Design

Experiment 1. Application of $GA_{4/7}$ and ABA: Seven treatments applied to 2 blocks x 10 cuttings; $GA_{4/7}$ @ 0.2 µg, 2 µg and 20 µg per cutting; ABA @ 0.2 µg, 2 µg and 20 µg per cutting; control = 96 % ethanol.

Experiment 2. Application of $GA_{4/7}$ with NAA: NAA applied at 0, 0.2 µg and 2 µg, with $GA_{4/7}$ applied at 0, 2 µg and 20 µg in a complete three x three factorial; control = 0 x 0 dosage. Treatments applied to 2 blocks x 10 cuttings.

Experiment 3. Application of $GA_{4/7}$ with ABA following 1 month later: Four treatments applied to 2 blocks x 10 cuttings; $GA_{4/7}$ @ 0.2 μg, 2 μg and 20 μg per cutting; control = 96 % ethanol. ABA applied @ 2 μg per cutting, one month later.

Experiment 4. Application of anti-cytokinin (4-Fluorophenyl N-(2-chloro-4-pyridyl)carbamate): Four treatments applied to 2 blocks x 10 cuttings; anti-cytokinin @ 0.02 μg, 0.2 μg and 2 μg per cutting; control = DMSO.

4.2.5 Morphological and anatomical assessment

At the time of first hormone application, and monthly thereafter, the height of each cutting was measured and recorded. At the same times morphological observations were made and recorded. These included the type of terminal bud present (enclosed or rosette), leaf type on each stem portion (fascicles, primary leaves, or no leaves/just cataphylls), presence and position of any branches, and any unusual occurrences such as a dead shoot apex.

At several times during the winter of 1999 all cuttings were assessed for male cone production, and details recorded. The development of male cones was carefully monitored, and cones were picked once they had fully "ripened" (when clear liquid no longer appeared when cones were crushed). Male cones from each cutting were stored in small paper bags. It was not necessary to dehydrate pollen samples as they were only stored for a few days before assessment. Pollen was extracted by lightly shaking the bags, and pollen viability assessment undertaken using the methods of McConnochie *et al.* (n.d.). Briefly, pollen was germinated on a sucrose medium incubated at 25° C for 48 h, and the germination rate of pollen tubes observed under a dissection microscope.

4.2.6 Statistical analysis

Cone production data were analysed using the SAS procedure GENMOD (SAS, 1990). A binomial distribution was specified in the MODEL statement. Where

necessary a CONTRAST statement was used to determine which independent variables (hormonal treatments) were significant in promoting male cone production.

4.3 Results

4.3.1 Experiment 1

$GA_{4/7}$ at 0.2 µg and 2 µg per cutting typically caused rapid stem elongation of cuttings during the period following application (Figure 4.1). The 20 µg per cutting dosage of $GA_{4/7}$ caused burning and subsequent death of the apical bud of most cuttings (Figure 4.2). On recognition of this problem a small experiment was undertaken in order to find its cause. Regulex™ is propylene glycol based, and at the 20 µg per cutting dosage it was not diluted with ethanol. Application of 20 µg/µl $GA_{4/7}$ in 96 % ethanol did not cause burning and subsequent death of the apical bud, whereas application of propylene glycol without $GA_{4/7}$ did.

Figure 4.1: *Stem elongation associated with application of $GA_{4/7}$ at 0.2 µg and 2 µg per cutting. 10/2/99.*

Figure 4.2: *Damage to stem apical bud associated with application of 20 µg per cutting dosage of $GA_{4/7}$. This problem was traced to the propylene glycol base of the commercial $GA_{4/7}$ preparation. 10/2/99.*

The effects of $GA_{4/7}$ or ABA application on male cone production are summarised in Figure 4.3. From the GENMOD procedure output, LR Statistics For Type 1 Analysis reveal that the different treatments had a significant effect ($p>\chi^2$ 0.0122) on male cone production, whereas the different application rates did not ($p>\chi^2$ 0.1215). The output of the CONTRAST statement reveals that ABA significantly increased ($p>\chi^2$ 0.0185) male cone production relative to the control, but $GA_{4/7}$ did not ($p>\chi^2$ 0.2799). A typical treatment cutting bearing male cones is shown in Figure 4.4. Application of ABA caused no other noticeable morphological effects.

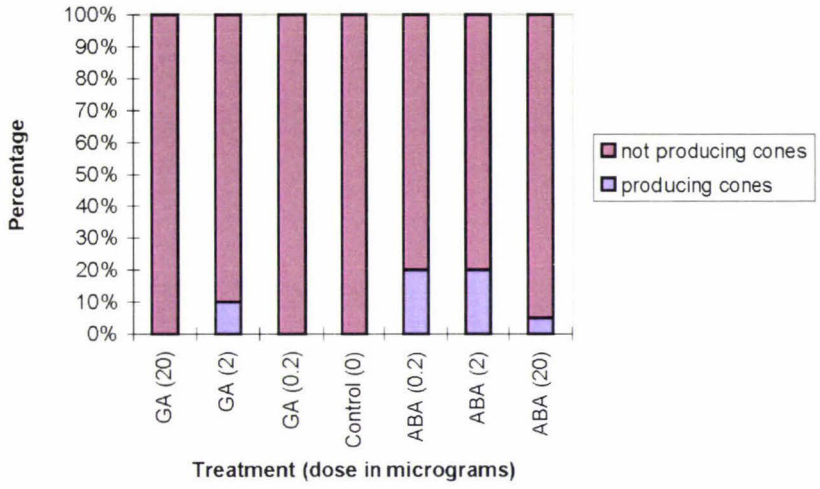

Figure 4.3: *Percentage of cuttings bearing male cones for each treatment in Experiment 1. Treatments: $GA_{4/7}$ at 20μg, 2μg and 0.2 μg per cutting, ABA at 20μg, 2μg and 0.2 μg per cutting, and control. Each bar represents pooled data for each treatment.*

4.3.2 Experiment 2

Application of NAA at 2 μg per cutting caused the formation of stunted brown appendages in place of normal leaves (Figure 4.5). Application of NAA at 0.2 μg per cutting had no noticeable effect.

Figure 4.4: *Typical treatment cutting bearing male cones.*

Figure 4.5: *Cutting showing stunted brown appendages which formed in place of normal leaves after the application of NAA at 2 μg per cutting.*

The effects of $GA_{4/7}$ combined with NAA on male cone production are summarised in Figure 4.6. From the GENMOD procedure output, LR Statistics For Type 1 Analysis suggest that at least one dose of $GA_{4/7}$ had a significant effect ($p>\chi^2$ 0.0466) on male cone production. Neither NAA ($p>\chi^2$ 0.1095) nor the combination of $GA_{4/7}$ and NAA ($p>\chi^2$ 0.6717) had a significant effect on male cone production.

The output of the CONTRAST statement reveals that $GA_{4/7}$ applied at either 2 μg ($p>\chi^2$ 0.1567) or 20 μg ($p>\chi^2$ 0.2376) per cutting did *not* in fact promote a significant increase in male cone production relative to the control. An iterative approach was used to further investigate this apparent anomaly (the apparent disagreement with the LR Statistics). This involved re-running the GENMOD procedure after one or other of the $GA_{4/7}$ treatments was temporarily removed. The information from the LR Statistics, that at least one dose of $GA_{4/7}$ had a significant

effect on male cone production, pertained to a pooled test of difference between the three doses (0, 2 μg and 20 μg). The investigation highlighted that the LR Statistics were detecting that the 2 μg dose was promoting a significant increase in male cone production relative to the 20 μg dose, rather than the control; in this case the 20 μg dose decreased cone production relative to the control, but this was not statistically significant.

Thus, none of the treatments in this experiment promoted a significant increase in male cone production relative to the control.

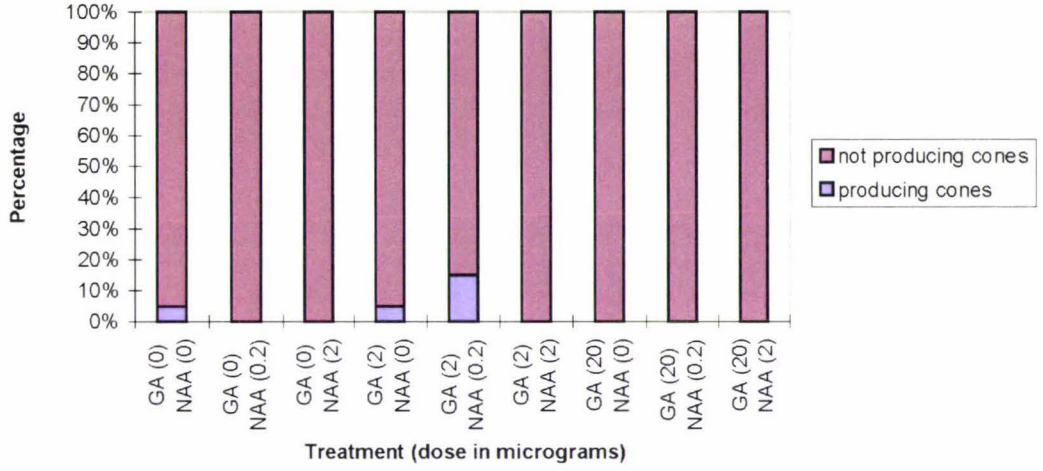

Figure 4.6: *Percentage of cuttings bearing male cones for each treatment in Experiment 2. Treatments: $GA_{4/7}$ at 0, 2μg and 20μg, in factorial combination with NAA at 0, 0.2 μg and 2 μg per cutting. Each bar represents pooled data for each treatment.*

4.3.3 Experiment 3

The effects of ABA application one month after the application of $GA_{4/7}$ on male cone production are summarised in Figure 4.7. From the GENMOD procedure output, LR Statistics For Type 1 Analysis suggest that at least one dose of $GA_{4/7}$ combined with ABA one month later had a significant effect ($p>\chi^2$ 0.0079) on male cone production.

Results of the CONTRAST statement reveal that $GA_{4/7}$ at 0.2 μg combined with ABA application one month later significantly increased ($p>\chi^2$ 0.0298) male cone production relative to the control, but other doses of $GA_{4/7}$ combined with ABA one month later did not ($GA_{4/7}$ at 2 μg + ABA one month later $p>\chi^2$ 0.5447; $GA_{4/7}$ at 20 μg + ABA one month later $p>\chi^2$ 0.2347). It should be noted that $GA_{4/7}$ at 20 μg per cutting caused burning and subsequent death of the apical bud of most cuttings, as in experiment 1.

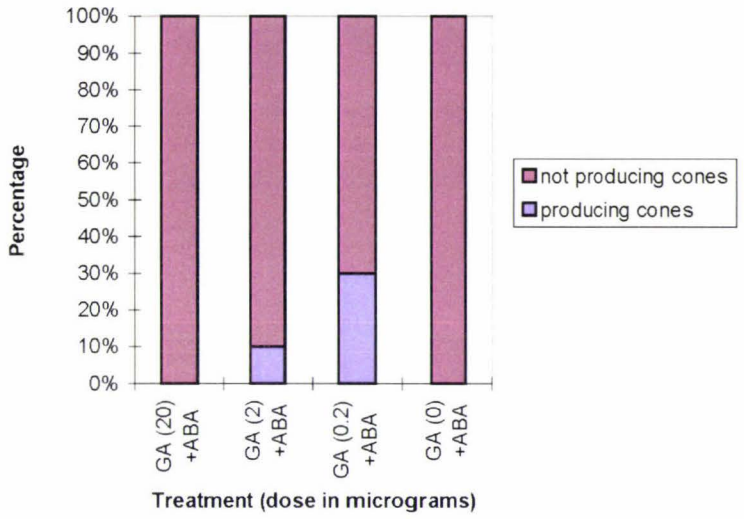

Figure 4.7: *Percentage of cuttings bearing male cones for each treatment in experiment 3. Treatments: $GA_{4/7}$ at 0, 0.2 μg 2μg and 20μg, with ABA applied one month later at 2 μg per cutting. Each bar represents pooled data for each treatment.*

4.3.4 Experiment 4

The anti-cytokinin (4-Fluorophenyl N-(2-chloro-4-pyridyl)carbamate) caused no observable morphological effects. The effects of anti-cytokinin on male cone production are summarised in Figure 4.8. From the GENMOD procedure output, LR Statistics For Type 1 Analysis reveal that neither treatment ($p>\chi^2$ 0.7170) nor dose ($p>\chi^2$ 0.1841) had a significant effect on male cone production.

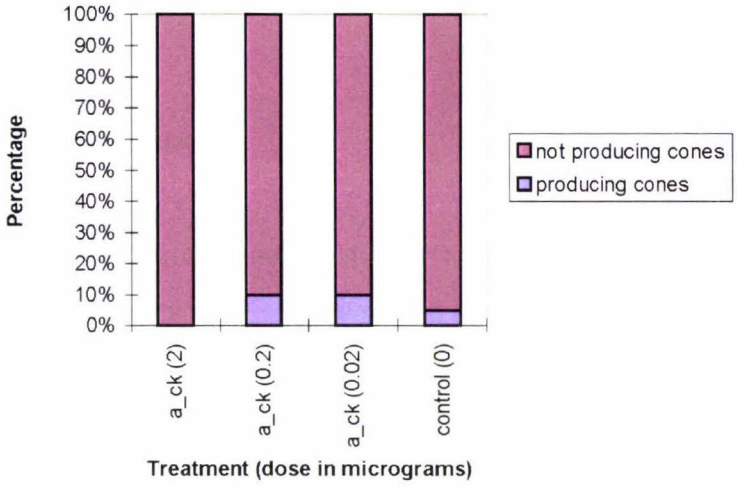

Figure 4.8: *Percentage of cuttings bearing male cones for each treatment in experiment 4. Treatments: anti-cytokinin (4-Fluorophenyl N-(2-chloro-4-pyridyl)carbamate) at 0, 0.02μg, 0.2 μg and 2 μg per cutting. Each bar represents pooled data for each treatment.*

4.3.5 Pollen viability

Across all experiments the mean pollen tube germination rate was 75.6 % with a standard error of 3.5 %. The mean was lowered considerably by one sample which had a pollen tube germination rate of only 30 %. With this outlier removed, the mean pollen tube germination rate becomes 80 % with a standard error of 2.2 %. The outlier came from a control sample, and besides this aberration there was no significant difference in pollen tube germination rate between samples from different treatments.

4.3.6 Tracheid length

Mean tracheid length was 0.97 mm for non-cone-producing cuttings and 0.99 mm for the cone-producing cuttings. An indicator of the relative number of cell divisions (see Chapter 3) undergone by each cutting was calculated by:

already occurring. There is not sufficient evidence to causally link an increase in endogenous ABA levels with increased male cone production. Nevertheless, the fact that both stress (which increases endogenous ABA levels) and exogenous ABA application appear to promote male cone production is sufficient evidence to suggest that these may be associated.

The mean relative cell number for cone-producing cuttings in this experiment was much lower (0.41) than for field grown trees (>3 for cone bearing trees; see Chapter 3). Hence, at the commencement of male cone production, considerably fewer divisions of the apical meristem have occurred in the stressed cuttings compared to field trees. This is very strong evidence for at least two separate pathways being involved in the transition to reproductive maturation. In field grown trees the "normal" pathway leading to male cone production appears to be switched on after the mean relative cell number exceeds three. In the case of the cuttings producing male cones a separate "stress" pathway is probably involved, and it appears that under suitable conditions this can be activated much earlier in ontogenetic development than the "normal" pathway.

It was observed that many of the cuttings in the buffer zone produced male cones. Presumably cuttings in the buffer zone received more sunlight than those inside the buffer zone. The reception of more sunlight would provide a higher level of photosynthetic activity, and greater accumulation of carbohydrate reserves in the buffer zone cuttings compared to others. Thus, it seems likely that accumulation of carbohydrate reserves may be important for promotion of cone production via the "stress" pathway. Evidence suggests that this is also the case for the "normal" pathway involved in cone production (see Chapter 2; Bernier *et al.*, 1981). This similarity suggests that there may be a degree of overlap between the hypothesised "stress" and "normal" pathways. It is possible that the upstream component of the two pathways is shared, with subsequent branching further downstream. Alternatively, the upstream parts of the two pathways might be separate, and converge downstream. Regardless of the actual arrangement, it is apparent that

two pathways is shared, with subsequent branching further downstream. Alternatively, the upstream parts of the two pathways might be separate, and converge downstream. Regardless of the actual arrangement, it is apparent that accumulation of carbohydrate reserves may be important for promotion of cone production via both of the hypothesised pathways.

Other species have also been observed to flower prematurely, apparently via a pathway other than the normal pathway. In *Citrus*, which normally flowers when five to ten years old, seedlings just a few months old sometimes flower and occasionally fruit containing viable seeds mature (Furr *et al.*, 1947). Wiesner (1902, cited in Jost, 1907) mentions oak which normally flowers in its sixtieth to eightieth year (presumably in Europe) sometimes flowers in its first year and then dies.

Chapter 5

Grafting of adolescent scions onto physiologically mature and seedling rootstocks

5.1 Introduction

Evidence from horticultural species, and to a lesser extent *Pinus*, suggests that certain rootstocks might provide worthwhile gains in cone production in the years immediately following grafting (Jayawickrama *et al.*, 1991). A predominant theme in the literature (from other *Pinus* species) is that a degree of incompatibility of the scion and rootstock tends to favour early ontogenetic cone production (see section 1.8.1.5). Therefore, it seems likely that judicious selection of rootstocks, presumably with the "right degree" of incompatibly could provide worthwhile gains in early ontogenetic cone production in *P. radiata*.

In addition, drought stress is commonly used to enhance cone production in conifers, both alone or in addition to other treatments (Pharis & Ross, 1986; Bonnet-Masimbert, 1987).

With the above information in mind, the trial described in this chapter was established to investigate the influence of grafting "adolescent" scion material onto various rootstocks. Several different clones of physiologically mature interstocks were investigated, as were seedling rootstocks. Additionally, the effects of drought stress on the grafts on physiologically mature interstocks, and application of $GA_{4/7}$ to the seedling rootstocks were considered.

5.2 Materials and Methods

5.2.1 Scion material

Scion material was collected in the winter of 1998, from seedlings of a wide variety of genotypes, planted in the winter of 1995. The collection area was located in Kinleith Forest, near the end of the first spur road off Pukerimu road (see Appendix B for details). A starting point was randomly assigned, 20 trees (ortets) were randomly selected and these were sequentially numbered from 201-220. Ten or more scions were snipped from the lower/middle crown region of each selected ortet. Each scion was comprised of a primary branch bud plus a portion of branch, totalling approximately 100 mm in length (Van Dorsser and Faulds, 1991). Scions derived from each ortet were immediately enclosed in a correspondingly numbered plastic bag, with a few milliliters of water to aid storage.

5.2.2 Grafts on reproductively mature interstocks

The plants used as "rootstocks" in this experiment were themselves grafted ramets (individual plants belonging to a clone; derivatives of the parent ortet). The scions for these ramets had been tip-cleft grafted (Thulin, 1957) onto seedling rootstocks several years prior to the commencement of this experiment. These scions were derived from "885" and "887" series ortets. Thus, as of 1998, the physiological age of these ramets was at least 13 years for the "885", and 11 years for the "887" series. The presence of both male and female cones on the ramets was evidence of their reproductive maturity. All ramets were growing in PB50 polythene bags, situated outside at the CHHF research greenhouse complex, Rotorua. From the point of view of this experiment, the physiologically mature scions of these ramets are effectively interstocks. For convenience these ramets will hereafter be referred to as the "mature interstocks".

During August 1998, one scion from each ortet (field tree) was grafted onto 10 different clones of mature interstocks. Ten replications were carried out utilising

scions from each ortet (field trees 201-210). Scions were grafted onto a lower lateral branch of each mature interstock using the tip-cleft method (Thulin, 1957). These plants will be referred to as the "grafts on mature interstocks". It should be noted that grafts were not made in some instances, due to poor interstock health. Additionally, some grafts were not successful and consequently died within a few weeks. Table 5.1 summarises the actual experimental design, as of 23rd September, 1998.

Watered treatments (grafts made with scions derived from ortets 206-210) were irrigated as required in order to supplement natural rainfall (Figure 5.1).

Figure 5.1: *Grafts on mature interstocks. Watered treatment showing overhead irrigation.Grafts for this experiment were made using the tip-cleft method (Thulin, 1957) on lower lateral branches of the interstocks (opposite pink tags).*

Table 5.1: *Grafting of adolescent scions onto physiologically mature interstocks. Experimental design, and graft success as of 23/9/98. Symbols used: * = Graft growing, ? = Healthy - not growing, X = Unhealthy - not growing, NG = Not grafted (poor root stock).*

		Ortet (field tree from which scions were derived)										
		Drought stressed					Watered					
		201	202	203	204	205	206	207	208	209	210	total
Mature interstock clone	887 715	?	*	*	*	*	*	*	?	*	?	7
	887 817	*	*	*	*	*	*	*	*	*	*	10
	887 846	*	*	*	*	*	*	*	*	*	*	10
	887 869	*	NG	*	*	*	*	*	*	*	*	9
	887 904	?	*	*	*	?	*	*	*	*	*	8
	887 924	NG	*	*	*	*	*	*	*	*	*	9
	885 004	*	*	*	*	*	*	*	*	*	*	10
	885 025	*	*	*	*	*	*	*	*	*	*	10
	885 046	?	*	*	*	*	*	*	*	?	*	8
	885 070	*	*	*	*	X	*	*	*	*	*	9
	total	6	9	10	10	8	10	10	9	9	9	

For drought stress treatments (grafts made with scions derived from ortets 201-205), watering was initially carried out at a bare minimum until graft unions were successfully established. During early October 1998 canopies were constructed over each drought stress treatment pot in order to exclude rain. Canopies consisted of plastic bags, which originally measured 635 x 900 mm, halved across the short axis and with the bottom seam slit. Hence, two plastic tubes approximately 450 mm tall resulted from each bag. These were lowered over each ramet, and the top of the tube sealed around the base of the ramet using duct tape. The bottom of each tube was then taped onto the pot loosely, so as to allow gas exchange (Figures 5.2a and 5.2b).

Figure 5.2a: *Grafts on mature interstocks Drought treatment showing canopies to exclude rainfall. Grafts for this experiment were made using the tip-cleft method (Thulin, 1957) on lower lateral branches of the interstocks (opposite pink tags).*

Figure 5.2b: *Canopies on drought treatment pots.*

Ortets (trees in the field) from which scions were derived were used as controls.

From February until September 1999 all mature grafts were assessed on a monthly basis for any signs of male cone production. Control ortets (field trees) were assessed for male cone production in the winter and spring of 1999.

An assessment of graft compatibility was undertaken in late November 1999. This involved:

1. measuring the length of the first leaf encountered on each scion i.e. the leaf closest to the graft union. This will be referred to as the scion leaf length (SLL).
2. measuring the length of the first leaf encountered on the interstock above the grafted branch. This will be referred to as the interstock leaf length (ISLL).
3. subjectively scoring the health of each graft, with respect to the grafts in Figure 5.3.

Figure 5.3: *Grafts used as standards in assessment of graft health scores. Top left: score 0, Top right: score 1, Bottom left: score 3, Bottom right: score 5.*

5.2.3 Grafts on seedling rootstocks

During August 1998, 10 scions from each of ortets (field trees) 211-220 were tip-cleft grafted (Thulin, 1957) onto a seedling rootstock. The seedling rootstocks were located in a stool bed at the Forest Research nursery. The grafted ramets remained there for the duration of the experiment.

During early October 1998, ramets derived from ortets 211-215 were treated with $GA_{4/7}$. $GA_{4/7}$ solution (Regulex™), was diluted to 1 μg/μl using 96% (v/v) ethanol (100 μl of Regulex™, to 900 μl of 96% (v/v) ethanol). Four μl of this solution was applied to each graft apex, using a 20 μl micropipette. Hence, each ramet received 4 μg of $GA_{4/7}$.

Regulex™ has a propylene glycol base. Control solution was comprised of 100 μl of propylene glycol, added to 900 μl of 96% ethanol. For ramets derived from ortets 216-220, 4 μl of this solution was applied to each graft apex, using a 20 μl micropipette.

These ramets will be referred to as "grafts on seedling rootstocks".

Table 5.2 summarises the experimental design.

Table 5.2: *Grafting of adolescent scions onto seedling rootstocks. Experimental design, and graft success as at 23/9/98.*
Symbols used: GA = received 4 μg GA_4 in 96% (v/v) ethanol
0 = control (received 96% (v/v) ethanol)
- indicates graft was not successful

	Ortet (field tree from which scions were derived)									
	211	212	213	214	215	216	217	218	219	220
Replicate										
1	GA	GA	GA	GA	GA	0	0	0	0	0
2	GA	GA	GA	GA	GA	0	0	0	0	0
3	GA	GA	GA	GA	GA	0	0	0	0	0
4	GA	GA	GA	GA	GA	0	0	0	0	0
5	GA	GA	GA	GA	GA	0	0	0	0	-
6	GA	GA	GA	GA	GA	0	0	0	0	-
7	GA	GA	GA	GA	GA	0	0	0	0	-
8	-	GA	GA	-	GA	0	0	0	-	-
9	-	-	GA	-	GA	0	0	-	-	-
10	-	-	GA	-	-		0	-	-	-
Total	7	8	10	7	9	9	10	8	7	4

5.3 Results

5.3.1 Grafts on reproductively mature interstocks

None of the grafts on mature interstocks produced any male cones, whereas all control ortets did (Figure 5.4).

Figure 5.4: *Typical control ortet in October 1999. Abundance of male cones present.*

Results of the compatibility assessment are summarised in Figure 5.5. Subjective graft health scores, and mean scion leaf length (SLL) / mean interstock leaf length (ISLL) are provided as possible indicators of graft compatibility. It is apparent from Figure 5.5 that the two different assessment methods do not provide identical indications of graft compatibility. Nevertheless, some general trends do emerge. Interstock clone 887 924 provides the best compatibility in most instances, while 887 715 provides generally poor compatibility. In general, compatibility appears to be better in the watered treatment, compared to the drought treatment.

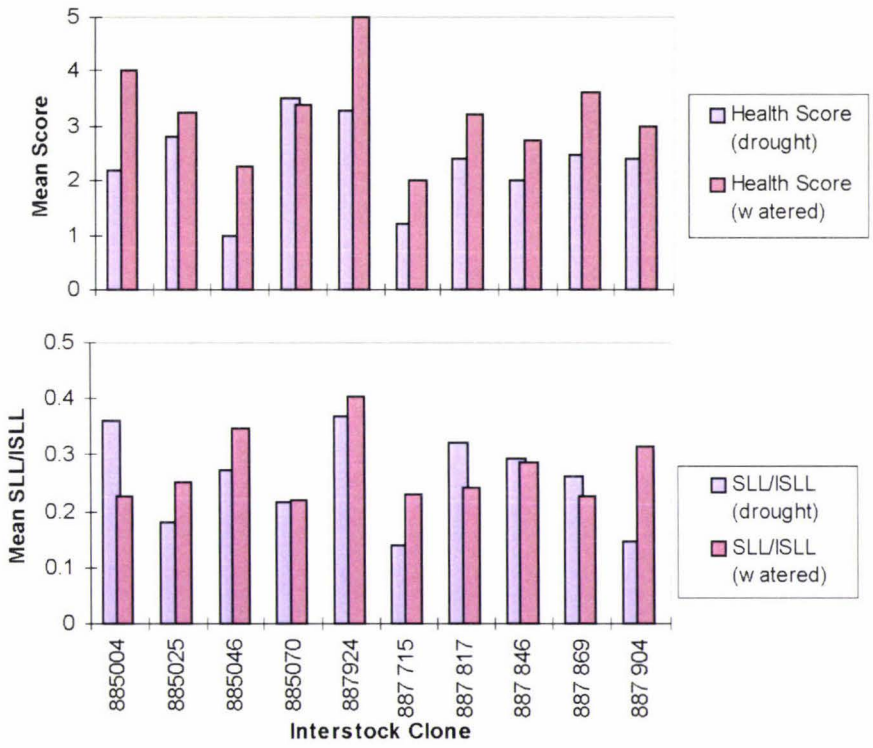

Figure 5.5: *Indications of graft compatibility between scions and interstocks. Top: subjective scoring of graft health as an indication of graft compatibility. Bottom: mean scion leaf length (SLL) / mean interstock leaf length (ISLL) as an indication of graft compatibility.*

5.3.2 Grafts on seedling rootstocks

None of the grafts on seedling rootstocks produced any male cones. Figure 5.6 shows a typical seedling graft in October 1999.

Figure 5.6: *Typical seedling graft in October 1999.*

5.4 Discussion

Grafting did not promote male cone production in these experiments. Indeed, the presence of male cones on control ortets (Figure 5.4) in 1999, in the mature interstock experiment indicates that grafting was detrimental to male cone production.

5.4.1 Grafts on reproductively mature interstocks

Evidence for graft incompatibility favouring cone production comes from observations of grafted *P. radiata* ramets. Relatively heavy male cone production is known to be associated with a high degree of graft incompatibility (Faulds, pers. comm.[14]). The ideal rootstock for a given clone would most likely provide a balance between sufficient incompatibility to provide gains in male cone production, in terms of precocity and cone numbers, while not causing excessive ramet mortality.

Subsequent to grafting for this experiment, a few of the potted mature rootstocks were observed to be producing male cones. These male cones were invariably located in a sub-terminal position of the main leader. In this experiment, all scions were grafted onto lower lateral branches, where male cones would normally be observed on field grown trees. In respect to the location of male cones, it appears that potted ramets display a crown architecture quite different from field grown trees.

As found in other studies (Copes, 1980), graft compatibility in this study appears to vary with rootstock genotype as evidenced by health scores and the ratio of scion leaf length to interstock leaf length (Figure 5.5). Of the watered treatments, the 887 924 rootstock appears to be extremely compatible with all scions tried. Conversely, other rootstocks such as 887 715 appear to be highly incompatible with the various scions tried. In most cases the drought treatment appears to have increased the severity of graft incompatibility symptoms (Figure 5.5).

[14] Trevor Faulds, Forest Research nursery, New Zealand. Personal communication 30 November 1999.

This trial utilised only ten different rootstock genotypes. Nevertheless, there appears to be wide variation in the degree of scion incompatibility associated with these different rootstocks. Furthermore, drought stress has been found to be a useful tool for further manipulating the degree of incompatibility associated with a given rootstock-scion combination. It is possible that the "right degree" of incompatibility for the successful promotion of male cone production was provided by one of these drought treated or watered rootstock-scion combinations. However, successful male cone promotion may have been thwarted by grafting scions onto an unsuitable position of the potted rootstocks. It is recommended that future grafting experiments for the purposes of promoting male cone production take this into account; scions should be tip-cleft grafted (Thulin, 1957) onto mid/lower lateral branches of field grown trees, or onto the leading shoot of potted ramets.

Tip-cleft grafting (Thulin, 1957) involves the complete removal of the rootstock shoot at the position where the graft is to be made and replacing it with the new scion. In cases where male cone production occurs in a sub-terminal position of the potted mature rootstock, it is obvious that this part of the plant possesses all the "requirements" for male cone production to occur. Removal of the leading shoot for a tip-cleft graft also removes this portion of the plant. A better strategy may be to leave the leading shoot with its associated "requirements" for male cone production to occur intact and use another grafting method to incorporate the scion, such as a bottle graft or veneer graft (Mergen and Rossoll, 1954, reproduced in Dorman, 1976). Using these methods it is possible to graft the scion directly onto the potted mature rootstock in the sub-terminal position of the leading shoot, without removing the leading shoot. According to Dorman (1976) the bottle grafting and veneer grafting methods provided by Mergen and Rossoll (1954, reproduced in Dorman, 1976) appear to be applicable to various (United States) southern pines. It seems likely that these methods could also be used for grafting *P. radiata*.

Additionally, it is apparent that tip-cleft grafting onto the leading shoot of the rootstock will irreversibly change the genealogy of its future development, commensurate with the genotype of the scion. This implies that subsequent future

grafts onto the leading shoot would be compromised. This would not be a problem if male cone production was realised and continued to occur for several years. However, if male cone production did not occur the rootstock would no longer be available (as the original genotype) for future work.

The bottle grafting and veneer grafting methods provided by Mergen and Rossoll (1954, reproduced in Dorman, 1976) could also be used to graft scions onto reproductively mature trees growing in the field. In this case scions should be grafted onto a sub-terminal position of branches in the middle of the crown where male cones usually occur (Figure 5.4).

Pinus contorta is renowned for precocious female and male cone production. A useful strategy for providing precocious male cone production in *P. radiata* might be to graft "adolescent" *P. radiata* scions onto *P. contorta* rootstocks. Grafting of *P. radiata* scions onto *P. contorta* rootstocks has been achieved successfully, although mortality due to incompatibility began after four to five years (Faulds Pers. Comm.[15]).

5.4.2 Grafts on seedling rootstocks

Scions grafted onto stool-bed seedling rootstocks grew much more rapidly than those grafted onto physiologically mature potted rootstocks (Figure 5.6 c.f. Figures 5.1 to 5.3). However, the accelerated growth and associated larger size of seedling grafts did not result in the production of any male cones.

Some evidence suggests that a certain ratio of mature to juvenile foliage must be reached before floral initiation can occur (Bernier *et al.*, 1981). An interesting strategy for future studies would be to graft mature interstocks into seedlings. This could be used to test the hypothesis that mature foliage is required for cone production to occur. In *P. taeda* interstocks are known to substantially affect male

[15] Trevor Faulds, Forest Research nursery, New Zealand. Personal communication 26 February 2000.

cone production (Schmidtling, 1983b). This strategy is similar to that used in the experiments with mature interstocks discussed above. However, the key difference is that the original rootstock of the seedling would be used.

Chapter 6

General Discussion and Conclusions

Early ontogenetic male cone production is of great interest to the tree breeding industry in order to reduce the time taken for successive generations to be bred. This thesis involved several studies which pertain to the aim of promoting early male cone production in *Pinus radiata*. An appraisal of trees at some contrasting locations around New Zealand was carried out, in order to determine whether certain locations favour precocious male cone production compared to others. In addition, a study was carried out to assess whether various morphological and anatomical characteristics were correlated with the onset of male cone production. Concurrently, various treatments including stress, plant growth regulator application and grafting were examined to determine whether any of these could be utilised to successfully promote early ontogenetic male cone production.

The regional study found male cone production to commence relatively late during ontogenetic development at Karioi and Northland, commencing at age 6, compared to age 4 at Northern and Southern Kinleith, and age 3 at Nelson. Findings suggest that high sunshine hours and low autumn and/or winter temperatures are of importance to precocity of male cone production. These climatic conditions are experienced in Nelson, and this appears to be associated with precocity of male cone production there, relative to other areas. Removal of either of these climatic conditions appears to be associated with a loss of precocity, as evidenced by findings for Karioi and Northland; Karioi experiences low sunshine hours, Northland experiences warm winter temperatures.

In order to substantiate the above hypothesis further studies in regions with similar climates to Nelson, such as Blenheim and the Mackenzie Basin are required.

A tentative logistic model was developed that may adequately describe cone production across all regions, and this is as follows:

$$P_{cones} = 1 / 1 + e^{-(-31.2343 + 3.3363age - 0.1506age^2 + 0.00874sun - 0.3225temp)}$$

Where: P_{cones} = Proportion of trees producing male cones
e = the base of the natural logarithm (2.7183)
age = age of trees in years
sun = mean yearly sunshine hours in region of interest
temp = average daily minimum July temperature in region of interest

A follow-up study is required to validate and fine tune the cone production model. This would involve collecting data for at least ten, but preferably twenty regions.

Morphological and anatomical characteristics of trees were used to develop a model which predicts the probability that a tree will *not* be producing male cones (the probability that a tree will be producing male cones can be easily found by subtracting this figure from one). Cell number was found to provide the greatest ability to predict whether or not a given tree will be producing male cones. Thus, the hypothesis that a certain number of cell divisions are required before male cone production commences in *Pinus radiata* is strongly supported by this study. Of the other independent variables, it was found that the number of branch clusters was the only one that significantly improved the single variable model based on cell number.

Height was also found to provide a good single independent variable model (although not as good as cell number) for predicting the probability that a tree will *not* be producing male cones. This is probably because height is strongly correlated with the number of cells (and therefore the number of divisions undergone by the apical meristem). The height model was not improved by the addition of further independent variables. Thus, as a rule of thumb taller trees have a higher probability of producing male cones than shorter trees, all else being equal. This point is of significant practical use in final selection of superior trees in trials. In cases where two trees are comparable in other characteristics, it may be judicious to favour the

taller of the two since it will have a higher probability of producing male cones. This method would eliminate the necessity of measuring tracheid lengths, required by the preceding model.

Grafting did not promote male cone production in the present study. However, relatively heavy male cone production in *Pinus radiata* has been observed to be associated with a high degree of graft incompatibility (Faulds, pers. comm.[16]). Additionally, a predominant theme in the literature regarding other *Pinus* species is that a degree of incompatibility of the scion and rootstock tends to favour early ontogenetic cone production (Schmidtling, 1973; Ahlgren, 1972; Dyson, 1975).

In the mature interstock experiment, it was apparent that scions may have been grafted onto an inappropriate crown position. Scions were grafted onto lower lateral branches, where male cones would normally be observed on field grown trees. Subsequently, male cones were observed on the interstocks in a sub terminal position. It is recommended that future grafting experiments for the purposes of promoting male cone production take this into account. Scions should be tip-cleft grafted (Thulin, 1957) onto the leading shoot of potted ramets. Alternatively, a different grafting method could be used to incorporate the scion, such as a bottle graft or veneer graft (Mergen and Rossoll, 1954, reproduced in Dorman, 1976).

These methods would allow incorporation of the scion of interest, without removal of the leading shoot of the interstock, which may possess some innate requirement for male cone production to occur, as evidenced by the presence of male cones in a sub terminal position of some interstocks. Similar methods could also be extended to grafting scions of interest onto field grown trees. In this case grafts should be made in a sub terminal position of branches in the part of the crown likely to bear male cones. A further useful strategy for promotion of precocious male cone production in *P. radiata* might be to graft "adolescent" *P. radiata* scions onto *P. contorta* rootstocks.

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[16] Trevor Faulds, Forest Research nursery, New Zealand. Personal communication 30 November 1999.

Male cone production on fascicle cuttings approximately two years old was promoted by growing them under stress in small pots (80 ml), with minimal watering and no fertiliser application. It is suggested that a "stress" pathway, distinct from the "normal" male cone production pathway is probably involved. It appears that under suitable stress conditions, the "stress" pathway may be activated leading to relatively early ontogenetic male cone production.

Male cone promotion in fascicle cuttings appears to be enhanced through the application of ABA, but not other plant growth regulators. Endogenous levels of ABA are known to increase in plants in response to various stresses (Bray, 1993; Kermode, 1997). It is suggested that exogenous ABA application might increase endogenous ABA levels, simulating greater stress than is actually occurring, thereby enhancing male cone production. Additionally, male cone production in fascicle cuttings appears to be enhanced by providing relatively high levels of light.

A promising strategy for Carter Holt Harvey Forests would be to identify potentially superior trees from trials as early during ontogenetic development as possible. Fascicle cuttings could then be produced from these ortets, and grown under stress conditions as in the present study. Provided they were established early enough the fascicle cutting ramets should produce male cones earlier than their parent ortets. At the time of final superior tree selection a source of small quantities of the required pollen would be available for further crossing.

A major outcome of this study is that, at least in some regions, male cone production commences at a younger age than was initially thought. It is now apparent that inadequate male cone production in young trees may not be the most critical impediment to the breeding program. Instead, the onus may be on early selection of superior genotypes. At present this cannot be accomplished reliably until approximately age six. It is apparent that marker aided selection will in the future probably become a very valuable tool in the early selection of superior clones. At this stage precocious cone production will again become critical.

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Glossary

Definitions of terms as used in this thesis:

ABA	Abscisic acid
BAP	6-benzylaminopurine, a synthetic cytokinin.
branch cluster	the region of attachment of branches to the stem.
cataphyll	non-photosynthetic bracts formed on the long shoot, referred to by Doak (1935) as sterile bracts.
cycle (of growth)	stem and its associated appendages, beginning with the region of sterile bracts which enclosed the bud (of the current cycle), followed by short shoots, and terminated by long shoots.
EFP	early flowering protein
evocation	Processes in the apex required for commitment to initiate flower primordia.
GA	Gibberellin
graft incompatibility	When a successful graft union occurs, but the plant later develops physiological and developmental malfunctions. It may occur soon, or several years after grafting.
graft success	The formation of a graft union between the scion and rootstock.

induction	Processes required for evocation to occur.
initiation (floral)	The production by the meristem of clearly recognisable flower primordia.
internode	The stem region between two successive nodes (nodes being defined as the part of the stem where cataphylls, or short shoots arise).
juvenile (phase)	Characteristics that occur early in ontogenetic development.
long shoot	Shoot forming either a branch, or female cone, at distal extremity of stem unit.
mature (phase)	Characteristics that occur late in ontogenetic development.
NAA	Naphthaleneacetic acid, a synthetic auxin.
node	the part of the stem where one or more leaves (cataphylls, or short shoots in the case of *Pinus*) arise.
ortet	The plant from which members of a clone (ramets) were originally derived.
photoperiodic	Responsive to changes in the ratio of light and dark in a 24-hour cycle.
provenance	Origin; locality where found or collected (Harlow, *et al.*, 1996)
range	The extent of an organisms natural distribution.

ramet	An individual plant belonging to a clone.
ripeness to flower	Attainment of sufficient carbohydrate and nutrient reserves for flowering to occur.
short day plant	Plants in which flower initiation is promoted by a reduction in daylength.
short shoot	Shoots forming either needle fascicles or male cones, along region of stem unit subtending terminal bud or long shoot.
stem unit	An internode, together with the node and nodal appendages at its distal extremity (Doak, 1935).
sterile	Regions of stem unit with no axillary components (Doak, 1935).
tree breeding	All measures directed towards the improvement of quality and yield of trees by the regulated production of desirable offspring (Thulin, 1957).

Appendix A

Killing and Fixing Solution

Foralin-acetic acid-ethanol (FAA)

- 95% Ethanol 350 ml
- Glacial acetic acid 25 ml
- Commercial formaldehyde25 ml
- Distilled water 100 ml

Appendix B

Atiamuri Scion Collection Area

Key	
x	Trees present, but not used for scion collection
2XX	Trees from which scions were collected
Skid	End of first spur off Pukerimu Road (approx 20 m from S.H. 30/ Pukerimu junction, to NW)