Micropropagation of

Paraserianthes falcataria

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ABSTRACT

Paraserianthes falcataria is a tropical, deciduous, leguminous tree. It is native to Papua New Guinea, West Irian, The Soloman Islands and The Moluccas. It is now widely grown in plantations of Philippines, Fiji, Asia, Australia and parts of America. The focus of this study was to establish micropropagation techniques for *Paraserianthes falcataria* , in order to introduce this species as a prospective source of timber and paper of the future. Seed germination experiments were investigated for seed pre-treatments with various agents, where warm (heated) water at 70^{0}C produced the maximum percentage (95%) of germination. Shoot initiation studies showed that zeatin between 0.1 to 0.5 mgL^{-1} supplemented in MS media produced the maximum number of shoots (25.4), while WPM supplemented with IBA between 0.1 to 0.25 mgL^{-1} produced the highest rooting percentage (21%). The explants were investigated for soil acclimation, with peat : perlite : coarse sand being the best soil conditions. In conclusion, *Paraserianthes falcataria* can be propagated *in vitro* and is a promising species for revegetation and agroforestry practices.

DECLARATION

I hereby declare that any material contained within this thesis is original or duly acknowledged and that no material from this thesis has been submitted for any other degree or publication at this point of time to my knowledge.

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LIST OF ABBREVIATIONS AND UNITS

ABA	Abscisic acid
$\mu Em^{-2}sec^{-1}$	Ampere per Metre square per second
ANOVA	Analysis of variance
&	And
et al	And others
Anon	Anonymous (author)
BAP	Benzyl amino purine
C	Carbon
Cm	Centimetres
Cm^2	Centimetre square
Conc	Concentrated
^0C	Degree Celsius
$	Dollar
=	Equal to
EDTA	Ethylene tetra acetic acid
e.g.	Example
ft	Feet
GA_3	Gibberellic acid
G	Grams
gL^{-1}	Grams per litre
GD	Gresshoff and Doy, 1972
h	Hour (s)
HCl	Hydrochloric acid
H	Hydrogen
pH	Hydrogen ion concentration
H_2O_2	Hydrogen peroxide
"	Inches
Inc	Incorporated
IAA	Indole acetic acid
IBA	Indole butyric acid
2ip	Iso pentenyl adenine
kcal	Kilo calories
$Kgcm^{-2}$	Kilogram per centimetre square
Kg	Kilograms
KOH	Potassium hydroxide
LP	Le Poivre, 1977
<	Less than
Ltd	Limited
L	Litres
M	Molar
M^3	Metre cube
µM	Micromoles
mM	Milimoles
Mg	Milligrams

MgL^{-1}	Milligrams per litre
Ml	Millilitres
Mm	Millimetres
>	More than
MS	Murashige and Skoog (1962)
NAA	Napthalene acetic acid
NaOCl	Sodium hypochloride
N	Nitrogen
Nm	Nanometre
No	Number
O	Oxygen
ppm	Parts per million
$m^{-1}sec^{-1}$	Per metre per second
$m^{-1}sec^{-1}$	Per metre per second
%	Percentage
P	Phosphorus
Pty	Propriety
Sec	Seconds
P	Significance probability
NaOCl	Sodium hypochlorite
s.e.	Standard error
H_2SO_4	Sulphuric acid
x	Times
Uv	Ultraviolet
U.S.A	United States of America
U	Units
V/v	Volume per volume
Wm^{-2}	Watts per metre square
W/v	Weight per volume
WPM	(Woody plant media) Lloyd and McCown, 1980

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Forest industries play an important role as the source of renewable raw materials and has important functions in the environment such as soil protection, carbon storage, carbon dioxide absorption, health improvement and recreational use (Chalupa, 1990). In face of rapid deforestation, depletion of genetic stocks and growing world population efforts are required to produce trees in short duration with rapid turnover of biomass and forest trees that are higher yielding, resistant to pests and diseases (Bajaj, 1986). Forests are being destroyed in Industrial Countries (such as Poland, Russia, and European countries) by air pollution, acid rain, drought, wind, snow, pests and diseases putting more demand on the forests as a resource (Chalupa, 1990).

1995 and 1996 had been marked by the commercialisation of the first genetically engineered plant oil and a number of groundbreaking publications with respect to plant biotechnology (Yuan and Knauf, 1997). The modification of plant components using transgenic technology is not just switching phenotypes from one host to another, rather, it is a means for producing valuable novel products that are normally not found (or are difficult to find) in plants. Active research is

being carried out with similar schemes in both academic laboratories and private biotechnology companies such as Florigene (Australia), Calgene (U.S.A) and Forbio (Australia). As a result, the traditional line that separates the 'basic' research of universities and the 'practical' work of industry is becoming blurred. Although many roadblocks such as cost and inability of certain plant species to be propagated through tissue culture techniques remain, judging from the progress made in the past two years the genetic engineering of plant components is heading towards a bright and exciting future (Yuan and Knauf, 1997).

Technologies in forest molecular biology and tissue culture could play an increasing role in the choice of genotypes for successful establishment of agroforestry practices (Klopfenstein and Kerl, 1995). Research areas such as micropropagation, somatic embryogenesis, genetic engineering, marker-aided selection or DNA fingerprinting and molecular diagnostics are merging with traditional forest biological studies to help identify and produce better-suited trees for agroforestry plantings (Teasdale, 1995). A combination of classical and molecular biological research could also be used to improve pest and stress resistance of selected genotypes, modify structure and function, and monitor pests of trees (Teasdale, 1995). This merger of approaches, as well as continued technological development, could accelerate the production and selection of suitable tree genotypes for agroforestry plantings (Klopfenstein and Kerl, 1995; Napoli and Klee, 1993).

Forestry is an important worldwide industry it therefore needs to be based on both natural and introduced plantations (Hall and Derose, 1988). Once an area has been logged, it requires replantation either by natural regeneration or seedlings. Natural regeneration - a cheap option has been extensively relied on in the past. There are number of disadvantages such as lack of control over the slow regeneration of seedlings, distribution of seedlings, species and level of genetic importance and therefore a more suited alternative has to be sought (Menzies, 1992). Hence, the importance of this project, which aims to provide adaptable techniques of micropropagation for forest vegetation species such as *Paraserianthes (Albizia) falcataria.*

1.1.1 Aforestation

Aforestation and revegetation of forest land are being encouraged and many queries have been raised about the environment and conservation of forests. The question still remains as to whether alternatives would be found soon and if the ecosystem would be balanced and returned to its original state (Kleinschmit and Meier-Dinkel, 1990; Le Roux and Van Staden, 1991a). Demands have exceeded production capacities for all forest products, with the exception of paper tissues, in Australia. Consumption in Australia is categorised as 90% for packaging, 60% for newsprint, and 40-50% for printings and writings (Bell *et al.*, 1995). State and Federal Governments have agreed to increase regeneration because: (a) access to new licence to clear forest is limited and (b) forestry

companies have access to only agriculturally cleared land which has salinity and low rainfall (Bell *et al.,* 1995). The ways and means to counteract such incidents have been experimented. Projects on alley cropping have been implemented in the Forest Zone of Cameroon (FZC), Africa (Duguma and Tonye, 1994). The main aim of the project was to identify agricultural constraints and to introduce alley cropping in the farming systems to improve soil fertility and crop yields. Surveys revealed that the main constraint in FZC was low soil fertility caused due to climatic conditions of the region (Duguma *et al.,* 1994). Thereby plant species such as *Leucaena leucocephala, Gliricidia sepium, Calliandra calothyrsus* and *Paraserianthes falcataria* were identified as promising tree species for alley cropping and results have proven that alley cropping has increased the yield of maize by approximately 52% in Africa (Tonye *et al.*, 1994). Aforestation activities have led revegetation to be a high priority for State and Federal Governments and Forestry companies such as North Forests, AMCOR plantations (formerly APM Forests) and ANM (Australian Newsprint Mills) Forest Management (Anon, 1996)

Loss of productive area is the greatest reducer of available volume, current, future and pending delays and deferrals. Comparison to native forests plantations are a greater risk of being greatly affected by natural disasters such as drought, flood, pests, diseases etc. due to their collectively smaller and compact areas (Boland *et al.,* 1994; Maghembe and Prins, 1994). Loss of growing stock and increment are greater in diaster prone areas because of the

limited capacity to refurbish and recover from death or defoliation. Replanting can restore the area however, the age and class differences that occur between plantations are irreparable, solutions which often are not entirely silvicultural manipulations of the destroyed areas. A good example of this problem can be seen with South Australian regions wherein the growing stock was damaged by fire in the year 1983. Therefore, revegetation with fast growing species is an absolute necessity (Lindey and Jones, 1989).

Exotic (introduced) forest estates comprise only 5% of the land area of New Zealand, forest products account for most of the domestic demands as well as providing 13.5% of export earnings (Smith *et al.,* 1994). In the year 1994, New Zealand Timber Industry was worth NZ $ 2.6 billion (Smith *et al.,* 1994; Wilcox, 1994). New Zealand made the transition from being dependent upon indigenous forest resources to reliance upon plantation forestry. Hence, New Zealand has not been dependent on its natural forests to supply the timber industry and plantation forests now supply the requirement of the industry (Smith *et al.,* 1994). Australia also is becoming a market for timber for the Asian countries such as Japan, Korea and China. Apart from this Asian interest, Australia is only a small distributor (28%) in the wood market for food products (Dargavel and Semple, 1991).

Revegetation can be achieved by conventional methods such as layering, grafting or cutting followed by clonal propagation of selected superior plant

material (Smith *et al.*, 1994). Desired plants are currently selected on phenotype (physical characteristics) analysis (volume growth, wood specific gravity and branching habit) all of which require several years for the completion of the breeding cycle making the entire process laborious, time consuming and expensive (Grattapaglia *et al.*, 1995). Cost of trait assessment in a traditional way is high and also the length of each breeding cycle and selection suitability (Phillips *et al.*, 1992). For example, the cost of processing specific gravity of *Pinus laeda* in laboratories vary from US$ 2.5 to US$ 5.0 per tree in the U.S.A. This excludes field collection costs and establishment and maintenance costs for genetic or associated tests for the entire breeding cycle which depends on the tree species that is being tested (Williams and Neale, 1992). Tree improvement programs brought about by White (1987) have deemed to reduce the time constraint of the conventional methods (Figure 1.1).

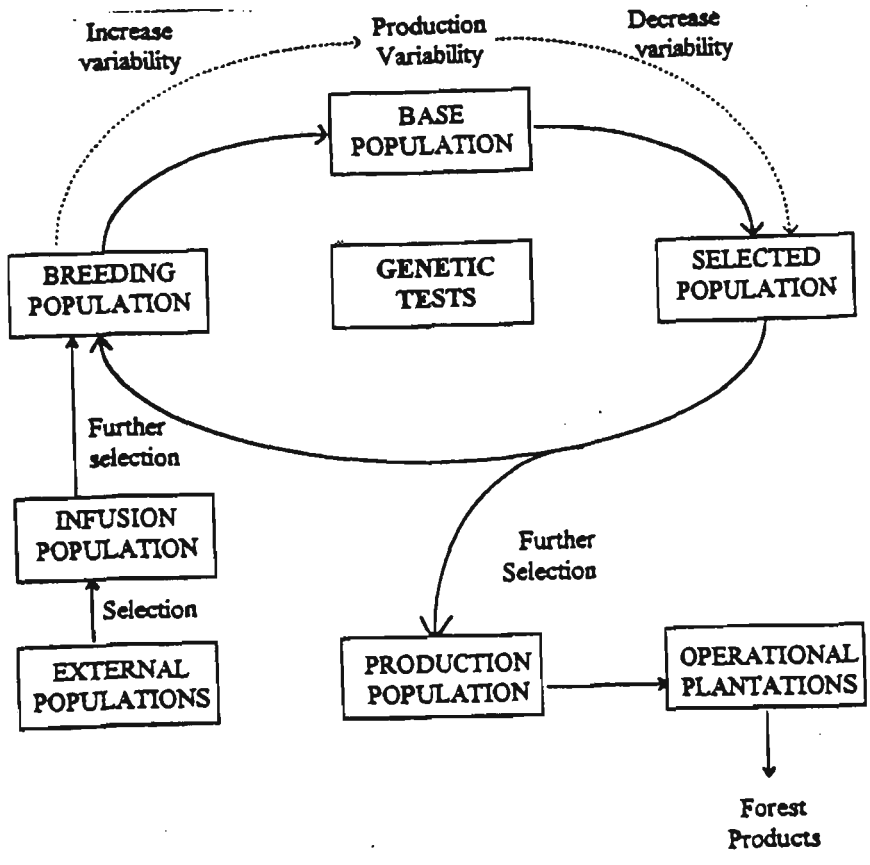

Figure 1.1 Major components and activities of the breeding cycle of forest tree improvement programs (White, 1987).

A range of steps determines the population of a species until the required phenotype is acquired. Figure 1.1 outlines the various processes involved in the acquiring of forest produces. The first step is the growth of the breeding population (parental stock) from which the base population (required stock) is selected based on its phenotype. The best of the base population becomes the selected population. Further breeding results in the breeding population from which the best traits are crossed to form the production population, which is ultimately grown in plantations (White, 1987).

1.1.2 Woody species

Herbaceous plants have been the centre of attraction for the enormous amount of research that has been carried out to reach this stage due to shorter regeneration time and less complexity of species. Therefore, woody species in comparison have not been concentrated in as much detail in the early 80's, however, more effort has been put into the research of woody plants in the past decade and the knowledge about these species has fast expanded (Murashige, 1974; Barlass, 1983). This advancement has been brought about by modern day techniques such as micropropagation, regeneration and DNA fingerprinting. One such species is *Eucalyptus*, that possess such qualities as rapid growth, highly desirable dense, hard and durable timbers (Goncalves *et al.*, 1984). *Paraserianthes falcataria* on the other hand has particular promise in reforesting sub- marginal sites, like idle and denuded hill lands (Anon, 1979; 1983; Walters,

1971). However, there are many difficulties associated with the vegetative reproduction of woody species because of the "waiting period" that accompanies the maturation of such species (Bonga and Durzan, 1982; Mehra- Palta, 1982).

1.1.3 Forest products

Looking at the Australian forest industry, depending on raw material sources, forest products can be categorised into the native forest sources, coniferous plantation resources, both sources or on imported wood either in processed or semi-processed form. 70% of Australian products are consumed domestically by Australians, mainly constitute domestic log; the remainder is imported as woodchip, pulp or paper products. Imports (37%) to Australia mainly include sawnwood, pulp or paper (Table 1.1)(Anon, 1991).

Table1.1 Australian paper and pulp products (Anon, 1991).

Product category	Softwood pulp (%)	Hardwood pulp (%)	Imported pulp (%)	Waste paper (%)	Additives (%)
Newsprint	73	22	5	A	-
Printing and writing	15	37	19	5	24
Tissues	54	-	46	B	-
Packaging and Industrial	14	17	6	61	2

A Nil until problem of de-inking resolved B Minor now, but reported to be increasing

Choice of a specific selection method on pulp cost, raw material production and quality is simulated through various factors as listed under Table 1.2. The demand for forest products is greater than supply, therefore, Australian imports from Asia make up approximately one-third of Australian requirements (Anon, 1992). For Australia to be self sufficient in this commodity, it is estimated that plantations will need to be established at a rate of 15,000 hectares per annum for at least the next 40 years (Chandler *et al.,* 1989). The country's strong demand for forest products and Australia's lack of capacity to supply it competitively has led to an annual deficit of A$ 1.4 billion (Anon, 1991; 1992).

Table 1.2 Simulation of the effect of choice of a specific selection method on pulp cost, raw material production and quality (Anon, 1992).

Production items, quality and cost of pulps and raw materials								
			Annual Volumetric Growth					
Used selection method	Pulp cost US$	Industrial Production /yr	Forest size ha	Specific consumption m^3	Pulp /ha/yr	Wood m^3/Ha/yr	Pulping yield %	Wood density Kg/m^3
Pulp cost	429.30	791,731	73,601	4.15	11.89	55.46	52.51	507
Industrial production	431.63	803,423	81,702	4.26	10.95	52.30	52.97	491
Forest dimension	438.73	765,335	70,196	4.30	10.86	57.20	51.17	502
Specific consumption	438.65	765,994	79,171	4.06	10.83	49.37	51.50	528
A.V.G./pulp	433.54	778,528	70,557	4.24	12.0	57.20	51.77	504
A.V.G./wood	437.70	763,295	73,592	4.50	11.55	58.23	51.67	474
Pulping yield	431.63	803,423	81,702	4.26,	10.95	52.30	52.97	491
Wood density	447.50	742,468	78,814	4.09	10.43	43.78	50.30	535

A.V.G. = Annual Volumetric Growth

The population of the world is growing and renewable resources of wood and non-renewable resources of coal and oil are decreasing at the same time. Therefore, the demand for wood and wood products is expected to rise in the future, due to the communities demand for paper, timber, tissue cardboard and furniture (Anon, 1992). Worldwide, the demand for wood in the year 1985 was 260 x 10^6 metric tonnes. This demand for industrial raw material is estimated to increase by 3 - 4% annually till at least year 2000 (Abdullah *et al.,* 1989).

1.2 Methods of propagation

Vegetative propagation dates back to the time of Theophrastus and Pliny (300 BC) who discussed the technique of tree propagation by means of cuttings and grafts (Rao and Lee, 1982). Since then, improved breeding programs and technologies are being developed via asexual techniques of vegetative propagation of selected material, to allow " screening " for the best plants. Most hardwood and softwood trees which are of economic value show variations in growth rate, frost hardiness, interspecific and intraspecific form during their lifetime (Mehra-Palta, 1982). The use of tissue and organ culture techniques, are possible methods of the future in terms of plant production (De Fossard, 1974, Teasdale, 1995). There was a great potential for the development of genetically improved strains through *in vitro* production, selection and multiplication because of the very long and complex processes of improvement by conventional techniques (Mehra-Palta, 1982). Such *in vitro* systems are of importance to large

scale forestry (Teulieres *et al.*, 1988).

The need to use tissue culture techniques to propagate *Paraserianthes falcataria* is to increase the normal output for the forest industry (Hartney and Svensson, 1992). This species has great capabilities that would enhance aforestation and revegetation due to its remarkable characteristics as discussed under Section 1.5. Thousands of trees can be commercially propagated from one seed in one year via *in vitro* techniques (Blomstedt *et al.,* 1991), while it takes 20 years for one seed to produce the same number of plants by the usage of conventional methods including cutting, layering and grafting. Tissue culture requires more skilled labour than the conventional methods and is also very expensive, however its advantages surpass the disadvantages. For instance, conventional propagation techniques do not guarantee a large number of plants and the plants have to grow to considerable lengths before they can be grafted or layered while in tissue culture the plants can be subcultured at shorter, regular intervals of 5 -6 weeks. Further, plants can be detected for diseases and can be manipulated to eradicate that character using tissue culture techniques (Draper and Scott, 1991). Therefore, suitable techniques and culture conditions must be established to achieve maximum growth with *in vitro* techniques and the need to grow these plants in initial plantation trials will be greatly reduced as these trials are rather cumbersome and time consuming. The total number required for final plantation in the fields can be acquired directly from the laboratories in a much shorter duration of time. (Hartney, 1980). Micropropagation also guarantees that

the characteristics of the source plant are conserved (Sankara and Venkateswara, 1985). Therefore, this project can be vital in providing more insight into *in vitro* propagation techniques of *Paraserianthes falcataria.* The micropropagation of *Paraserianthes falcataria* would be suggested as the change required by the forests to survive and be established as an alternative solution for forest revegetation.

1.2.1 Conventional breeding

Conventional techniques confine the breeder to the variability present in the normal gene pool but transformation techniques leads to the creation of desirable varieties containing completely man made genes. The sexual process in transformation techniques is bypassed which speeds up the whole process of plant improvement (Draper and Scott, 1991). Compatibility is very important for successful grafts, cutting or layers. However, due to the different genotypes, the method of conventional breeding used, inhibitors present, incompatibility may arise (Hartney, 1980). Other problems with traditional methods are due to low rooting capacity (Mehra-Palta, 1982) and low seed production (Cheng and Voqui, 1977).

Layering: Layering is generally used when the root formation from cuttings is slow (Hartney, 1980). During the rooting process the layer remains attached to

the plant: the internal phloem of *Eucalyptus* allowing metabolites to be continuously supplied. Grafting adult scions from adult trees onto rootstocks followed by layering of the scion is one way to layer. A second way involves the layering of basal epicormic shoots of adult trees. Several *Eucalyptus* species such as *Eucalyptus robusta* and *Eucalyptus camaldulensis* have been found to produce great variation in root forms during such growth. Their production was found in the case of *Eucalyptus robusta* to be dependent largely on climate (Pryor, 1976). The rate of rooting of air layers of *Eucalyptus camaldulensis* varied from 64 % in February to 27 % in April and 15 % in July (in Italy) showing that a link in seasonal conditions exists (Hartney, 1980).

Grafting: Grafting is the technique used, when specific characteristics of a species are required. For example resistance to root and shoot diseases and salinity tolerance (Hartney, 1980). Grafting is also turned to when other methods such as layering do not succeed or become too difficult (Hartney, 1980). Methods traditionally developed for horticultural crops have been successfully used on *Eucalyptus* species. Grafting consists of two main components, namely the stock and the scion. The stock provides the root system for the new plant while the scion provides the shoot system. (Hartney, 1980). Despite the performance of such techniques, they are labour intensive and ultimately produce high cost trees. In addition, a graft union when established does not always mean future compatibility. Thulinn and Faulds (1983) observed that developing buds on the scion could help graft union and the ensuing growth. It is

thought that growth regulators such as cytokinins produced in growing buds assist in the grafting process. It has also been found that if a large leaf area remained on the root stock graft success could also be improved. The high vigour of some rootstocks of *Eucalyptus ficifolia* was found to be advantageous in grafting attempts, but high nitrogen content of rootstocks were found to be detrimental. A further disadvantage like that found in *Eucalyptus* species is that they are very sensitive to the bruising and entry of air imparted by the graft process. Very little is known about the physiological causes of graft incompatibility but factors such as root stocks, scion genotypes, grafting methods and root inhibitors found in leaves and bark of adult trees of certain species may be involved (Hartney, 1980). The mechanism of root formation is also very poorly understood because of the wide and varied effects of growth regulators. For example, the effect of cytokinesis that cytokinins exert cannot be considered in isolation of the role that auxins play as the two are intertwined. Root formation is inhibited by sclerenchyma rings on cuttings acting as a mechanical barrier for root emergence in woody species (Hartney, 1980). By the time a tree has displayed its superior qualities in growth rate, resistance to other areas, it is morphologically unable to produce tissues capable of vegetative reproduction. The introduction of epicormic buds (via fire) which provide tissue that can be rooted or the use of grafting techniques can give rise to clones from the desirable individuals. But, the time and cost of these techniques make them viable only for special purpose propagation such as seed orchard establishment (Hartney, 1980). Trees are a difficult crop to manipulate because by the time

they have displayed desired characteristics; they are not capable of vegetative reproduction. This may then mean induction of suitable tissue is required where possible. Traditional methods such as grafting prove too costly in these situations. The basic strategy required for tree improvements is to capture existing genetic gains by cloning methods and then introduce new variations through both traditional and modern techniques. Improvement of woody perennial species by conventional breeding is a long and difficult process. The generation time from seed to flower for most woody species can take many years and therefore conventional breeding may prove to be a big strain. Tissue culture techniques can make a substantial input to breeding techniques by reducing the interval between pollination and the identification of useful hybrids. It also reduces the interval between generations (Barlass, 1983).

1.2.2 *In vitro* techniques

In vitro germination is the process by which thousands of trees are generated from a single elite clone outside the living plant (White, 1987). Plant tissue culture is the process by which small pieces of living tissues, explants are isolated from plants and grown aseptically for indefinite periods on semi-defined or defined nutrient medium (Mantell *et al.,* 1985; Smith *et al.,* 1980). The starting material for tissue culture is generally juvenile tissue such as embryos, cotyledons, hypocotyls, bud explants from seedlings and bud meristems (Ahuja, 1993). Thorpe and Patel (1984) also used epicots, dormant buds, needle

fascicles, basal and lateral shoot tips and stem segments with buds for tissue culture. Micropropagation is an *in vitro* process and was developed through the failure of classical methods of multiplication such as budding, layering and grafting techniques and their associated problems with incompatibility (De Fossard, 1974).

According to Goncalves *et al.* (1984) and Le Roux and Van Staden (1991a) clonal propagation through tissue culture techniques is a potential method for overcoming traditional vegetative problems. The use of *In vitro* techniques for clonal or asexual propagation is the most advanced application of plant tissue culture and offers advantages for research in several areas of forest biology (Libby, 1974). The use of plant tissue culture allows rapid gains to be made for instance as the long complex lifecycles of tree species may be avoided (Durzan, 1988). Tissue culture techniques has numerous advantages over conventional propagation. The most important advantages are the higher rates of multiplication, greater degree of control over most facets of plantlet growth such as nutrients, light and temperature (George and Sherrington, 1984).

Micropropagation is one form of plant propagation techniques which now firmly established as a commercial method for the propagation of many species of herbaceous ornamentals, orchids and ferns. The degree of control that can be exercised over virtually every stage of development is the most influential feature of multiplying clones by aseptic culture (Le Roux and Van Staden, 1991b). Many

research areas such as basic studies of cell cycle, the production of secondary plant products with pharmaceutical importance and monitoring of virus cell interaction are offered by tissue culture (Barlass, 1991). Micropropagation reduces the high risk of genetic variation due to chromosomal changes that occur in callus cultures, but it is potentially very useful in increasing the gene pool of some species (Le Roux and Van Staden, 1991a). Micropropagation is being researched as a parental source that could increase the availability of genetically superior stock (Horgan, 1987; Lorz and Brown, 1986). Genetically controlled traits such as timber shape, fibre and fast growth rates are sought via clonal propagation of trees displaying desired traits (Durzan, 1988). Micropropagation involves four basic stages. First the growth of shoots *in vitro* from pre existing meristems in the shoot tip or from axillary buds is induced. Second, the shoots need to be maintained and proliferated through a series of subcultures. Third, individual shoots are induced to initiate adventitious roots and the last stage involves acclimation of the plantlets to free living conditions (Hutchinson *et al.,* 1992; Mantell *et al.,* 1985).

An important aspect of tissue culture is the use of any part of the plant as the inoculum, segments of stem and root, leaf and petiole sections, inflorescence portions, seed embryos and seedling parts such as cotyledons, epicotyls and hypocotyls. Further, the more juvenile the material, the more easily will be the *in vitro* organ propagation. However, the best explant for each species must be determined experimentally (Thorpe and Patel, 1984). Superficial or epidermal

explants have served as inoculum in a limited number of species (Tran Thanh Van and Trinh, 1978). The stages of development include multiplication to rooting up to establishment. The buds that arise such, may not be stable with regard to trueness to type, depending on the species. It is important to recognise these differences so that plants after regeneration are thoroughly checked for stability (Thorpe and Patel, 1984). Clones can be stored and then manipulated using various media to produce shoots or roots throughout the year according to the demands of the market. Enhancing axillary bud breaking, somatic embryogenesis and production of adventitious buds can be achieved by rapid asexual multiplication. Roots, stems, leaves and flowers are ultimately derived from specialised regions (meristems) of plants. Unlike the situation in animals, new organs are produced from meristems throughout the life of the plant. Plant development is also modulated by external environmental influences including light, temperature and gravity and internal plant hormones (Newbigin *et al.*, 1995). Adventitious shoots or roots can be induced to form on tissues that normally do not produce these organs. Adventitious meristems which are formed *de novo* in response to growth regulators, that can arise directly from the explant or from an intermediate stage of organised tissue developed in culture from the explant. This process has far more potential for mass clonal propagation of plants than multiplication from axillary buds. In addition it is more common than somatic embryogenesis. Adventitious shoot and roots can be produced directly on the explants or on callus derived from primary explants (Thorpe and Patel, 1984). It is important to recognise that the choice of culture procedures and

conditions are based primarily on the objective or purpose of the investigation. Success in the technology and application of *in vitro* methods is largely due to the nutritional requirements of cultured cells and tissues (Gamborg *et al.*, 1976; Murashige, 1974; Street, 1977; Thorpe and Patel, 1984). Explant origin and general nutritional milieu are the two factors that determine the success of cultures (Gamborg and Shyluk, 1981). Current sources of germplasm are seed orchards and long range breeding programs which have been demonstrated to be costly and time consuming (Durzan, 1988).

Techniques involved with tissue culture, which are becoming more important in the production of new genotypes, are somatic embryogenesis and particularly protoplast cultures. Plant tissue culture technology is divided into three broad classes depending on the type of materials (Table 1.3) used. The three main classes are protoplast culture, organ culture and callus culture.

Protoplast cultures: Protoplast (naked plant cell) culture incorporates the aseptic isolation and culture of plant protoplasts from cultured cells or plant tissues (Teulieres, 1988; Gamborg and Shyluk, 1981). Protoplast cell culture is a culture of cells in liquid media (Ammirato and Steward, 1987). Over all the study of protoplast cultures showed that the yields of protoplasts are independent of the species used and modifications of basic procedures (ie. enzyme concentrations) allows protoplasts to be obtained from most *Eucalyptus* species (Gamborg and Shyluk, 1981; Teuliers *et al.*, 1989). The development of

protoplast culture has facilitated the genetic manipulation of cells, of particular importance to secondary metabolite production. The availability of embryogenic cell lines for use in protoplast cultures may mean gene introduction and expression of desirable traits is possible.

Organ culture: Meristem (organ) culture and morphogenesis covers the aseptic cultures of shoot meristems or other explant tissue on nutrient media for the purpose of growing complete plants. Organ culture includes the aseptic culture of embryos, anthers (microspores) ovaries, roots, shoots or other plant organs (De Fossard, 1973; Muralidharan *et al.*, 1989). Organ culture in *Eucalyptus* differs from tissue culture in that it aims to induce roots and shoots virtually immediately from pieces of organs; nodes; leaves; and stems. Organ cultures move the tissues away from such inhibitors (endogenous cytokinins and auxins) and therefore initiation and development are possible (Gamborg and Shyluk, 1981; De Fossard *et al.*, 1974).

Embryogenesis is an alternative method to *in vitro* propagation technique using cultures and adventitious bud formation (Muralidharan *et al.*, 1989). Somatic embryogenesis is the process by which somatic cells develops throughout the stages of embryogenesis without the actual fusion of gametes, to give rise to a whole plant. Embryogenic cultures provide a lack of uniformity due to the lack of synchronisation of development with in an embryo population (Ammirato and Steward, 1987). Advancements and technologies such as cryopreservation of

embryogenic cell lines allow the maintenance of germplasm until the progeny and specific tree clones, have proven economically viable and reliable. Embryogenesis can therefore, provide a high frequency of embryogenesis through a subcultured embryogenic mass without involving callus, retention of embryogenic competence over a relatively long period of time, the possibility of low temperature storage (10^0C) without subculture while ensuring high rates of plantlet regeneration and survival (Gamborg and Shyluk, 1981; Muralidharan *et al.*, 1989). In this study, organ culture was used in the propagation of seeds and then meristem culture in the propagation of shoots and roots.

Callus culture: Callus culture in which masses of cells are cultured on agar media and are produced from an explant of a seedling or other plant source such as shoot, root, anther portions (Le Roux and Van Staden, 1991a). Callus formation in tissue culture of *Eucalyptus* occurs in a number of species. Callus development is observed on various organs. These include seeds, hypocotyls, cotyledons, stem, segments, apical shoots, lignotubers, anthers, bark explants and leaf blades (Lignotubers are the swellings which are sometimes produced at the base of a young tree) (Gamborg and Shyluk, 1981; Goncalves *et al.*, 1984; Le Roux and Van Staden, 1991a).

When working with woody species such as *Eucalyptus* regeneration from callus culture becomes increasingly difficult because of the formation of differentiated callus cells. In addition, as the callus ages the amount of regeneration

decreases giving fewer cells capable of regeneration. The concentration of sugars in the medium also influences callus formation. At sucrose levels of 2 % (w/v) profuse callusing is noted on *Eucalyptus nova-anglica* bud explants (Mehra-Palta, 1982). As the concentration of sucrose in a Nash and Davis (1972) medium was increased to 2.5 % (w/v), an increase in both fresh and dry weight of leaf blade callus of *Eucalyptus grandis* was observed. Dry weight increase continued through to 4 % (w/v) sucrose (Goncalves *et al.*, 1984). Concentration and form of nitrogen also influence callus growth; for example ammonia as the only nitrogen source gave consistently lower fresh weight values than callus grown on comparable nitrate concentrations.

Tests performed by De Fossard *et al.* (1977), involving auxin/ cytokinin ratios utilised *Eucalyptus bancroftii* stem calluses which were 1.5 years old; unsuccessful regeneration attempts led to the belief that as a callus ages it looses its ability to regenerate somatic tissues (ie. looses its differentiating ability). Work by Warrag *et al.* (1991) showed that callus from *Eucalyptus* hypocotyls displayed morphogenesis.

Table.1.3 *In vitro* methods of propagation used in propagation of plant species.

Culture methods	Explants used
Callus culture	Mass of callus (organ varies with type of research conducted)
Cell culture	Cells from explants (varies with type of research) in liquid media
Organ culture	Anthers, Root, Stem, Seeds, Embryos, Ovaries (research dependent)
Embryogenic culture	Subdivision of organ culture, but has reached a high level of acceptance
Meristem culture or morphogenesis	Aseptic culture of shoot meristems
Protoplast culture	Protoplasts from organs (research dependent)

1.3 Culture environment

There is an interplay between the inoculum, the medium and the culture conditions, in the formation of the plantlets. Each of the components has to be assessed for optimum organogenesis. There are four distinct processes in the process of plantlet formation via organogenesis, namely, induction of shoot buds, development and multiplication of the buds, rooting of the shoots and hardening of the plantlets. Experiments should be conducted for the optimum requirements of each stage (Thorpe and Patel, 1984).

Many aspects of culture environment can influence growth and organised development. These variables include physical form of the medium, pH, humidity and gas atmosphere, light and temperature.

Physical form of medium: This factor plays a very important part in growth and differentiation irrespective of whether it is solidified or a liquid. Callus maintained on medium solidified with agar (0.6-1.0 % (w/v)) grows slowly, with the new cells formed mainly on the periphery of the existing callus mass. Cell suspension cultures on the other hand, tend to grow more rapidly, as the single cells or small cell clumps are constantly exposed to the nutrient media. Most success in organogenesis is achieved with explants, callus or plated cell suspensions on solid medium (Thorpe and Patel, 1984). However, a liquid phase during plantlet formation, in which the tissue is slowly agitated may be beneficial (Murashige, 1977).

pH: The pH of the medium is usually set at approximately 5.0 for liquid formulations and between 5.2 to 5.8 for agar-gel media (Thorpe and Patel, 1984). Blueberry callus was grown on pH 4.8, however, the pH scale varies according to the explant and the type of media used (Muralitharan *et al.,* 1993).

Humidity and gas atmosphere: Relative humidity is rarely a problem except in arid climates, where rapid drying of the medium occurs. This can be reduced by the use of tightly closed containers, covering closure such as foam or cotton

wool plugs with aluminium foil or another material and sealing petri dishes with a household plastic covering (eg., Handi wrap, cling wrap) or parafilm. In climates with high humidity, dehumidifiers in the culture rooms may be useful, but the major problem would be the growth of fungi and other microorganisms. The gas atmosphere, which includes ethylene, ethanol, carbon-dioxide and acetaldehyde, can inhibit morphogenesis if gaseous exchange does not occur. In some cases in the instance of urban atmospheres, it may be necessary to filter the air entering the culture rooms, for example filters such as that of charcoal will be useful in the removal of dust and spores as well (Thorpe and Patel, 1984).

Light: Light has been a major influence on growth, development and morphology of plants (Ellis and Webb, 1993). Light is shown to have a major effect on the organised development of *in vitro* cultures. Light requirements involve a combination of several components, including intensity, daily light period and quality and is necessary for certain photomorphogenic events (Murashige, 1974). Although maximum callus growth occurs in darkness, low light intensity (eg., 10 - 80 $\mu E.m^{-2}. sec^{-1}$) may enhance organogenesis. In some cultures, higher light intensities (eg., 90 $\mu E.m^{-2}.sec^{-1}$) are needed for optimum shoot formation, multiplication and development. Lower intensities of light are beneficial for rooting. During plantlet hardening even higher light intensities (eg., 300 $\mu E.m^{-2}.sec^{-1}$) may be needed for subsequent survival. Light sources with electromagnetic spectra closer to those of sunlight are probably best. However, good results have been obtained with normal fluorescent lamps with or without

supplementary incandescent lamps (Raven *et al.,* 1992; Thorpe and Patel, 1984).

Temperature: Generally cultures are kept at a constant temperature of 20°C to 30°C depending on the species. The optimum temperature for growth and differentiation for a particular species should be determined as different species have different optima (Hughes, 1981). Thermoperiod and temperature pre-treatments (including a chilling temperature have been shown to affect morphogenesis) (Cheng and Voqui, 1977; Rumary and Thorpe, 1984). Higher temperature (30°C and above) favours shoot initiation and cooler temperature (15°C and below) favours shoot elongation (Aitken – Christie and Thorpe, 1984).

Sterility: There is a tendency to carry out rooting under non-sterile conditions (Biondi and Thorpe, 1982). Shoots are often dipped in IBA solutions or commercial rooting powders and planted in sterilised soil mixes or supports (eg., Vermiculite, Perlite). Watering with half-strength mineral salts and use of fungicides is common. This type of approach is not only simpler but often produces superior roots and facilitates planting out (Thorpe and Patel, 1984).

It is important to note that the conditions of form of media, pH, humidity, light, temperature, sterility, site preparation and growth hormones are essential for the successful micropropagation of plant species. Hence, these conditions were carefully considered for micropropagation of *Paraserianthes falcataria.* A

summary of the conditions of the main environmental conditions required for *in vitro* techniques are listed under Table 1.4.

Table.1.4. Summary of culture conditions required for the growth of tissue cultured plant explants.

Culture Environment	Types
Physical form of medium	a. Solid b. Liquid
PH	a. Liquid – scale of 5 b. Agar based – scale of 5.8
Gas atmosphere	Charcoal filters
Light	a. Low light – 90 $\mu Em^{-2}sec^{-1}$ b. High Light –10 – 80 $\mu Em^{-2}sec^{-1}$
Temperature	$20^{0}C$ to $30^{0}C$ (species dependent)
Sterility	Dipping of shoots in IBA solution or commercial rooting powders

Growth hormones: The word hormone comes from Greek, which means "to set in motion". The response to the particular regulator in plants depends not only on the chemical structure but on the mechanism and efficiency of transcription by the target tissues, that is the same hormone can elicit different responses in different tissues or at different times of development in the same

plant. Five main groups of hormones are recognised (Raven *et al.*, 1992) and are briefly described in Table 1.5. Ethylene, the fifth growth hormone was not relevant to this present study and hence was not discussed nor experimented in this study and therefore, was excluded from discussion. Each hormone type is discussed in detail in the relevant topics: abscissic acid and gibberellin (Section 2.3.4), Cytokinins (Section 3.3) and Auxins (Section 4.3).

Table 1.5 Growth hormones and their action (Raven *et al.*, 1992).

Hormone	Nature	Sites of Biosynthesis	Transport	Effects
Auxin	Indole-3-Acetic acid is the only known naturally occurring auxin. It is synthesized primarily from tryptophan.	Primarily in leaf primordia and young leaves and in developing seeds.	IAA is transported from cell to cell and transport is undirectional (polar).	Apical dominance; tropic responses; vascular tissue differentiation; promotion of cambial activity; induction of adventitious roots on cuttings; inhibition of leaf and fruit abscission; stimulation of ethylene synthesis; inhibition or promotion (in pineapples) of flowering; stimulation of fruit development.
Cytokinins	Adenine derivatives,	Primarily in root tips.	Cytokinins are	Cell division; promotion of shoot

	phenyl urea compounds. Zeatin is the most common cytokinin in plants.		transported via the roots to shoots.	formation in tissue culture; delay of leaf senescence; application of cytokinin can cause release of lateral buds from apical dominance.
Abscisic acid	"Abscisic acid" is a misnomer for this compound, for it has little to do with abscission. It is synthesized from mevalonic acid.	In mature leaves, especially in response to water stress. May be synthesized in seed.	ABA is exported from leaves in the phloem	Stomatal closure; induction of photosynthesis transport from leaves to developing seeds; induction of storage protein synthesis in seeds; embryogenesis; may effect induction and maintenance of dormancy in seeds and buds of certain species.
Gibberellic acid	Gibberellic acid (GA_3), a fungal product, is the most widely available. GA_1 is probably the most important gibberellin in plants. Gas are synthesized from mevalonic acid.	In young tissues of the shoot and developing seeds. It is uncertain whether synthesis also occurs in seeds.	Gibberellins are probably transported in the xylem and phloem.	Hyperelongation of shoots by stimulating both cell division and cell elongation, producing tall, as opposed to dwarf, plants; induction of seed germination; stimulation of flowering in long-day plants and biennials; regulation of production of seed enzymes in cereals.

The concept that plant hormones help to coordinate growth and development by acting as chemical messengers between cell comes in part from numerous examples of the observable influences of plant hormones on the rate of cell division and on the rate and direction of cell expansion (Table 1.6) (Raven *et al.*, 1992)

Table 1.6 Hormonal influences on basic cellular processes (Raven *et al.*, 1992).

Hormone	Rate of cell division	Rate of cell expansion	Direction of cell expansion	Differentiation (Gene expression)
Auxin	+	+	l	+
Cytokinin	+	*	*	+
Abscisic acid	-	-	*	+
Gibberellin	+	+	l	+

+ = positive effect - = negative effect * = little or no effect l = longitudinal expansion

1.4 Genus description (*Albizia*)

Albizia are big, compound- leafed trees that are adapted to a wide variety of soils and environments. They are well suited to cultivation and show very rapid early growth and appear to be efficient nitrogen fixers (Anon, 1979; Binkley *et al.*, 1997; Debell *et al.*, 1989). *Albizia (*syn. *Paraserianthes) falcataria* and *Albizia lebbek* have been planted extensively; the others remain restricted in their distribution and largely untested. Minute differences in flower structure (variations in androecium and gynoecium) is the only source to distinguish the genus *Albizia* from the genus *Acacia* (Anon, 1979). The wood of this species is similar to that of black cottonwood (*Populus trichocarpa*) (Gerhards,1966), the wood of *Albizia lebbek* resembles walnut (Anon, 1979). The wood are non-silicaceous, usually light coloured with some open pores and produce a sawdust that may cause sneezing (Anon, 1979). *Albizia* are characteristic as pioneers of

forest regrowth because abundant levels of seeds and establish readily in the open and are very robust, thriving well in diverse climates and altitudes. They are easy to propagate and handle in plantation production (Anon, 1979). Table 1.7 shows the flowering dates of the species of the species *Paraserianthes falcataria.*

Table1.7 *Paraserianthes falcataria* : Flowering, fruit ripening and seed dispersal dates (Palit, 1980)

Location	Flowering Dates	Fruit Ripening Dates	Seed Dispersal Dates
Hawaii	April - May	June - August	June – August
Buxa Forest Division	May	June - July	June - July

1.5 Species introduction (*Albizia* syn. *Paraserianthes falcataria)*

Paraserianthes falcataria can be considered as an alternative because of its special characteristics as one such tree species. This species is considered to be the world's fastest growing tree species, with incredible rates of growth and coppicing reaching upto 45m in its lifetime of 17 years (Anon, 1992). They are also regarded to have particular promise for reforesting sub-marginal sites, like idle and denuded hill lands. The open, spreading canopy of *Albizia* suits agroforestry (Anon, 1979; 1983; Walters, 1971). *Albizia falcataria* grows so fast

that it is regarded as a cash crop and is sponsored by a World Bank funded project for pulpwood since 1970 (Anon, 1992).

1.5.1 Characteristics of *Paraserianthes falcataria*

The most important attribute of *Paraserianthes falcataria* 's is its rapid growth rate. It is one of the world's fastest growing tree species and is considered as the miracle tree due to its extraordinary height and pace of growth (Anon, 1983; Walters, 1971). In the first year, it reaches a height of about 7m followed by 13m - 18m in three years, 30m in 9 -10 years and reaches a final height of about 45m in 17 years (Anon, 1979, 1983; Walters, 1971)

Botanical name: *Paraserianthes falcataria* (L.) Nielson (earlier *Albizia falcataria* (L.) Fosberg).

Synonyms: *Paraserianthes falcataria, Albizia falcataria* Fosberg, *Albizia falcata* (L.) Backer, *Albizia moluccana* Miq.

Common names: Batai, Molucca albizia, Moluccan sau, Djeungjing, Sengon, Falcata, Vaivai, Puah, White albizia, Kayu macis, Tamalini, Mara, Placata, Plakata.

Family: Leguminosae and sub-family Mimosoideae (Anon, 1979; Palit, 1980).

Main attributes: Because of its rapid growth, vigorous coppicing and usefulness when grown in combination with agricultural crops, it is considered for firewood irrespective of its low calorific value and specific gravity.

Habitat: About 100 species of *Albizia*, and they are found in Africa, Asia and tropical America (Anon, 1979). *Paraseriathes falcataria* is a deciduous tree having its natural habitat in Indonesia (Anon, 1979; Palit, 1980). *Paraserianthes falcataria* is native to Papua New Guinea, West Irian, The Solomon Islands and the Moluccas. It has been grown in plantations in the Philippines and in Fiji (Anon 1979; 1983). It is now commonly found in the tropical and sub-tropical Asia, Africa, Australia and parts of the United States of America (primarily in Hawaii) (Palit, 1980). It has also been used in a variety of plantation trials in India, Sri Lanka, Taiwan and Western Samoa. It has been naturalised in urban areas of Sabah. In Western parts of Java, it is planted in home gardens and in gardens mixed with herbaceous and trees crops as a source of timber and firewood (Anon, 1979; 1983; 1992).

Habit: Most *Albizia* species are attractive evergreens, with sprays of graceful foliage. When grown in the open, its crown spreads to form a large umbrella shaped or flat-topped or funnel-shaped canopy (Plate 1.3), but in

plantations it has a narrow crown (Plate 1.1 and 1.2). In mature species, the branch tips droop and the trees become massive, greyish white trunks topped by a gigantic umbrella of foliage. Flowers are creamy white and have a slight fragrance, they also have long pods that rattle in the breeze. The trunks are thornless and unbuttressed (Anon, 1979; 1983).

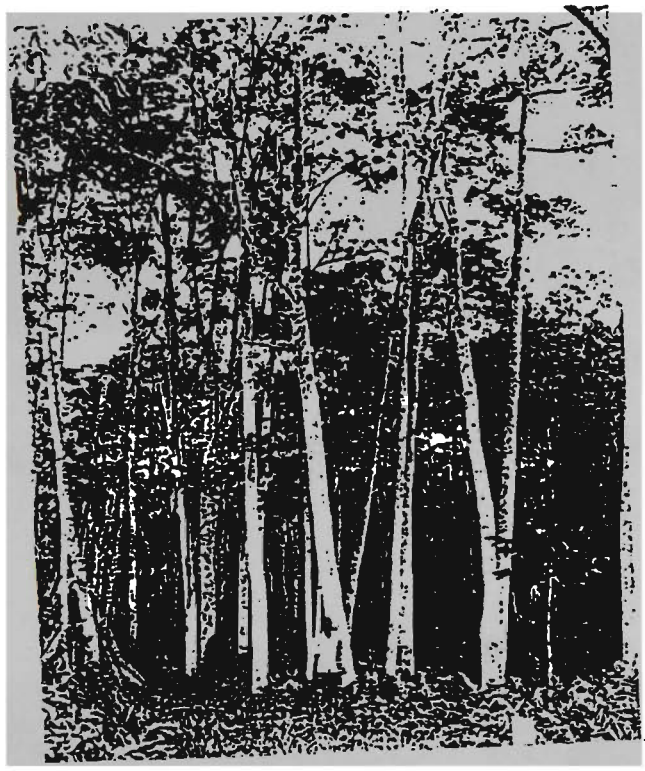

Plate 1.1 *Albizia falcataria*, 9 years old, Ivory Coast (Anon, 1979).

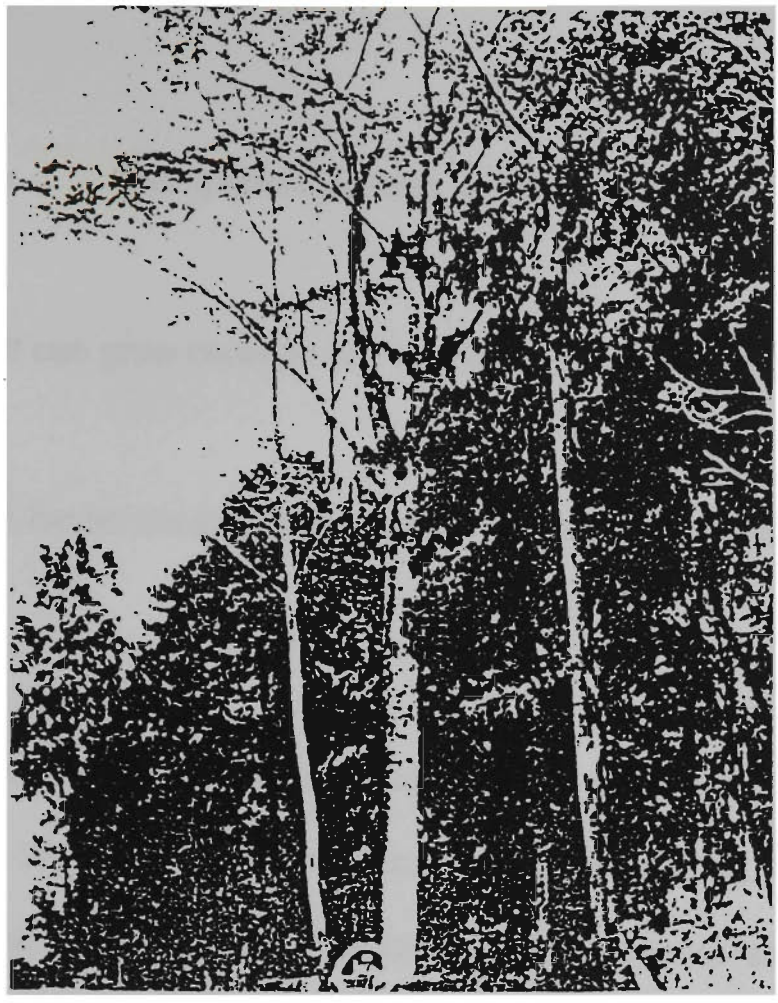

Plate 1.2 *Albizia falcataria.* Lindbergh tree, Surigao, eastern Mindanao, Philippines, 17 years old, 96cms diameter. Note person (circled) for scale (Anon, 1979).

Plate 1.3 *Albizia adianthifolia.* Kruger National Park, South Africa (Anon, 1979).

Temperature: This species requires an optimum temperature of 22 – 29^{0}C.

Altitude: It can grow between 800 to 1500 m in altitude.

Climate: A humid tropical climate with no more than a slight dry seasons (0-2 months).

Rainfall: In Hawaii, the most suitable areas are those with a rainfall of 2000 - 5000 mm (80 - 200") in an altitude of less than 600 m (2000 ft). The best growth has been achieved on sites in the Philippines with an annual rainfall of 4,500 mm and no dry season, but it can grow well in rainfalls ranging from 2,000 - 2,700 mm per year with at least 15 rain days during the driest four months (Anon, 1979; 1983).

Soil: They grow well in a wide range of soil types from heavy clay to volcanic ash. Well-drained, deep soils with reasonably high fertility are well suited. They grow best in fertile soil, but generally have the ability to grow rapidly on infertile sites that are not waterlogged (Anon 1979; 1983). The most suitable soils are deep, moist but free- draining, including free draining clays. Dry, sterile and sandy or compacted soils have been found unsuitable for the species (Palit, 1980). Being a leguminous plant, it grows better on alkaline soils than on acidic soil and can tolerate acid soils down to a pH of 4.5 (Anon, 1979).

Usage in plantations: *Paraserianthes falcataria* is a relatively easy species to propagate in the open and is also used widely on a commercial basis in the tropics. In spacing and fertiliser trials, young plantations have annually produced in excess of 50 m^3 of wood per hectare, but a mean increment of 25 - 40 m^3 per hectare in a 8 - 10 year rotation is the expected range. It is also grown in home gardens and public gardens along with fruit and herbaceous trees in parts of Western Java (Anon, 1979; 1983). Due to their soil improving (caused due to its nitrogen fixation abilities) and light shade characteristics that their airy foliage provide, this species have been extensively planted in Southeast Asia as a shade and nurse crops for coffee, cocoa, tea, banana, patchouli and young timber plantations as well as for support for pepper vines. In Java, *Albizia falcataria* is used in small farms. Both annual and perennial crops, such as hot peppers, upland rice, pineapple grass, banana. bamboo, coffee or fruit trees can grow normally under this tree thus providing a productive, three-tier combination. Further, this species is useful as a shade and avenues tree (Anon, 1979; 1983; Vincent *et al.*, 1964). *Paraserianthes falcataria* can be a noxious plant due to its aggressive growth. It has the ability to seed prolifically beneath competing species in plantations and then overtake and shade them out, that has occurred in two high rainfall, low elevation areas in Hawaii (Anon, 1979).

Survival in adverse conditions: In trials conducted in Hawaii in the year 1968, Batai *(Albizia falcataria* Fosberg) was planted in a site about two miles from a volcanic crater that had erupted in 1960 and about one and a half mile

away from the edge of the concurrent lava flow. The eruption site was covered with 3 to 8' of cinders. Eventhough the conditions of growth were far from ideal, *Paraserianthes falcataria* developed quickly. If this tree is planted widely and grow as quickly as shown under the adaptability trials, the supply of wood may soon meet the demand (Walters, 1971). *Albizia falcataria* has been successfully established even on tailings left after tin mining, it can also be grown in land lost to weeds (Anon, 1979).

Competing against weeds: The ability to survive competition from weeds is another credit of this plant. Weeds are unable to compete with *Paraserianthes falcataria* because of its fast pace of growth by which it completely shades them out. The young plants can be closely spaced in plantations (1,000 - 2,000 trees per hectare), which not only increases the number of trees produced per hectare, it also makes them grow straight and reduces the volume of the crown to provide more shade. Thus, inhibiting the growth of weeds such as the tenacious *Imperata cylindrica*, when managed carefully it overtops and kills the grass by cutting of the light. Therefore, weeding, can be limited to one complete weeding and three spot weeding during the first year of planting (Anon, 1979; 1983).

Paper production: The greatest promise for *Paraserianthes falcataria* is in the manufacture of paper. Inspite of its low specific gravity (0.24 - 0.49) and calorific value (2, 865 - 3,357 kcal per kg), it is well suited as a low-density pulp

producer. The ground wood pulp of this species because of its pale colour and fibre length makes it even better. Because, the wood is soft and easy to chip, it reduces considerable amount of labour and time. The pale colour requires minimum bleaching and therefore reduces the utilisation of chemicals. The strength properties of kraft and soda pulps are comparable to good quality eucalypt pulp (Anon, 1979). In the Philippines, it is successfully being used for newsprint (Anon, 1983). Some of the critical requirements for good quality paper pulp are listed in Table 1.8.

Table 1.8 Critical fibre properties for paper and pulp manufacture (Dean, 1995

	Copy Paper	Offset Printing	Coating Base
Bulk	Yes	Yes	Yes
Opacity/Colour	Yes	Yes	-
Sheet Roughness	Yes	Yes	Yes
Porosity	-	Yes	Yes
Stiffness	Yes	-	-
Formation	-	Yes	Yes

Wood: The low specific gravity and calorific value of the wood of *Paraserianthes falcataria* makes it useful as fuel in parts of Western Samoa and Java. The wood is light and soft and lacks strength, due to this it is an excellent for the production of charcoal. The wood is used in the manufacture of fibre and

particle board, and also as veneer core stock and for light weight pallets, crating, furniture components, matches, matchboxes, packing cases, tea chests and shelves (Anon, 1979; 1983, Vincent *et al.*, 1964). Studies have shown the wide uses of the pulpwood potential, usefulness as hardboard, particleboard and flakeboard (Chen, 1982; 1987; Logan *et al.*, 1984; Semana *et al.*, 1982). The low crown is an adverse characteristic for a plantation species grown for timber, because it reduces the length of the trunk, which may prove to be a disadvantage for the wood and paper industry (Anon, 1979).

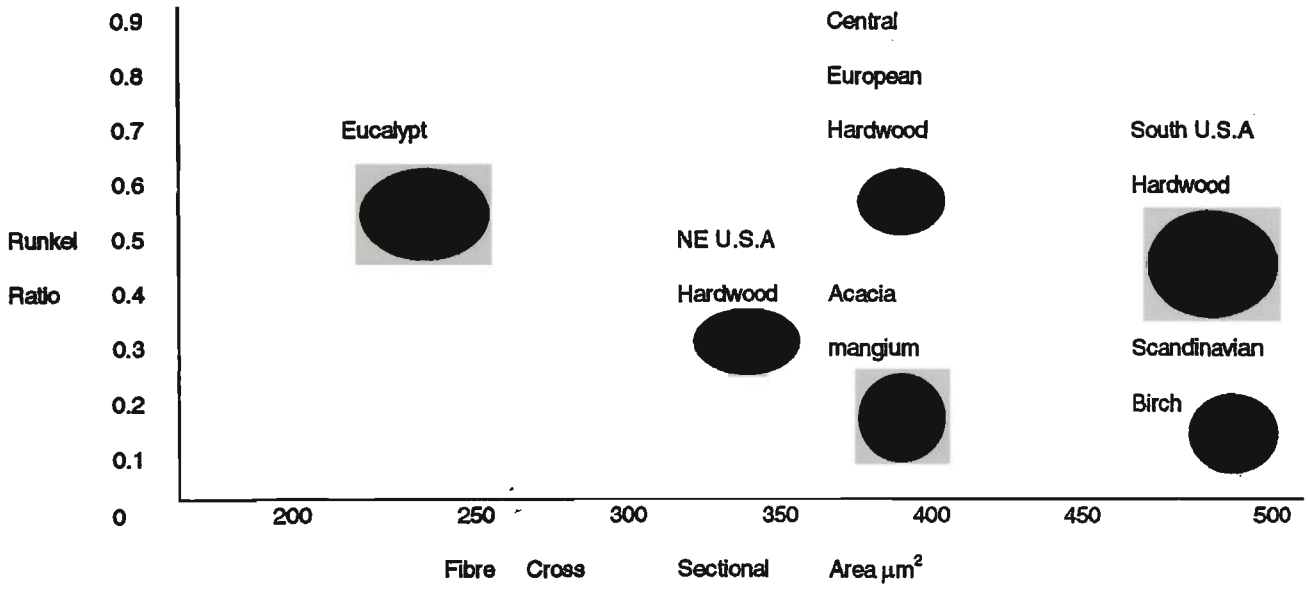

Figure 1.2 Fibre shape versus size for hardwood pulp (Dean, 1995).

Figure 1.2 represents two parameters for a range of hardwood pulps, although the presentation should be taken as merely indicative of typical pulps (Dillner, 1979; Andtbacka *et al.*, 1990). Fibre size is represented by the cross sectional area. Wall thickness relative to diameter is indicated by the Runkel Ratio which

measures 2 x w/l, where w is the wall thickness and l is the lumen diameter (Dean, 1995).

The wood of *Paraserianthes falcataria* is light in weight (27 pounds per cubic foot air dry, specific gravity 0.43). It is moderately weak in bending or compressing strength, moderately soft and moderately limber. The plantation-grown material from Hawaii and Malaysia has been tested for timber and those from Malaysia for pulpwood as well. The timber can be sawed easily, even though it is a bit fibrous. Papers made from sulphate pulp obtained from 43-46 cm diameter trees from Malaysia compared favourably with those made from coniferous kraft pulp (Palit, 1980). The bark of *Albizia* trees exude a water-soluble gum when the trunk is damaged, and also contains tanning compounds, these gums and barks are commercial useful. The foliage is foraged by livestock (Anon, 1979).

Nitrogen fixation: The roots of *Paraserianthes falcataria* bear abundant nodules, their ability to fix nitrogen along with the plants prodigious nitrogen fixing capacity they make good soil improvement, cover and green manure crops. The natural drop of leaves, pods and small branches contributes nitrogen, organic matter and minerals to the upper soil layers (Kadiata *et al.,* 1996a, b). The plants extensive root system improves soil conditions by breaking up heavy soils and provide drainage and aeration (Anon, 1979). Productivity of *Eucalyptus saligna* Sm, plantations is commonly limited by low levels of available nitrogen (N). Synthetic nitrogen (N) fertiliser applications are costly and sometimes

impractical and therefore, mixed species plantations of *Eucalyptus saligna* Sm and by *Paraserianthes falcataria*, were used achieving 66% more growth in the biomass of the *Eucalyptus* strains. Over time, the benefits of mixed plantations have increased and include diversity in stand structure (Binkley, 1997; Debell *et al.*, 1989; Kadiata *et al.*, 1997).

Research had been carried out on the nitrogen fixing abilities of this species along with other species like *Eucalyptus* species, *Leucaena leucocephala* in plantations in Hawaii and have proven successful. By using *Eucalyptus - falcataria* mixed plantations the need for application of synthetic nitrogen fertiliser was eliminated on the test site after the first year (Binkley *et al.*, 1992; Debell *et al.*, 1985, 1987, 1989, 1997; Moloney *et al.*, 1990; Schubert *et al.*, 1988). The growth rates, production (Panjaitan *et al.*, 1993), nutrient cycling and resource use efficiencies of *Paraserianthes falcataria* were intensively studied (Binkley *et al.*, 1992). *Albizia* seedlings fixed >95% of the nitrogen (N) and took up less soil nitrogen (N) than the performance of *Eucalyptus* seedlings. The overall pattern indicated that *Albizia* increased soil nitrogen (N) supply and growth of *Eucalyptus* seedlings, although the higher supply of nitrogen (N) was not enough to remove nitrogen (N) limitation on growth. *Albizia* also reduced the phosphorus (P) supply of the soil regardless of the plantation species. The long-term productivity of tropical plantations depends on continued supplies of nutrients, and these supplies may be strongly influenced by the species selected for plantation use (Awonaike, 1996; Binkley, 1997).

Forage: Forage production of *Albizia procera* was significantly higher than the other species in all seasons. Cell wall components were comparatively higher in *Albizia procera* than the other three species tested (*Albizia lebbeck, Albizia saman* and *Paraserianthes falcataria).* Potential DM (Dry matter) and Nitrogen degradations of *Albizia lebbeck* and *Albizia saman* were significantly higher than *Paraserianthes falcataria* and *Albizia procera.* The results suggest that *Albizia lebbeck* and *Albizia saman* provenances used in this study have relatively higher feed value than *Albizia procera* and *Paraserianthes falcataria* (Larbi *et al.*, 1996).

Wind damage: The major disadvantage of this species is that they are highly prone to wind damage. Their rapid growth creates inherently weak limbs and with shallow often-exposed roots, they are highly susceptible to uprooting and breakage of branches and crowns. The trees possess a massive root system which instead of holding the soil together seem to have exactly the opposite effect. They cause soil erosion and therefore, should be avoided from hillsides (Anon, 1979; !983)

Pests: The bark and foliage of *Albizia* species can be attacked by several insects that cause defoliation and slow tree growth in cultivation. Frequent attacks by caterpillars, monkeys and deer have been experienced in Indonesian plantations located adjacent to rain forests. However, the tree recovers well from

defoliation by sprouting multiple branches if the leader stem is broken (Anon, 1979; 1983). Barking by squirrels, defoliation by *Semiothisa* and *Eurema* larvae and bark feeding by *Arbelid* moth larvae are common occurences. Young plants are browsed by wild animals like elephants, gaur, etc. (Palit, 1980). Harmful insects are *Eurema blanda, Eurema hecabe, Semithise spp., Xystocera festiva* (Wood borer) and *Penthicodes jarinosa* (Anon, 1983).

Diseases: White and brown rots are commonly occurring diseases in the *Albizia falcataria* plantations. These diseases normally follow wind damage. Plantings are attacked by the fungus *Coiticum salmonicolor*, causing " albizia canker" (Anon, 1983)

1.6 Main Objectives

The knowledge of micropropagation in *Paraserianthes falcataria* is very limited, the main objective was to establish a micropropagation *in vitro* technique for the propagation of this species. This was achieved by seed germination, shoot initiation and multiplication, root initiation and plant acclimation studies *in vitro*. The adaptation of these *in vitro* grown cultures were investigated in field conditions. This was achieved by growing the cultures in polypots. The appropriate conditions for germination of *Paraserianthes falcataria* seeds was achieved by altering light, temperature and pre-treatment of seeds. To effectiveness of different media (MS, LP, GD and WPM) supplemented with

cytokinins (Zeatin, BAP, 2iP and Kinetin) for shoot initiation and auxins (IAA,, IBA and NAA) for root initiation from nodal explants of *in vitro* grown seedlings were also investigated. This was achieved by growing explants from seed germination experiments in the above said media with concentrations of cytokinins ranging from 0.1 to 2.5 mgL^{-1}. The same was carried out for root initiation experiments using explants from shoot initiation experiments. Finally, successful transfer of *in vitro* grown rooted microcuttings into the plant acclimation studies were also tested.

CHAPTER 2

IN VITRO GERMINATION

2.1 Introduction

Long generation times and the capacity to change their growth rate and morphology are two main characteristics that make breeding and selecting plant material a difficulty with forest trees (Hartney, 1980). This means that quantitative genetic change as a result of selection will be slower for desirable characteristics and cannot be carried out until the trees are several years old. Both of the mentioned characteristics restrict commercial breeding of forest trees in seed orchards, which is an extremely slow process (Hartney, 1980; Le Roux and Van Staden, 1991a).

Seeds of such forest trees can be germinated using either *in vivo* or *in vitro* conditions. The particular condition a species requires for germination depends largely on the province which itself depends upon environmental conditions (Richards and Beardsell, 1987). The germination of seeds depends upon temperature, light, water availability and seed quality which in turn are influenced by genetic factors (Bonga, 1991). Obviously the viability varies for each species. Fruits when mature releases a mixture of viable seeds and chaff, which includes unfertilized ovules and ovuleds. It is this mixture which makes up

the commercial seed mixtures (Richards and Beardsell, 1987). Such mixtures vary in the percentage of viable seeds they contain, but commonly include approximately around 20% viable seeds. In general, each mature seed pod releases only 2 to 10 seeds capable of developing into plants (Boland *et al.*, 1980).

2.2 Objectives

The aim of this chapter was to ascertain the viability of commercially purchased seeds of *Paraserianthes falcataria* from Multipurpose Trees and Shrubs, Sources of Seeds and Innoculants, Victoria. The best method for sterilisation of the seeds for *in vitro* germination was also investigated.

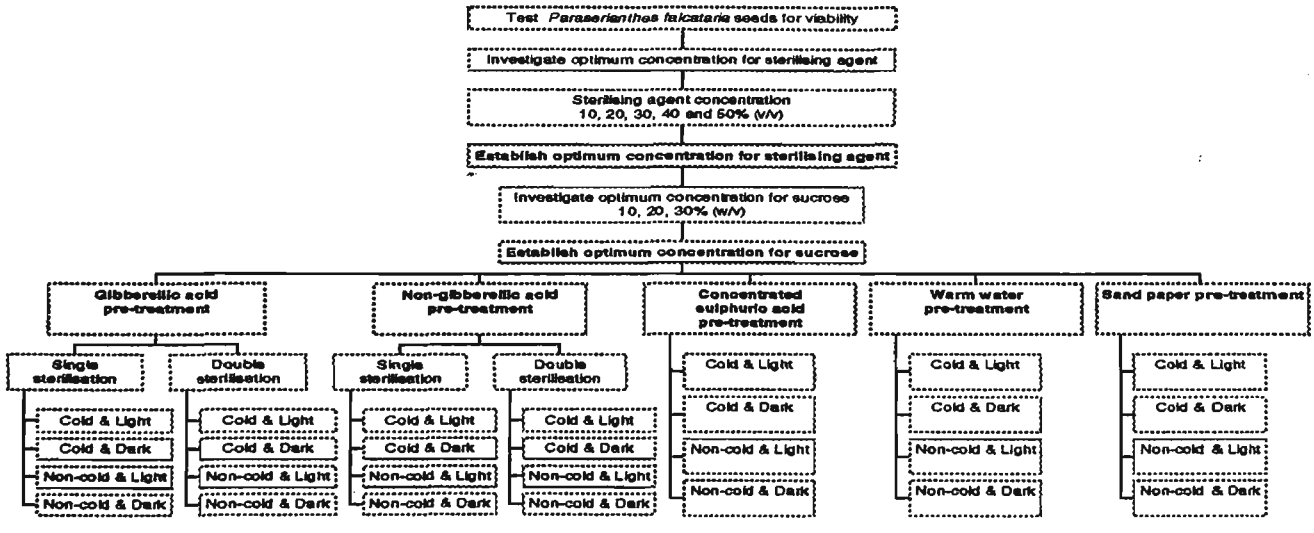

Figure 2.1 Overall experimental design for determination of seed germination in *Paraserianthes falcataria.*

The optimum seed germination percentage was determined by various pre-treatment techniques. Sucrose and direct sowing pre-treatment, Sodium hypochlorite pre-treatment, gibberellic acid and single sterilisation pre-treatment, gibberellic acid and double sterilisation pre-treatment, hot water pre-treatment and sand paper pre-treatment and their influence on *in vitro* seed germination in *Paraserianthes falcataria.* Finally, the optimum conditions for *in vitro* seed germination were investigated in *Paraserianthes falcataria* as outlined in Figure 2.1.

2.3 Requirements for seed germination

Germination of seedlings is initiated by a range of conditions that vary from species to species. It is essential that the conditions are favourable for the endogenous hormones in seeds to trigger growth. Some of these aspects have been discussed as follows. Dormancy of a seed is the failure to germinate as a result of a blockage of the biochemical and physical process which occur during germination (Krishnamoorthy, 1977; Richards and Beardsell, 1987). Dormancy is important in terms of species survival in adverse conditions but in many cases is not desirable, especially when the species is of commercial value such as *Eucalyptus.*

The fracturing of hard seeds is a common occurrence during bush fires and explains the sudden germination of hard seed species after fires. Mechanical

restriction of seed germination by means of physical restriction by the seed coat is thought to be associated with the dormancy of many hard, woody species. The dormancy of *Eucalyptus pauciflora*, and *Eucalyptus delegatensis* seeds is thought to be due to mechanical resistance and the application of gibberellic acid (GA_3) is thought to promote the enzymatic degradation of the hard coat thus allowing germination to take place (Raven *et al.*, 1992). The action of GA_3on *Ruellia humilis* was on the other hand suggested to be centered around its ability to increase the growth and thus expansive ability of the embryo (Longkamp, 1987).

Embryo dormancy is another type of dormancy again which is encountered when excised embryos still fail to germinate despite their removal from adverse conditions. Dormancy breakage may be achieved in many different ways, all of which will depend on the species present (Burza *et al.*, 1994). The mechanisms used include leaching, temperature and or light changes, physical removal of seed coverings and chemical treatments. Various pre-treatments used in other woody species have been listed in Table 2.1.

Table 2.1 Pre-treatment techniques for seed germination in various plant species.

Plant species	Pre-treatments	Author
Eucalyptus pauciflora	GA_3	Bachelard, 1976
Ruellia humilis	GA_3	Baskin and Baskin, 1971
Melia azedarach	GA_3	Domecq, 1988
Eucalyptus polybractea	Sucrose	Yamaguchi *et al.*, 1986
		Damiano *et al.*, 1985
Eucalyptus regnans	Sucrose	Blomstedt *et al.*, 1991
Eucalyptus regnans	Sodium hypochlorite	Blomstedt *et al.*, 1991
Eucalyptus sideroxylon	Sucrose	Cheng *et al.*,1992
Eucalyptus delegatensis	GA_3	Raven *et al.*,1992

Temperature of growth environment: Seasonal fluctuations influence seed germination (Raven *et al.*, 1992). In general, seeds of plants that endure cold winters will not germinate unless there is a period of low temperature, which is usually above freezing (Longkamp, 1987). For instance, incubation at 4^0C for 1-2 weeks may help for species requiring low temperatures as pre requisites for germination. Otherwise germination fails or is much delayed, with the early growth of the seedling often abnormal (Ridgeway and Kingsley, 1989). The ecological significance of the tests would be that species which have seed dormancy mechanisms capable of delaying germination until the cool temperature, winter rainy period of Mediterranean-type climate would be more likely to survive than if germination followed summer rain showers or the first,

intermittent rains of autumn.

Seeds that are referred to as being imbibed often require exposure to cold to break their dormancy (Richards and Beardsell, 1987). The process is ideally performed at between 0 and 50^{0}C for 30 to 100 days and is also known as stratification. The subsequent exposure of stratified seeds to higher temperatures stimulates germination; some alpine species of *Eucalyptus* will only germinate after such treatment (Pryor, 1976). Such germination is also thought to be associated with deep embryo dormancy and will obviously avoid winter germination. For example, *Eucalyptus pauciflora* seeds collected from high altitudes required stratification for germination, while those from lower coastal regimes did not. It has also been shown that seeds which had the outer covering removed did not require as lengthy a cold treatment to break dormancy (Pryor, 1976; Richards and Beardsell, 1987).

Germination conditions however vary with each plant species (Chinearivera, 1995). Many woody species and herbaceous species are said to germinate readily when exposed to a period of cold (Longkamp, 1987). Species native to Western Australia representing a range of plant species (e.g. *Eucalyptus, Allocasuarina, Regelia, Xanthorrhoea, Bossiaea, Acacia, Kennedia, Hovea, Hardenbergia, Paraserianthes)* life-history strategies, fire response syndromes and seed weights were tested for germination. The combinations of constant temperatures of 15 or 23^{0}C, constantly dark or 12hour diurnal white light conditions and with or without addition of gibberellic acid (GA_3), 50 mgL^{-1} were

tested (Bell *et al.*, 1995).

Exposure to light: Survival of seedlings could be enhanced if germination of seed was restricted to the positions protected from high light, higher temperatures and lower soil moisture by the presence of a forest canopy (Bell *et al.*, 1995). Light and its effect on germination involves the interaction of photosensitive sites in the embryonic axis (Boisand and Malcoste, 1970) and also the effect of this on seed coat membrane. Few *Eucalyptus* species have high light requirements for germination (Richards and Beardsell, 1987).

Growth hormones for seed germination: Treatments such as stratification have become unnecessary with associated exposure to GA_3 (e.g. *Corylus avellana*) (Bradbeer, 1988). Dormancy may also be controlled by a balance of growth promoters and growth inhibitors in seeds such as abscissic acid (ABA), shown in Table 2.2 (Ridgeway and Kingsley, 1989).

Table 2.2 Table showing the site of production and method of translocation of seed germination hormones (Ridgeway and Kingsley, 1989).

Hormone	Site of production	Method of translocation
Abscissic acid	Older leaves, root caps and stems	Vascular tissue
Gibberellic acid	Young leaves and shoot, apical meristems, embryos in seed	Unknown

Cytokinins are not as effective in the induction of germination as auxins; Table 2.3 shows the effect of some different combinations of auxins on germination. Gibberellin was first isolated in 1960 in bean seeds. Gibberellins are involved in many normal functions of plants (Table 2.2). They are involved in the germination process in many plant species. Seed embryo gibberellins trigger physiological responses involved in germination (Raven *et al.*, 1992; Salisbury and Ross, 1992).

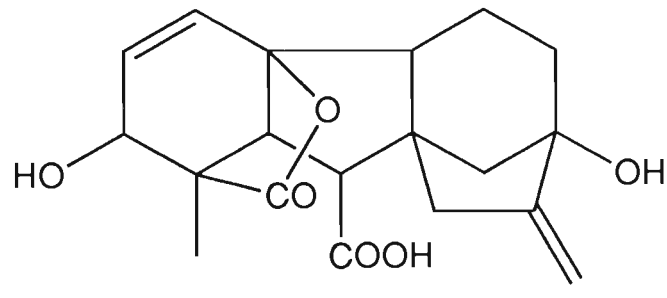

Figure 2.2 Structure of gibberellic acid (Raven *et al.,* 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

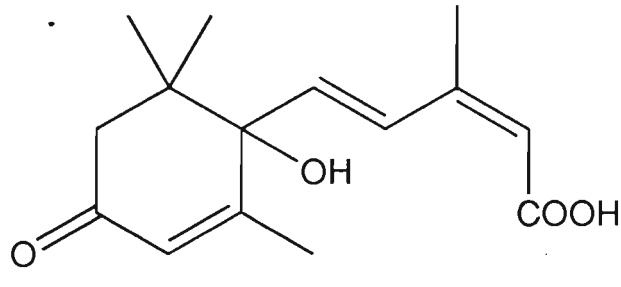

Figure 2.3 Structure of abscissic acid (Raven *et al.,* 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

Gibberellins have a complex chemical structure composed of five rings (Figure 2.2). More than 70 naturally occurring gibberellins have been discovered, they all have the same basic structure but differ slightly in the number of double bonds and in the location of certain chemical groups. These structural differences have pronounced effects on plant growth on some plants, whereas are inactive in others (Delarosaibarra *et al.,* 1994). Some of the interactions between plant hormones during various aspects of plant growth are shown in Table 2.3 (Raven *et al.,* 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

Table 2.3 Physiolgical activity of abscisic acid and gibberellic acid (Raven *et al.,*1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

Physiological activity	Gibberellin	Abscissic acid
Seed germination	Promotes	Inhibits
Growth of seedling into mature plant	Cell Division and elongation	--
Initiation of reproduction (flowering)	Stimulates flowering in some plants	--
Fruit development and ripening	Development	--
Winter dormancy of plants	Breaks	Promotes
Seed dormancy	Breaks	Promotes

The levels of GA_3 present in seeds has been shown to increase during stratification, but there has been no conclusive evidence that GA_3 alone is responsible for the breaking of dormancy (Richards and Beardsell, 1987).

Investigations of the effects of various GA_3 on the germination of barley seeds have shown that the action of the hormone is intricately involved in the stimulation of enzyme production, which provides the embryo with nutrients. This activity is not isolated to action on a single enzyme though as shown in Table 2.4 (Jacobs, 1979, Richards and Beardsell, 1987).

Table 2.4 Khan's model of dormancy and germination depending on the presence or absence of various hormones (Richards and Beardsell, 1987).

Gibberellin "primary"	Cytokinin "permissive"	Inhibitor "preventive"	Outcome
+	+	+	Germination
+	+	-	Germination
+	-	+	Dormancy
+	-	-	Germination
-	-	-	Dormancy
-	-	+	Dormancy
-	+	-	Dormancy
-	+	+	Dormancy

+ = positive effect - = negative effect

Structure of abscissic acid is underlined in Figure 2.3, but was not experimented with in this study. However, abscissic acid was discussed in this section due to the endogenous role played by the hormone in relation to seed dormancy and germination.

Combinations of pre-treatments: Variation in viability and germination percentages were apparent in some cases where more than one provenance was available for testing, indicating that further aspects, such as seed age, maturity collection, storage conditions and depth of seed dormancy, remain to be considered (Bell *et al.*, 1995).

Species such as *Eucalyptus, Allocasuarina, Regelia, Xanthorrhoea, Acacia, Bossiaea, Kennedia, Hovea* and *Hardenbergia* tested in Western Australia that require heat- shock treatment to overcome dormancy due to an impervious testa were pre-treated prior to imposition of temperature, light and GA_3 conditions. The test environmental conditions related to differences between winter and autumn temperatures. For all the species that were tested no combination of temperature, acid or light induced all viable seeds to germinate.

The greatest percentage of germination under any combination of temperature, light and acid treatments was 71% for all species. Large seeds (>10 mg seed weight) had greater germinability. Germination of the dominant tree representatives namely *Eucalyptus* was indifferent to trial conditions of temperature, light and germination, except for *Eucalyptus marginata.* GA_3 also overcame the inhibition resulting from exposure to light in some understorey species (e.g. *Allocasuarina, Regelia,* and *Xanthorrhoea*). Germination of many hard-seeded, understorey shrub and herbaceous perennial species, especially those with small (< 10 mg) seeds (e.g. *Bossiaea* and *Acacia*) was greater at the

lower trial temperature and in the dark. Some large (> 10 mg) seeded, understorey species (e.g. *Acacia, Kennedia, Hovea and Hardenbergia*) germinated in high percentages in both temperatures, but maximum germination percentages still tended to be at 15^{0}C. According to Palit (1980), the best germination of *Paraserianthes falcataria* seeds occur after treatment with sulphuric acid for 12 minutes. Concentrated sulphuric acids soften up the seed coat by dissolving waxy substances that impede entry of water (Raven *et al.,* 1992). Seed pre-treatments were carried out on the seeds of *Bowdichia virgilioides* belonging to the family Leguminosae with immersion in 98% (v/v) sulphuric acid for a range of times beginning at 1 to 20 minutes which showed the highest germination rate at 5 and 10 minutes (Deandrade *et al.,* 1997). Further treatment with boiling water for 15 minutes can also increase the strike rate of *Paraserianthes falcataria* seeds (Raven *et al.,* 1992). Dendrade *et al.* (1997), experimented with water at 100^{0}C for 1, 2 and 3 minutes and 30^{0}C water for 24 hours.

2.4 Seeds

The seeds of *Paraserianthes falcataria* are about 1 to 1.5 cm in length and about 1 cm in width. The most viable seeds weigh about 40 mg. Seeds are inexpensive, easy to transport and store and may be kept for long periods and produce plants that are disease free (Chinearivera, 1995; Palit, 1980). The seeds have a thick seed coat, which are very common with legumes (Raven *et*

al., 1992). The major disadvantages are the long time required for new plants to develop and the possibility of the heterozygote (dominant and recessive allele) seed possessing unwanted, recessive characteristics not apparent in the parent plants (Bonga, 1991; Murashige, 1978).

Seeds in Philippines average 50 mg in weight and usually germinate within 4 to 5 days in the field, being complete within 15 to 30 days, the actual period varies with the time of sowing. Experiments conducted have shown that sowing in April is the best. The seeds are dark brown in colour with a hard seed coat (Anon, 1979; 1983; Palit, 1980). The seed coat helps primarily in withstanding adverse environmental conditions like high temperatures or forest fires. Therefore, seed germination poses a problem in species with hard seed coats due to the inability of water and oxygen intake. In order to overcome adverse environmental conditions affecting plants growing in their natural habitat and to trigger germination by breaking down dormancy various pre-treatments have been conducted with as shown in Table 2.5.

Table.2.5 Summary of conditions for seed germination techniques for pre-treatment of seeds to aid in the breakage of dormancy.

Techniques for seed pre- treatment	Authors
Viability testing	Anon, 1976
Direct sowing	Palit, 1980
	Lorilla, 1992
Sodium or Calcium hypochlorite	Blomstedt *et al.*, 1991
Gibberellic acid	Raven *et al.*, 1992
	Bradbeer, 1988
Concentrated sulphuric acid	Palit, 1980
	Dendrade *et al.*, 1997
	Bell *et al.*, 1995
Temperature (15 - 23 ^{0}C)	Ridgeway and Kingsley, 1989
	Longkamp, 1987
Hot water (90^{0}c – 100^{0}C)	Palit, 1980
Exposure to light	Ridgeway and Kingsley, 1989
	Raven *et al.*, 1992
Mechanical scarification	Palit, 1980
Incubation (4 ^{0}C)	Ridgeway and Kingsley, 1989
	Longkamp. 1987

Seeds of *Albizia* species require certain pre-treatments. The pre-treatment depends on the species that is to be micropropagated. Some of the pre-treatments for each species are listed in Table 2.6.

Table 2.6 Summary of pre-treatment techniques conducted for seed germination in *Albizia* species (Caulovitz, 1995).

Species name	Number of seeds per kg	Usual germination rate in %	Pre-treatment applied
Albizia adiantifolia (Schum) W.Wight	24000 – 40000	30 - 70	N
Albizia amara (Roxb.)Boivin spp.sericocephala (Benth.)Brenan	9500 - 18000	30 - 70	N
Albizia carbonaria Britton	24000 - 40000	25 - 65	N
Albizia chinensis (Osbeck) Merr. (syn. *A.stipulata*)	21300 - 36000	50 - 85	N, CW3
Albizia falcataria (L.) Fosb. (syn. *A.moluccana)*	40900 - 50000	60 - 90	N,HW1,SC1,BW1
Albizia guachapele (Kunth) Dugand	20000 - 22000	95	N
Albizia julibrissin Durazz	20000 - 28600	70 - 100	N
Albizia lebbeck (L.) Benth	6000 - 16000	50 - 95	N,A1,A2,CW1,CW3, BW1,BW
Albizia lopantha (Willd.) Benth.	10600 - 14000	60	N
Albizia odoratissima (L.f.) Benth	8700 - 23700	30 - 70	CW1

A1: Soak in Conc. H_2SO_4 for 5-15 minutes; A2: Soak in Conc. H_2SO_4 for 20-30 minutes; BW: Immerse in boiling water; BW1 - BW: remove from fire and allow to soak for 1minute; CW1: Soak in cold/tepid water for 24 hours; CW3: Soak in cold/tepid water for 48 hours; HW1: Soak in hot water for 12 hours; N: none; SC1: Remove part of seed coat.

2.5 Methods and Techniques

2.5.1 Sterilisation of equipment and materials

Sterilisation of instruments, transfer areas were sterilised using either methylated spirits or 40% ethanol (carried out in the laminar flow). Nutrient media and distilled water were sterilised by autoclaving at 1.05 $kgcm^{-2}$ and $120^{0}C$ for 20 minutes.

2.5.2 Preparation of germination basal media

The basal media MS (Murashige and Skoog, 1962) (Table 2.7) half strength was utilized for seed germination studies. Half strength MS micro and macro salts supplemented with 30 gL^{-1} sucrose (Sigme, U.S.A) and 3.2 gL^{-1} phytagel (Sigma, U.S.A) was prepared. The pH of the media was maintained at 5.6 by adding 0.1 M KOH or HCl and media was hormone free.

One litre half strength Murashige and Skoog Basal medium (1962) was prepared for 100 seeds. 10 ml of prepared stock (Major, minor, vitamins) solutions (10x)(Sigma, U.S.A) was added to the volumetric flask (the contents of each stock solutions are shown in Table 2.7). 30 gL^{-1} of sucrose was dissolved and sterile distilled water was added upto the 1 litre mark. The pH of the solution was adjusted to 5.6.10 ml of the dissolved solution was poured into 50 ml plastic

tissue culture tubes and then autoclaved for 20 minutes at 1.05 kgcm^{-2} and 120^{0}C. The tubes were allowed to cool and solidify before they were stored in the cold room (4^{0}C) to be taken out for further use.

Table 2.7 MS medium (half strength) for plant tissue culture (Murashige and Skoog Medium 1962)

Solution	Ingredients	Formulae	MgL^{-1}
Major salts	Ammonium Nitrate	NH_4NO_3	825
	Potassium Nitrate	KNO_3	950
	Magnesium sulphate	$MgSO_4.7H_2O$	185
	Calcium nitrate	$Ca(NO_3)_2.4H_2O$	220
	Potassium Hydrogen Sulphate	KH_2PO_4	85
Minor salts	Sodium EDTA	Na_2 EDTA	19
	Ferrous sulphate	$FeSO_4.7H_2O$	14
	Cobalt chloride	$CoCl_2.6H_2O$	0.013
	Copper sulphate	$CUSO_4.H_2O$	0.013
	Sodium Molybdate	$NaMoO_4.H_2O$	0.13
	Boric acid	H_3BO_3	3.1
	Potassium Iodide	KI	0.4
	Manganese sulphate	$MnSO_4.4H_2O$	11.2
	Zinc sulphate	$ZnSO_4.7H_2O$	4.3
Vitamins	Inositol		50
	Nicotinic acid		0.25
	Thiamine HCl		0.05
	Pyridoxine HCl		0.25
	Glycine		1.0

2.5.3 Viability testing and seed separation

The seeds of *Paraserianthes falcataria* were subjected to experiments to test the viability of the seeds by immersing the seeds in 1% (v/v) aqueous solution of 2,3,5 - triphenyl - tetrazolium - chloride (pH 6.5 - 7.0) and maintained at a temperature of 30^{0}C in complete darkness for 24 hours. At the end of the period tetrazolium solution is decanted, the seeds are rinsed with water, spread on a petri dish and observed under the light microscope (Olympus) while still wet (Anon, 1976).

In the biochemical test for viability, the evidence of the reduction process which takes place in living cells is provided by the reduction of an indicator. The indicator used is a colourless solution of 2,3,5 - triphenyl - tetrazolium - chloride salt imbibed by the seed. Within the seed tissues the indicator interferes with the reduction process of living cells and accepts hydrogen from the dehydrogenases. By hydrogenation of,3,5 - triphenyl - tetrazolium - chloride a red, stable and non-diffusable substance (triphenyl - formazan) is produced in living cells. This makes it possible to distinguish the red coloured living parts of the seeds to the colourless dead ones. In addition to completely stained viable seeds and completely unstained non-viable seeds, partially stained seeds may occur. The position and size of the necrotic areas in the embryo and/or endosperm determine whether such seeds are classified as viable or non - viable (Anon, 1976). Such partially stained seeds were omitted from the

experiments in this study. Seeds from seed banks that showed a viability of 50% or more were chosen for this study.

2.5.4 Sodium hypochlorite single sterilisation pre- treatment

100 viable (Section 2.5.3) seeds per treatment were single sterilised in 10, 20, 30, 40 and 50% (v/v) sodium hypochlorite (40 gL^{-1} available chlorine present as sodium hypochlorite, with 9 gL^{-1} sodium hydroxide) at 25^{0}C for 30 minutes in a sterile laminar flow cabinet. The seeds were rinsed three times in sterile distilled water to remove all traces of the bleach. 100 seeds were placed in a 50 ml glass vials along with sodium hypochlorite (10, 20, 30, 40 and 50% (v/v)), plus 2-3 drops of Tween 80 (which acts as a wetting agent). The seeds were rinsed thrice in sterile distilled water and planted in 50 ml tissue culture tubes which contain half strength germination medium (30 gL^{-1} sucrose, 3.2 gL^{-1} phytagel, half strength MS basal media (Table 2.7)). The seeds were then placed in germination conditions of cold, non-cold, light and dark (Table 2.8) and were checked daily for germination and results were tabulated. The experiment was repeated three times with 100 seeds per treatment.

Table 2.8 Conditions of growth for *Paraserianthes falcataria.*

Conditions	Optimum
Temperature (Non-cold)	25°C Day - 18°C Night
Temperature (Cold)	4^{0}C
Light	16h photoperiod ($29Wm^{-2}$)
Light Intensity	Top shelf 40 $\mu Em^{-2}sec^{-1}$
	Bottom shelf 30 $\mu Em^{-2}sec^{-1}$

2.5.5 Direct sowing and different levels of sucrose treatment

Carbon source is an important constituent for active growth of an explant and the variation in the amount of carbon provided can alter the morphogenesis of the explant. Therefore, it was important to identify the optimum amount of carbon that is required for each species. 100 viable seeds per treatment (Section 2.5.3) of *Paraserianthes falcataria* were mildly sterilised in sodium hypochlorite (10% (v/v)) for 15 minutes and rinsed three times in sterile distilled water and planted in MS half strength basal media (Section 2.5.2) supplemented with varying concentrations of sucrose (1, 2 and 3% (w/v)), 3.2 gL^{-1} phytagel. The seeds were placed under controlled conditions of cold, non-cold, light and dark as shown in Table 2.8. The experiments were performed three times with 100 seeds per treatment .The results were recorded.

2.5.6 Sodium hypochlorite and double sterilisation pre-treatment

100 viable (Section 2.5.3) seeds were double sterilised, the seeds are first single sterilised (30% (v/v)), rinsed in sterile distilled water, then incubated on petri plates (containing 4 gL^{-1} bacterial agar) at 37^{0} C for 4 hours. This enables the growth of contaminants such as bacteria and fungi. Seeds were treated again with 10% (v/v) sodium hypochlorite and thoroughly rinsed three times in sterile distilled water and planted on germination basal media in a laminar flow cabinet. The seeds were placed in conditions of cold, non-cold, light and dark (Table 2.8) and checked daily for any signs of germination.

2.5.7 Gibberellic acid and single sterilisation pre- treatment

100 viable (Section 2.5.3) seeds were treated with gibberellic acid for 12, 18 and 24 hours. The seeds were then single sterilised with the optimum bleach concentration obtained from previous sodium hypochlorite pre-treatment (Section 2.5.4). The seeds were then rinsed three times in sterile distilled water and planted in half strength MS basal media (Section 2.5.2)and placed under conditions of light, dark, cold and non-cold (Table 2.8).

2.5.8 Gibberellic acid and double sterilisation pre-treatment

100 viable (Section 2.5.3) seeds were treated with gibberellic acid for 12, 18 and

24 hours. The seeds were then single sterilised with the optimum bleach concentration obtained from previous sodium hypochlorite and double sterilisation pre-treatment (Section 2.5.4). The seeds were then rinsed three times in sterile distilled water and incubated at 37^{0}C for 4 hours as indicated in Section 2.5.6. The seeds were further sterilised in 10% bleach (v/v), rinsed three times in sterile distilled water and planted in half strength MS basal media (Section 2.5.2).

2.5.9 Concentrated sulphuric acid pre- treatment

100 viable (Section 2.5.3) seeds were treated with 20, 40, 60 and 80% (v/v) concentrated sulphuric acid. Seeds were sterilised with optimum bleach concentration (v/v) (Section 2.5.4) and planted in half strength MS basal medium (Section 2.5.2).

2.5.10. Warm water pre-treatment

100 viable (Section 2.5.3) seeds were pre-treated in warm water ranging in temperature of 60, 70, 80 and 90^{0}C. The seeds were left for three minutes in the warm water and sterilised with optimum bleach concentration (Section 2.5.4) and planted in half strength MS basal medium (Section 2.5.2).

2.5.11 Sand Paper pre-treatment

100 viable (Section 2.5.3) seeds were given mild abrasive treatment with sand paper for 15 minutes and then sterilised with optimum bleach concentration (Section 2.5.4) and planted in half strength MS basal medium (Section 2.5.2).

2.5.12 Shoot initiation and maintenance

Seedlings were observed daily for germination, any contaminated seedlings were promptly removed and replaced to maintain the number of 100 seeds per treatment. Seedlings that had reached 5 cms or over in height were removed for subculture and shoot initiation experiments.

2.6 Statistical Analysis

Seed germination percentage calculated based on biostatistical analysis of Zar (1984) and ANOVA (Version '95) Systat package.

2.7 Results

In general, percentage germination was highest with seeds exposed to conditions of non-cold and dark for all pre-treatments. The first emergences of plumule and radicle were observed approximately within 2 - 3 days after planting

in non-cold and dark culture conditions. The seeds that were subjected to various pre-treatments but that which failed to germinate in a week did not germinate even after a month and were discarded. Error bars have been included in the diagrams. Due to the error being between 0.01 and 0.03, the bars are not visible in the graph.

2.7.1. Sodium hypochlorite single sterilisation pre-treatment

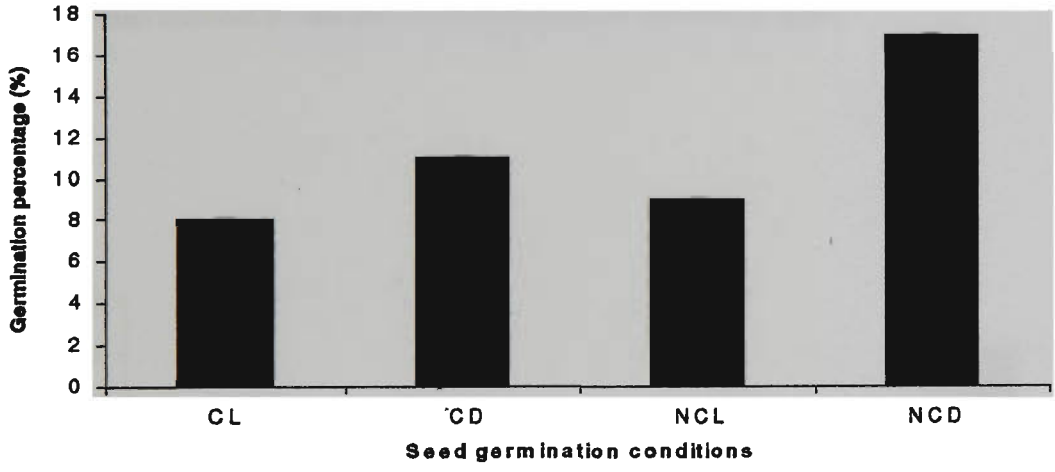

Figure 2.4 The effect of 10% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times

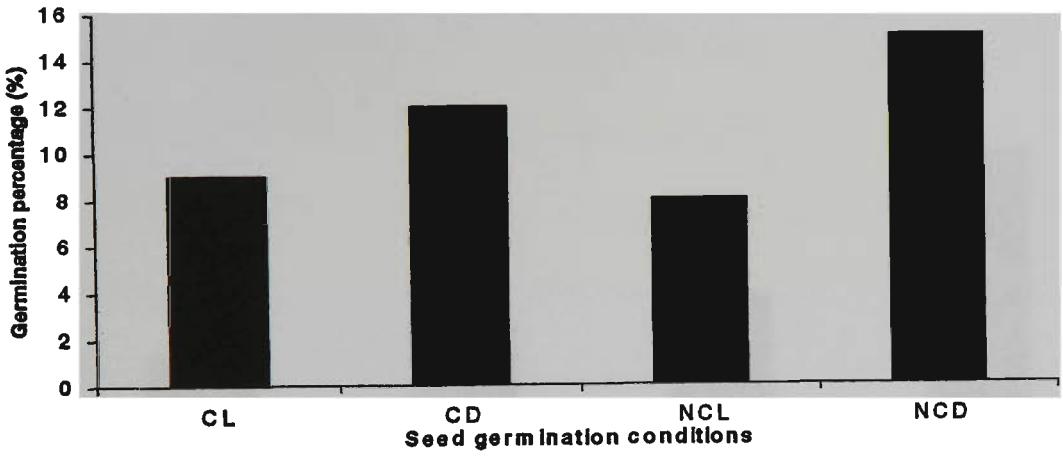

Figure 2.5 The effect of 20% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

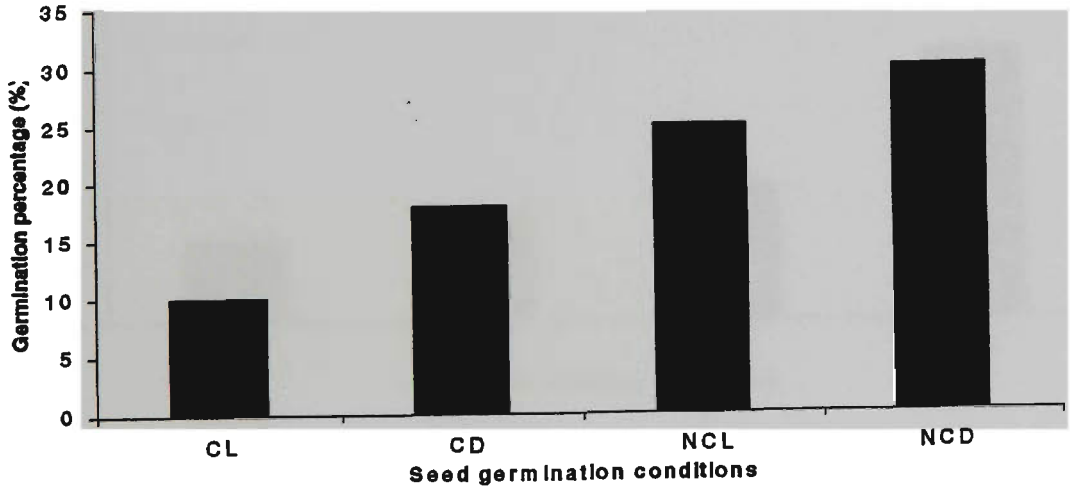

Figure 2.6 The effect of 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

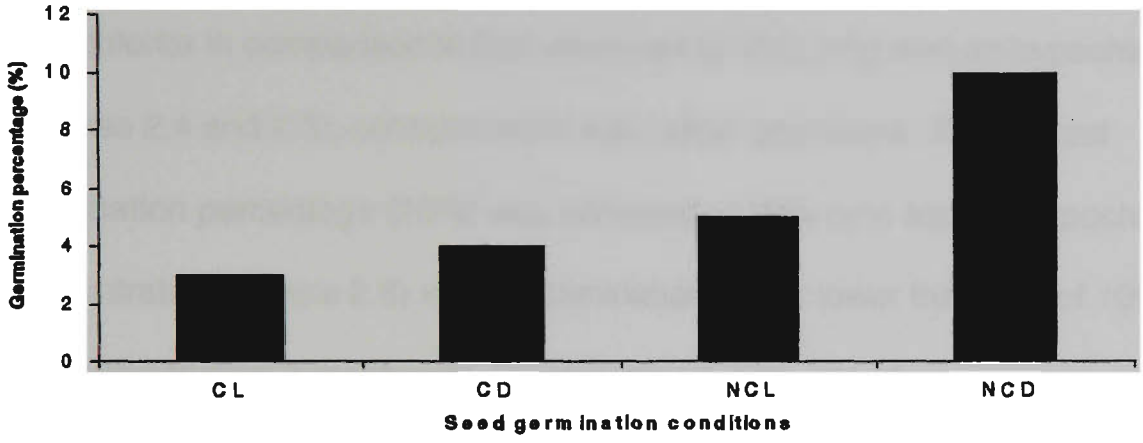

Figure 2.7 The effect of 40% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

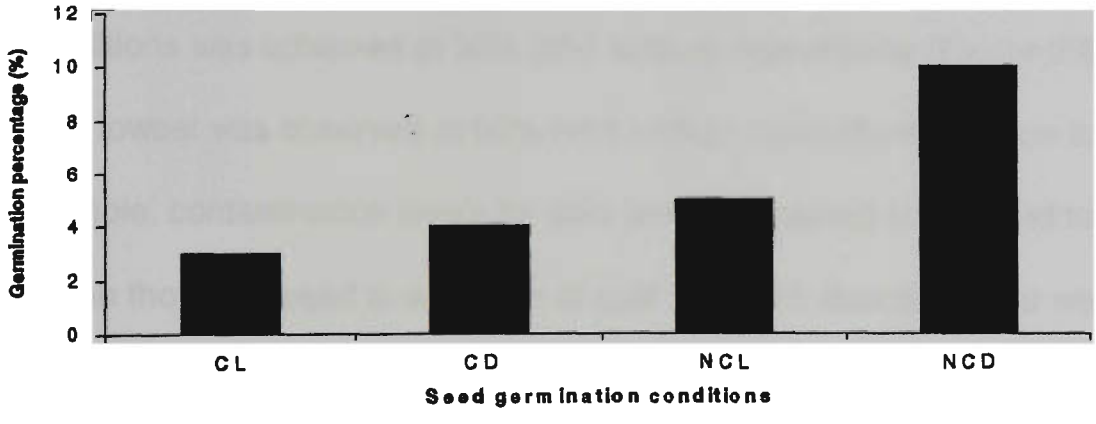

Figure 2.8 The effect of 50% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

Excessive contamination was observed at 10% (v/v) sodium hypochlorite in cold and light conditions with a germination rate of only 8% (Figure 2.4). Although the percentage of germination was slightly higher (9%) at 20% (v/v) sodium hypochlorite in comparison to that observed at 10% (v/v) sodium hypochlorite (Figures 2.4 and 2.5), contamination was rather prominent .The highest germination percentage (10%) was achieved at 30% (v/v) sodium hypochlorite concentration (Figure 2.6) with contamination levels lower than that of 10% and 20% (v/v) sodium hypochlorite concentrations. The rate of germination dropped to 5% (Figure 2.7) at 40% (v/v) sodium hypochlorite and this concentration was deemed excessive although the contamination levels were minimal. 50% (v/v) sodium hypochlorite concentration proved detrimental to the seeds with germination percentage as low as 3% (Figure 2.8). However, least contamination was observed at 50% concentration in cold and light conditions. Similar to cold and light conditions the highest percentage of germination (18%) in cold and dark conditions was achieved at 30% (v/v) sodium hypochlorite (Figure 2.6) whilst the lowest was observed at 50% (v/v) sodium hypochlorite (Figure 2.8). On the whole, contamination levels for cold and dark treated seeds tend to be higher than those exposed to condition of cold and light. Seedlings that were grown from seeds exposed to dark conditions showed abnormal elongation of the internodes. Non-cold and light treatments resulted in a much higher rate (25%) of seed germination was again achieved at 30% (v/v) sodium hypochlorite concentration (Figure 2.6). The seedlings that were subjected to light treatment resulted in small but thick stems and spreading leaves due to the availability of

light contrary to that observed with dark treatments. Non-cold conditions for both light and dark proved to be beneficial for the seeds. Plants grown in the dark were found to have long slender stems reaching upwards against gravity in their bid to reach light for photosynthesis. The highest strike rate of 30% in non-cold and dark conditions was achieved at 30% (v/v) sodium hypochlorite concentration (Figure 2.6).

2.7.2 Sodium hypochlorite and double sterilisation pre-treatment

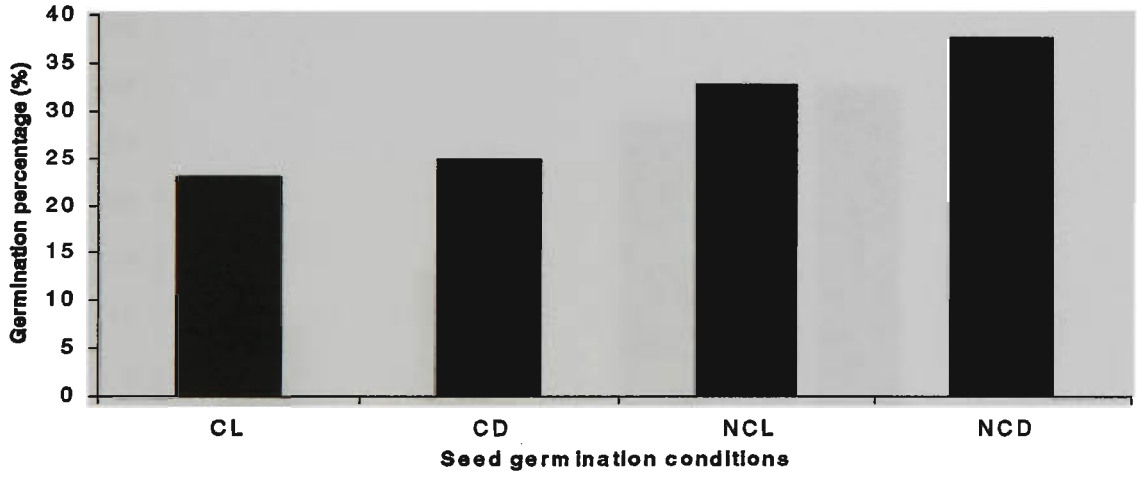

Figure 2.9 The effect of double sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

The results obtained from double sterilisation pre-treatments were comparitively better than single sterilisation for all conditions tested. The lowest germination

percentage (23%) was observed when the seeds were subjected to cold and light treatment (Figure 2.9), while, the highest percentage (38%) was attained with conditions of non-cold and dark treatment (Figure2.9). Moreover, the level of contamination was relatively lower in comparison to single sterilisation pre-treatments. Seeds subjected to cold and dark treatment attained the germination strike rate of 25% whilst the non-cold and light treatment had a relatively higher strike rate of 33% (Figure 2.9).

2.7.3 Direct sowing and sucrose pre-treatment

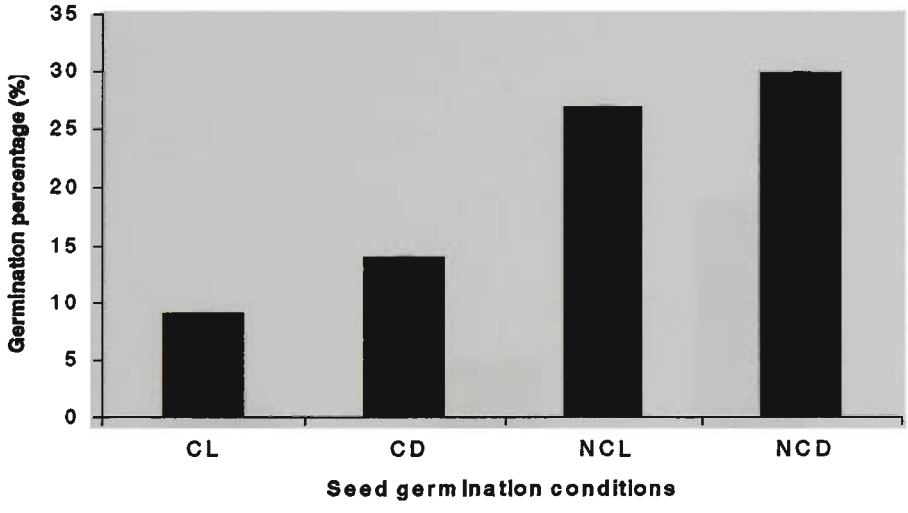

Figure 2.10 The effect of Direct sowing and 1% (w/v) sucrose pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

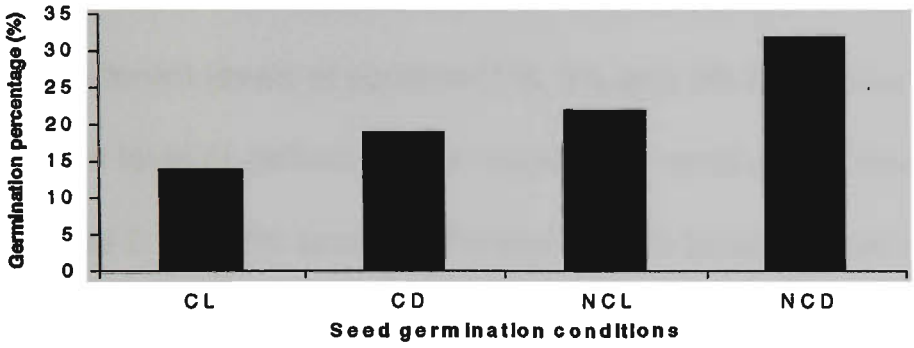

Figure 2.11 The effect of Direct sowing and 2% (w/v) sucrose pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

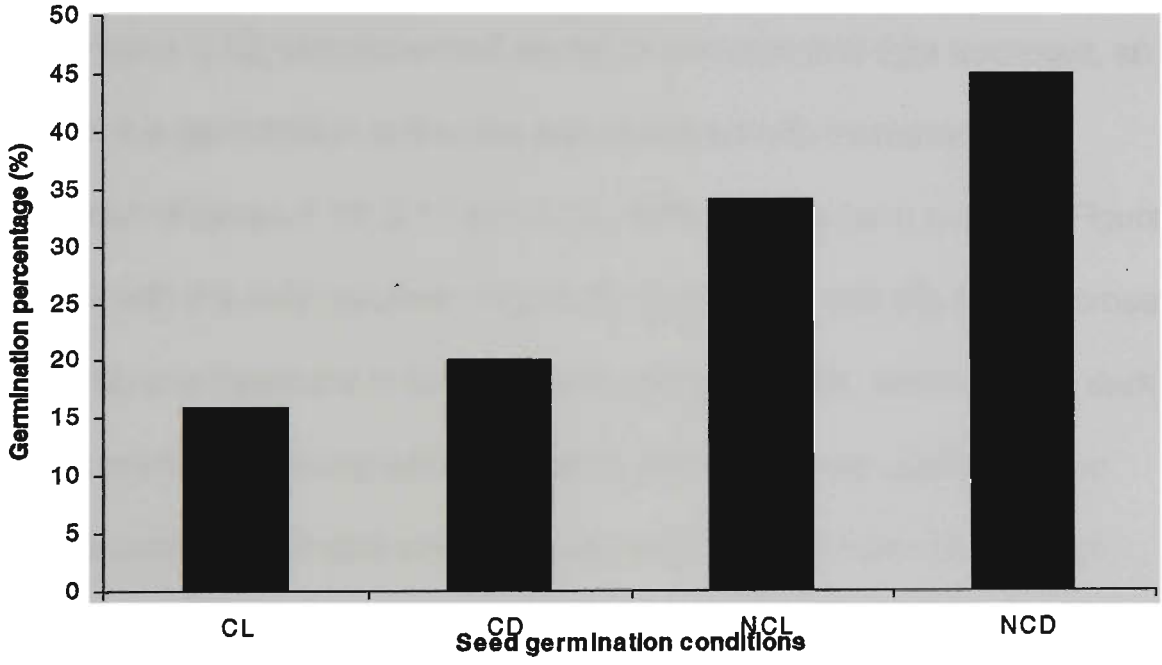

Figure 2.12 The effect of direct sowing and 3% (w/v) sucrose pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

In the following method seeds were mildly sterilised with 20% (v/v) sodium hypochlorite for 15 minutes and sown directly into half strength MS media supplemented with 10%, 20% and 30% (w/v) sucrose. Direct sowing of seeds on three different levels of sucrose (1%, 2% and 3% (w/v)) was aimed to obtain the optimum level of carbon source required for seed germination (Figures 2.10, 2.11 and 2.12). The seeds of *Paraserianthes falcataria* had a germination strike rate of 9% with 1% (w/v) sucrose (Figure 2.10), 14% germination with 2% (w/v) sucrose (Figure 2.11). A slightly higher germination percentage of 16% in 3% (w/v) sucrose (Figure 2.12) supplemented half strength MS media in cold and light conditions. However, in the cold and dark treatment a higher germination rate was observed with a germination strike rate of 14% at 1% (w/v) sucrose (Figure 2.10), 19% at 2% (w/v) sucrose (Figure 2.11) and 2% at 3% (w/v) sucrose (Figure 2.12) supplemented media. In non-cold and light treatment, an increase in the germination strike rate was observed with increasing concentration (Figures 2.10, 2.11 and 2.12). 27% with 1% (w/v) sucrose (Figure 2.10), 22% with 2% (w/v) sucrose (Figure 2.11) and 35% with 3% (w/v) sucrose (Figure 2.12) pre-treatment in non-cold and light conditions. Non-cold and dark conditions produced the highest germination percentage with comparison to other three conditions of cold and light, cold and dark and non-cold and light conditions with all three sucrose concentrations (1,2 and 3% (w/v)). 30% (Figure 2.10), 32% (Figure 2.11) and 45% (Figure 2.12) germination was observed with 1, 2 and 3% (w/v) sucrose concentrations respectively.

2.7.4 Gibberellic acid and single sterilisation pre-treatment

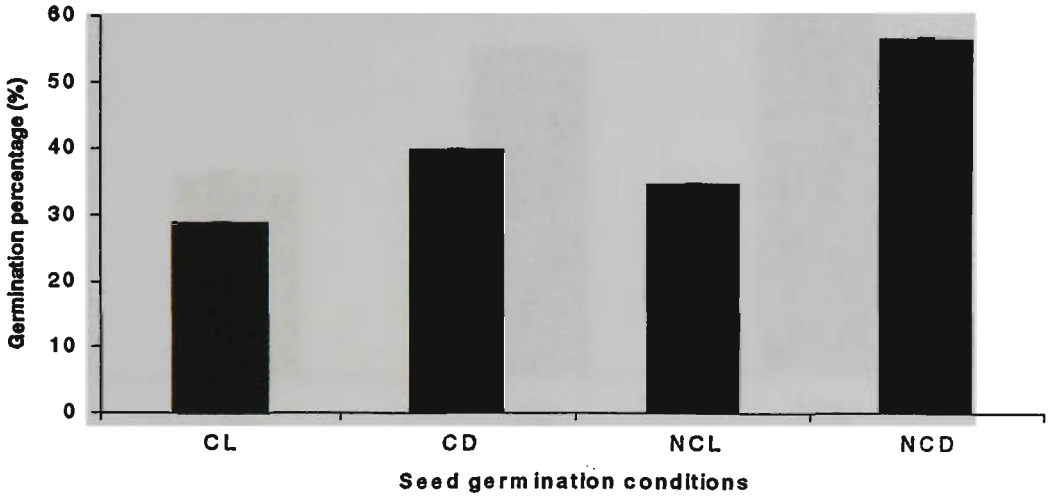

Figure 2.13 The effect of 12 hour gibberellic acid and 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

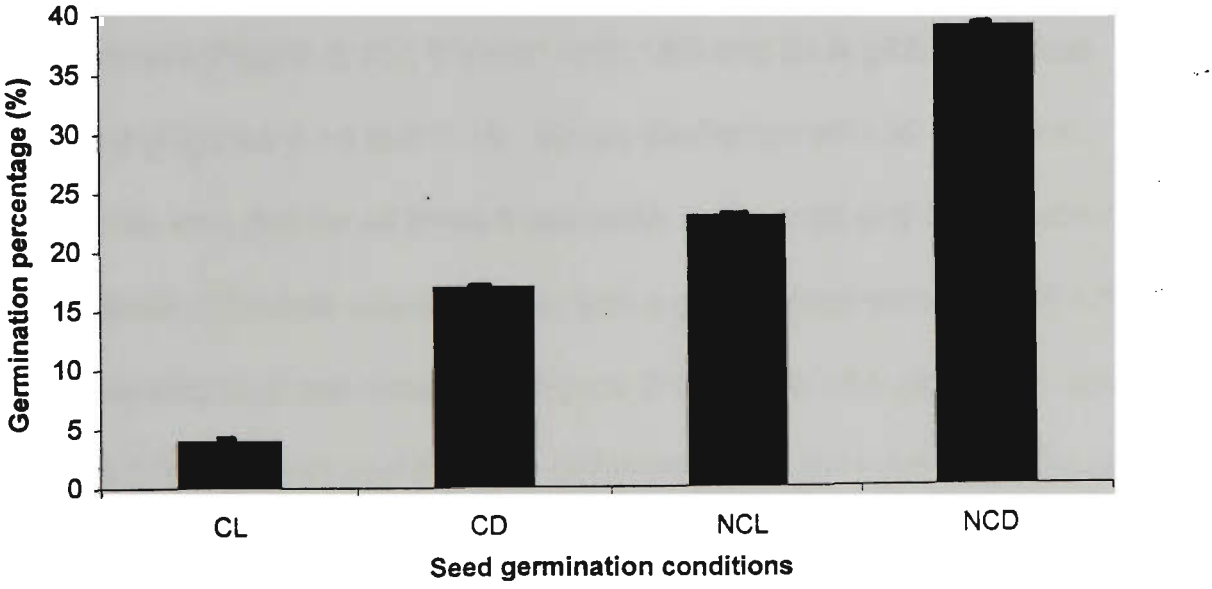

Figure 2.14 The effect of 18hour gibberellic acid and 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

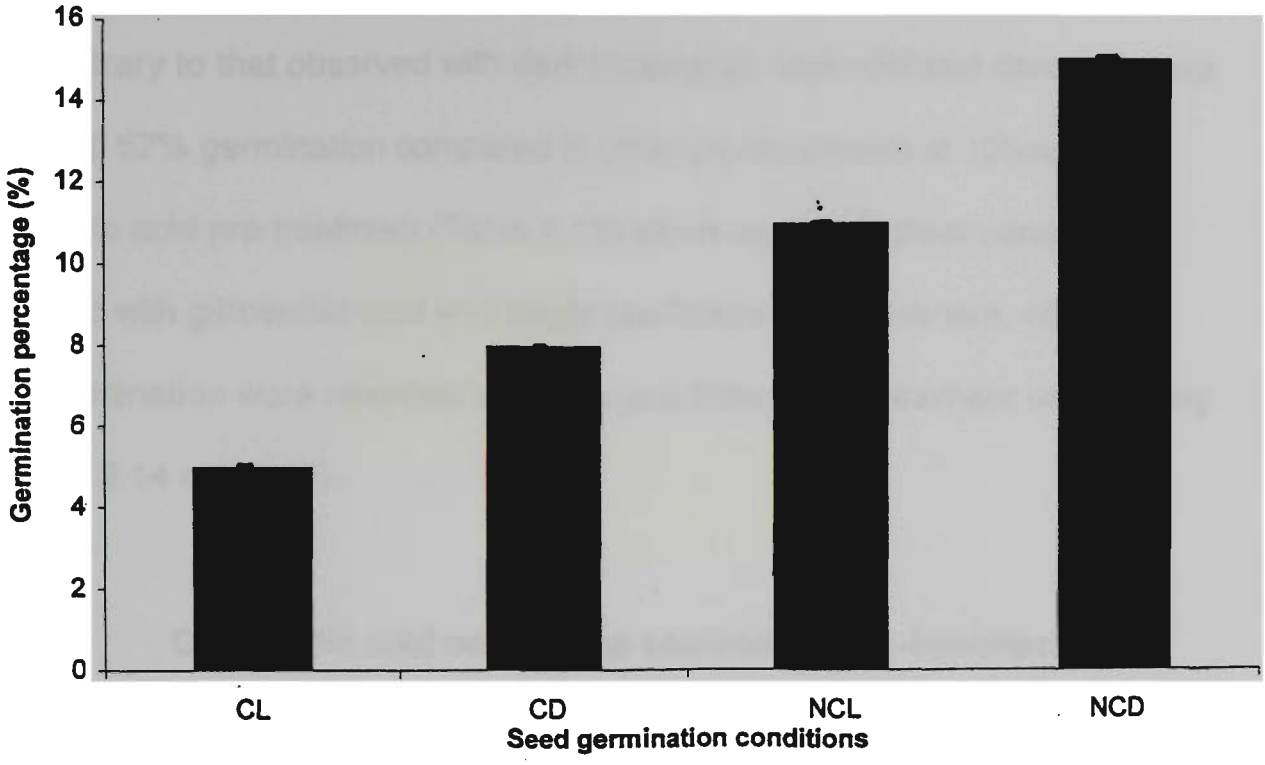

Figure 2.15 The effect of 24hour gibberellic acid and 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

30% growth was achieved with cold and light treatments with 12 h Gibberellic acid treatment (Figure 2.13), 5% with both 18 h and 24 h gibberellic acid treatments (Figures 2.14 and 2.15). Single steriliation with 30 % sodium hypochlorite was use for all three treatments. In the cold and dark treatment a higher germination rate was observed with a germination strike rate of 42% with 12 h gibberellic acid pre-treatment (Figure 2.13), 18% 18 h gibberellic acid pre-treatment (Figure 2.14) and 8% 24 h gibberellic acid pre-treatment (Figure 2.15) supplemented media. Non-cold and light treatments resulted in a much higher rate (11%) of seed germination which was achieved at 24 hour Gibberellic acid pre-treatment (Figure 2.15). The seedlings that were subjected to light treatment resulted in small but thick stems and spreading leaves due to the availability of

light contrary to that observed with dark treatments. Non-cold and dark treatment produced 57% germination compared to other pre-treatments at 12hour gibberellic acid pre-treatment (Table 2.13) which was the highest percentage observed with gibberellic acid and single sterilisation pre-treatment. 40% and 16% germination were recorded with 18 h and 24hour pre-treatment respectively (Figures 2.14 and 2.15).

2.7.5 Gibberellic acid and double sterilisation pre-treatment

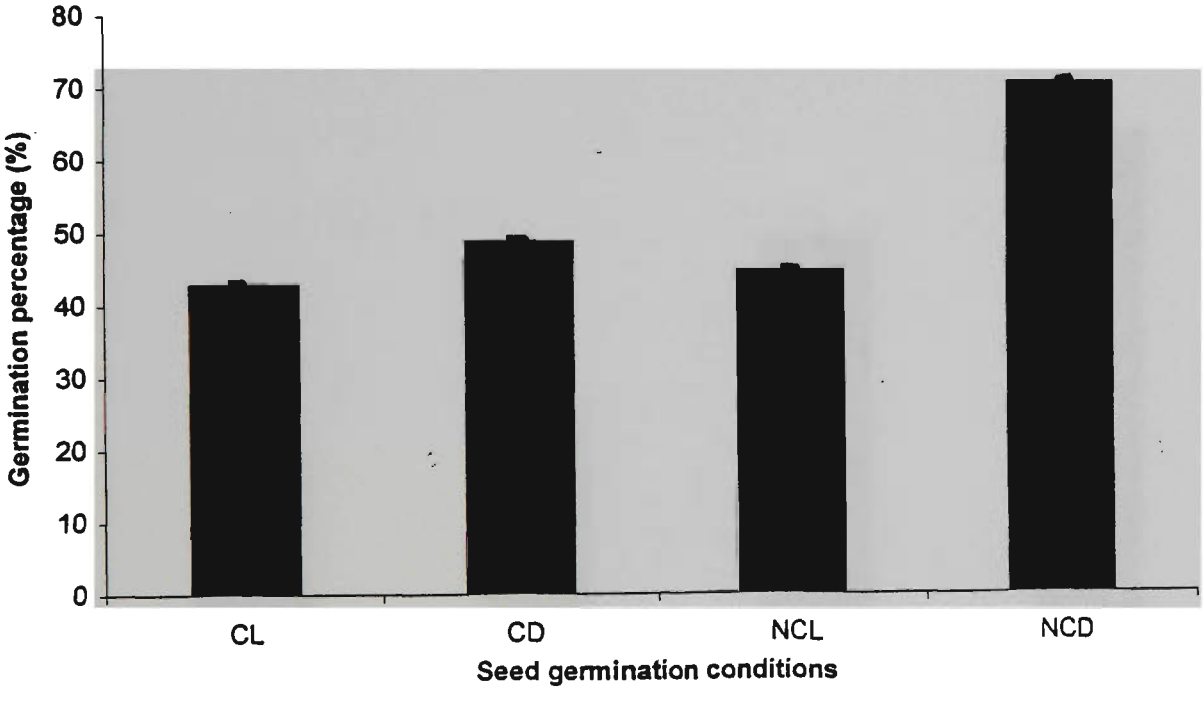

Figure 2.16 The effect of 12hour gibberellic acid and double sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

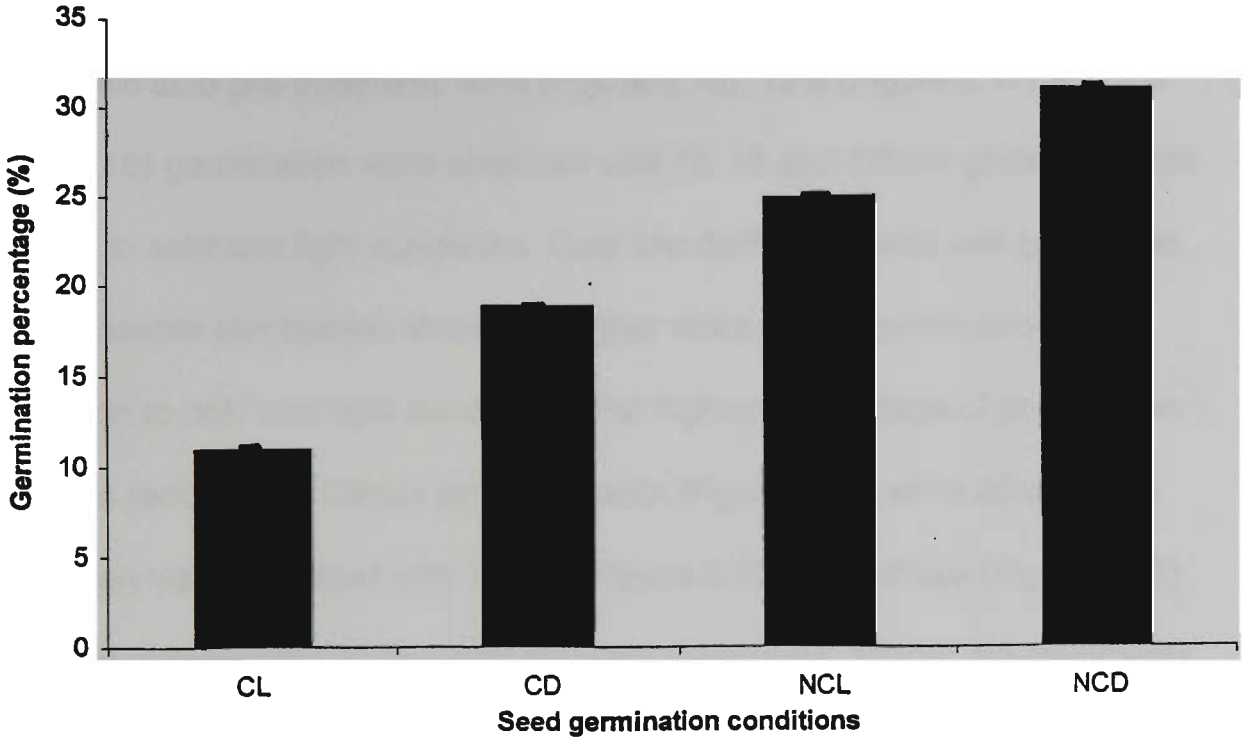

Figure 2.17 The effect of 18hour gibberellic acid and double sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

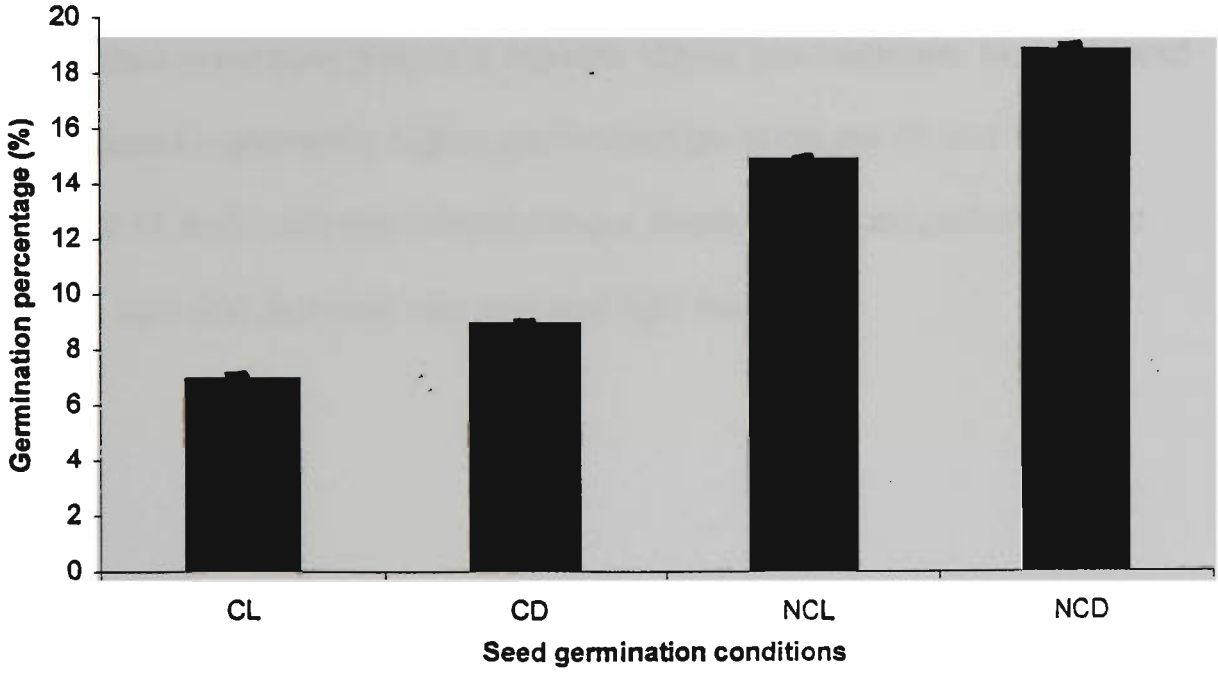

Figure 2.18 The effect of 24hour gibberellic acid and double sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria.* Each experiment contained 25 replicates per experiment and was repeated four times.

71% of seeds germinated under non-cold and dark treatment (Table 2.16) in 12

h gibberellic acid pre-treatment. 45% (Figure 2.16), 12% (Figure 2.17) and 7% (Figure 2.18) germination were observed with 12, 18 and 24hour gibberellic acid treatment in cold and light conditions. Cold and dark treatments with gibberellic acid and double sterilisation showed a higher strike rate of germination in comparison to cold and light conditions. The highest percentage of germiantion (50%) was recorded at 12hour pre-treatments (Figure 2.16) while 20 and 10% germination were tabulated with 18hour (Figure 2.17) and 24hour (Figure 2.18) treatments respectively. In non-cold and light treatments, 12hour pre-treatments with gibberellic acid and double sterilisation produced the highest (40%) germination percentage (Figure 2.16). Similar to gibberellic acid and single sterilisation pre-treatment (70%) germination percentage was observed with non-cold and dark conditions (Figure 2.16) with 12hour pre-treatment. Non-cold and dark produced significantly higher germination percentages 32 and 18% (Figures 2.17 and 2.18) with 18 and 24hour treatments in comparison to cold and light, cold and dark and non-cold and light conditions.

2.7.6 Concentrated Sulphuric acid pre-treatment

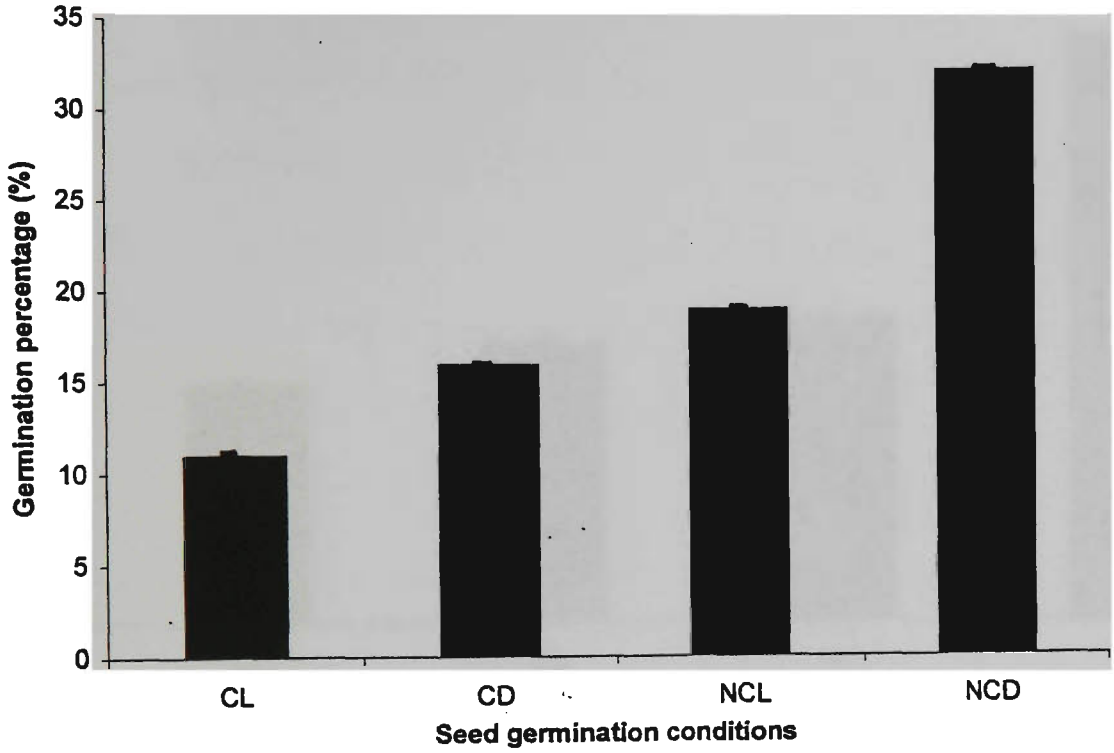

Figure 2.19 The effect of 20% (v/v) concentrated sulphuric acid pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

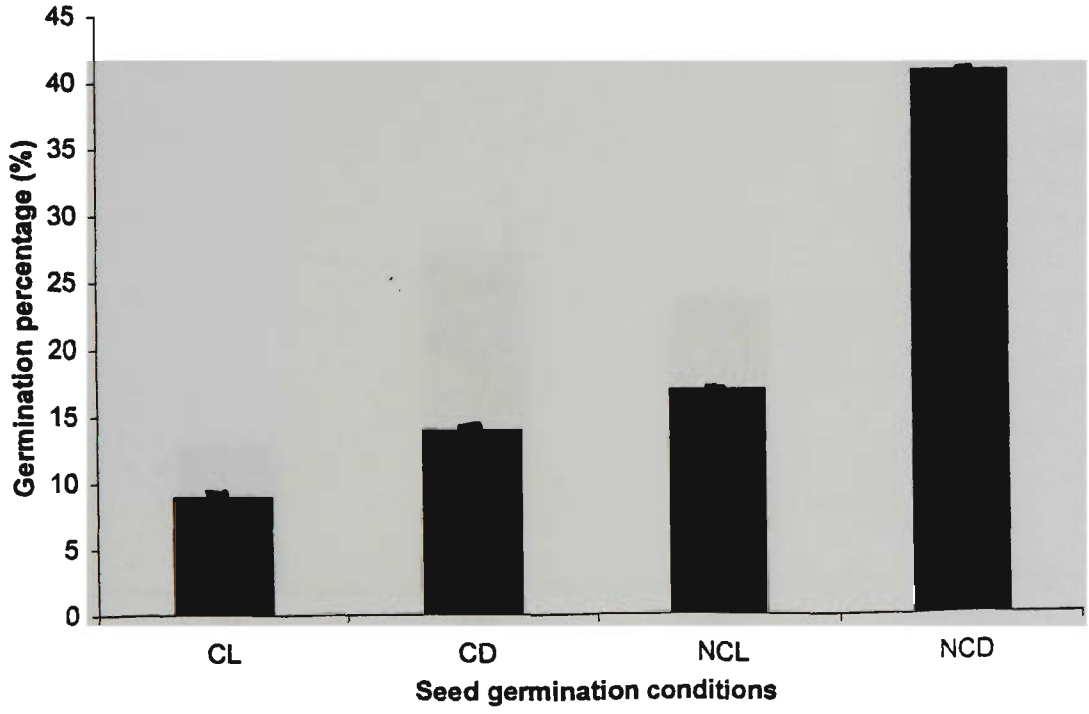

Figure 2.20 The effect of 40% (v/v) concentrated sulphuric acid pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

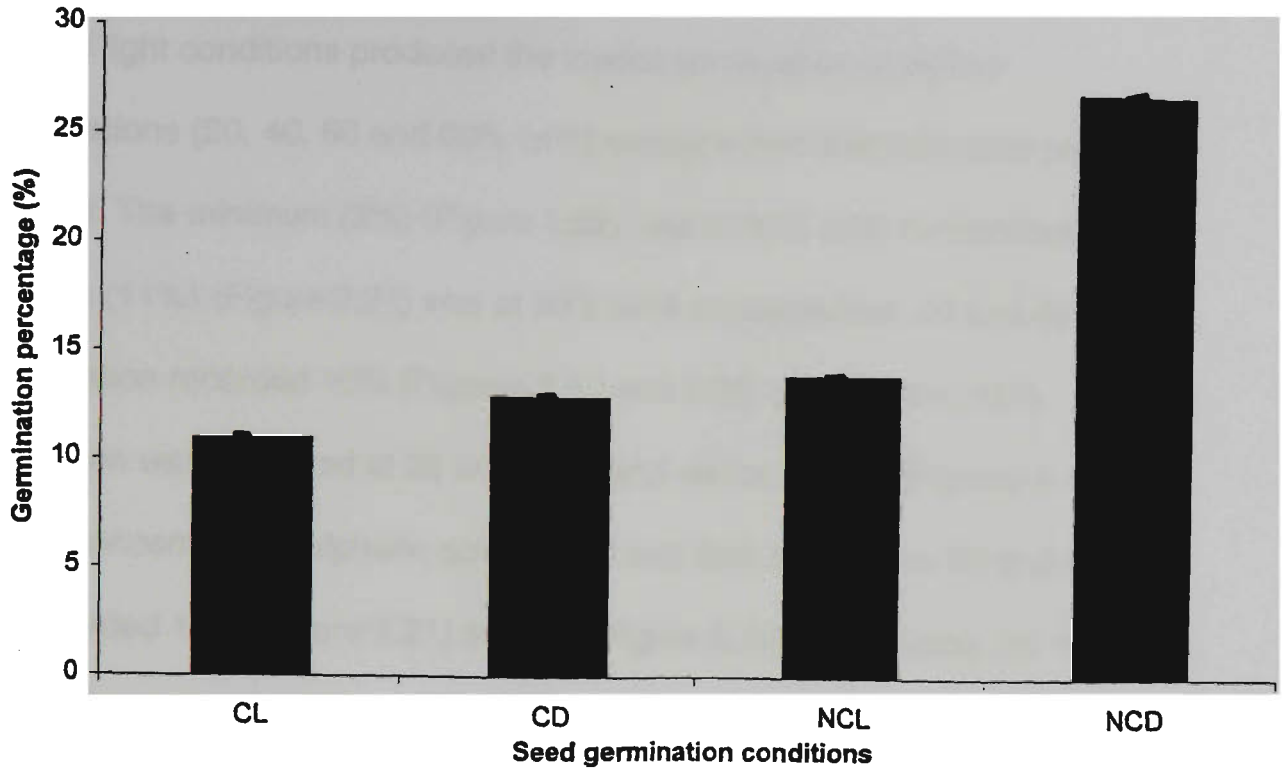

Figure 2.21 The effect of 60% (v/v) concentrated sulphuric acid pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

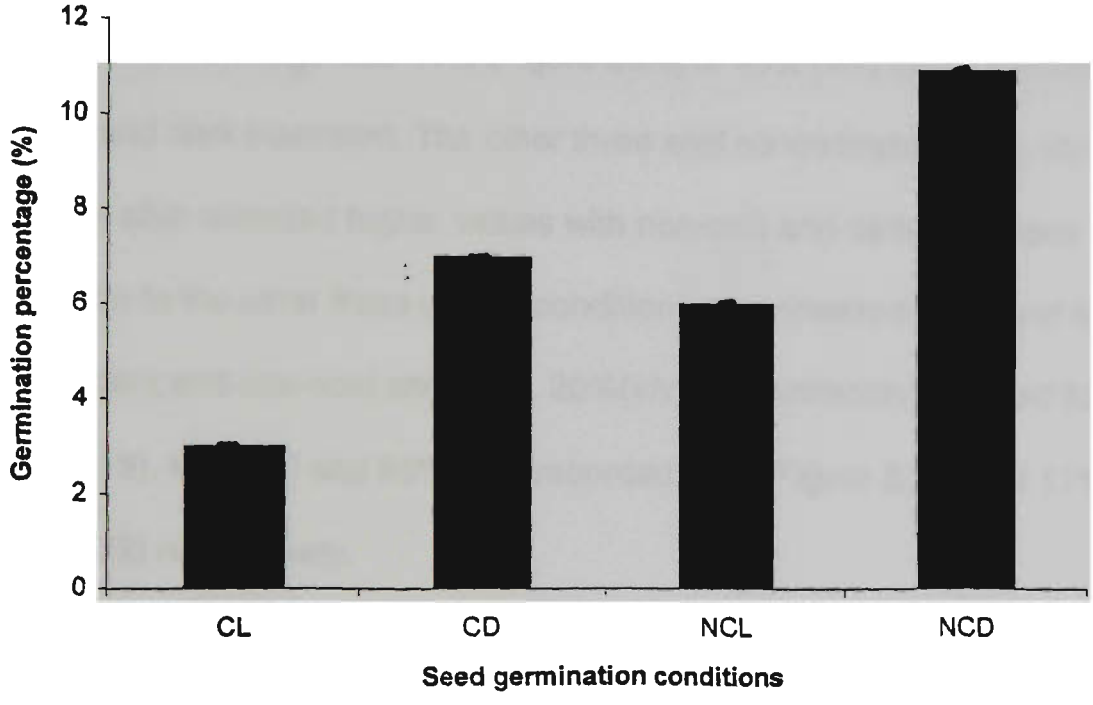

Figure 2.22 The effect of 80% (v/v) concentrated sulphuric acid pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

Cold and light conditions produced the lowest germination at all four concentrations (20, 40, 60 and 80% (v/v)) concentrated sulphuric acid pre-treatment. The minimum (3%) (Figure 2.22) was at 80% (v/v) concentration, the maximum (11%) (Figure 2.21) was at 60% (v/v) concentration. 20 and 40% (v/v) concentration recorded 10% (Figures 2.19 and 2.20) germination. 15% germination was observed at 20 and 40% (v/v) concentration (Figures 2.19 and 2.20) of concentrated sulphuric acid at cold and dark conditions. 60 and 80% (v/v) recorded 14% (Figure 2.21) and 7% (Figure 2.22) respectively. 20 % was the highest percentage recorded with non-cold and light conditions. This percentage was recorded with 20% (v/v) sulphuric acid concentration (Figure 2.19). 40 and 60% (v/v) recorded 16% (Figures 2.20 and 2.21). The least percentage (6%) was tabulated at 80% concentration (Figure 2.22). The highest germination percentage was 41% (Figure 2.20) at 40% (v/v) acid treatment with non-cold and dark treatment. The other three acid concentrations (20, 60 and 80% (v/v)) also recorded higher values with non-cold and dark conditions with comparison to the other three growth conditions experimented (cold and light, cold and dark and non-cold and light). 20%(v/v) concentration recorded 33% (Figure 2.19), while 60 and 80% (v/v) recorded 28% (Figure 2.21) and 11% (Figure 2.22) respectively.

2.7.7 Warm water pre-treatment

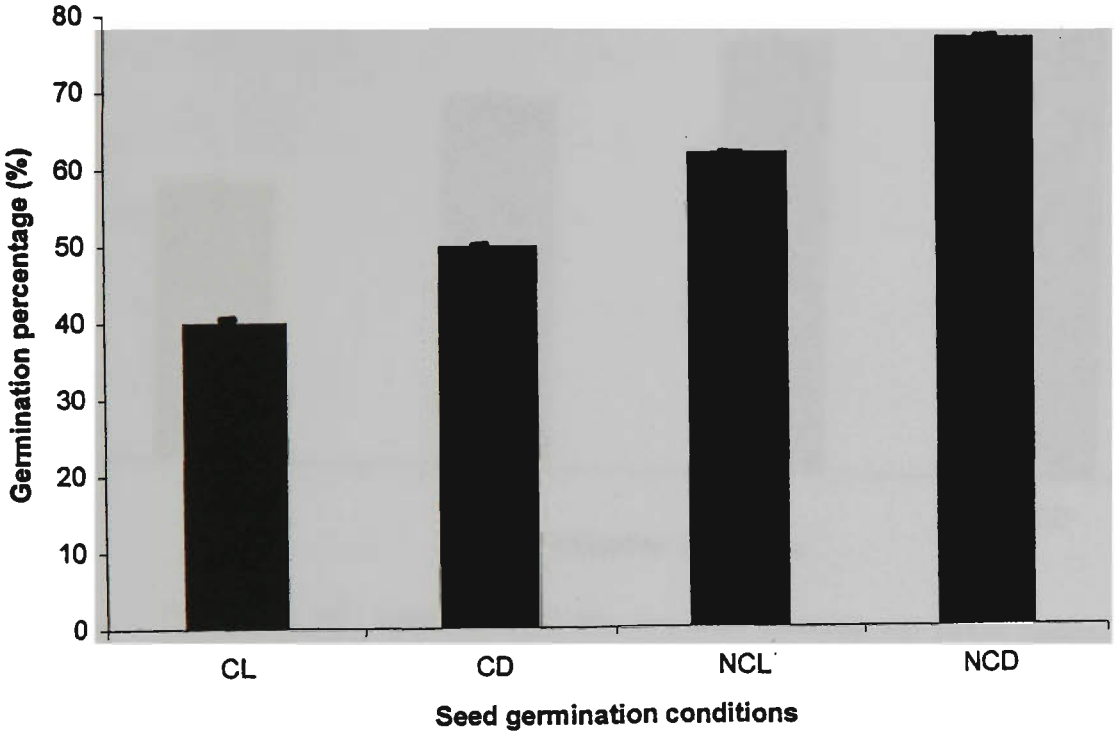

Figure 2.23 *The effect of 60°C heated water and 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of Paraserianthes falcatari.* Each experiment contained 25 replicates per experiment and was repeated four times.

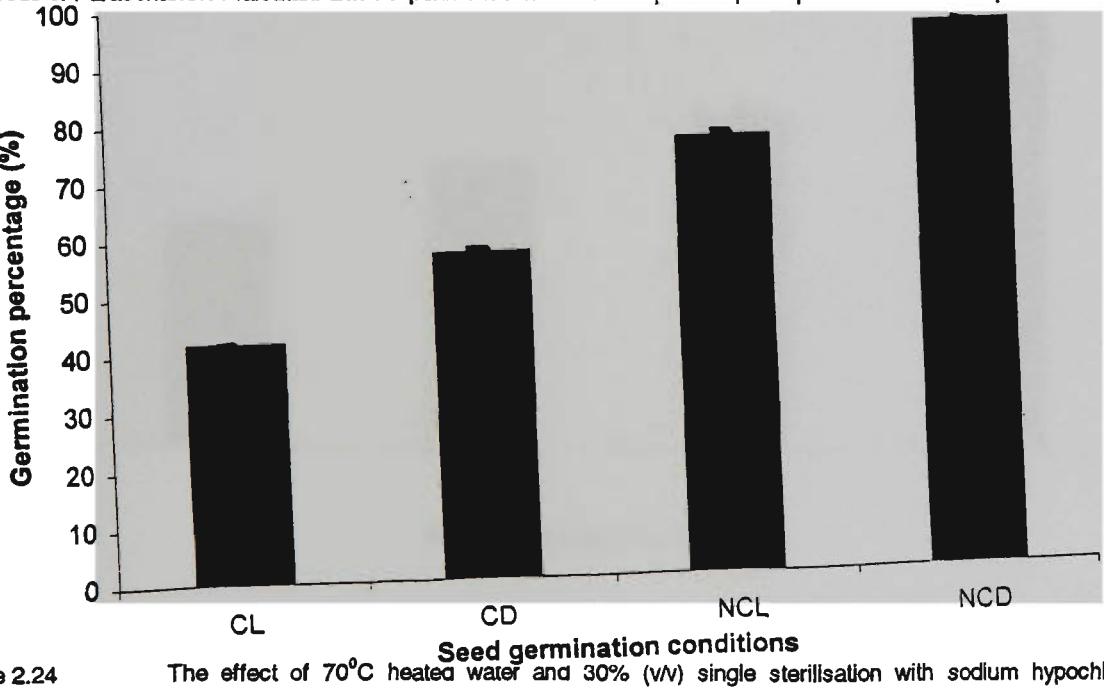

Figure 2.24 The effect of 70°C heated water and 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcatari.* Each experiment contained 25 replicates per experiment and was repeated four times.

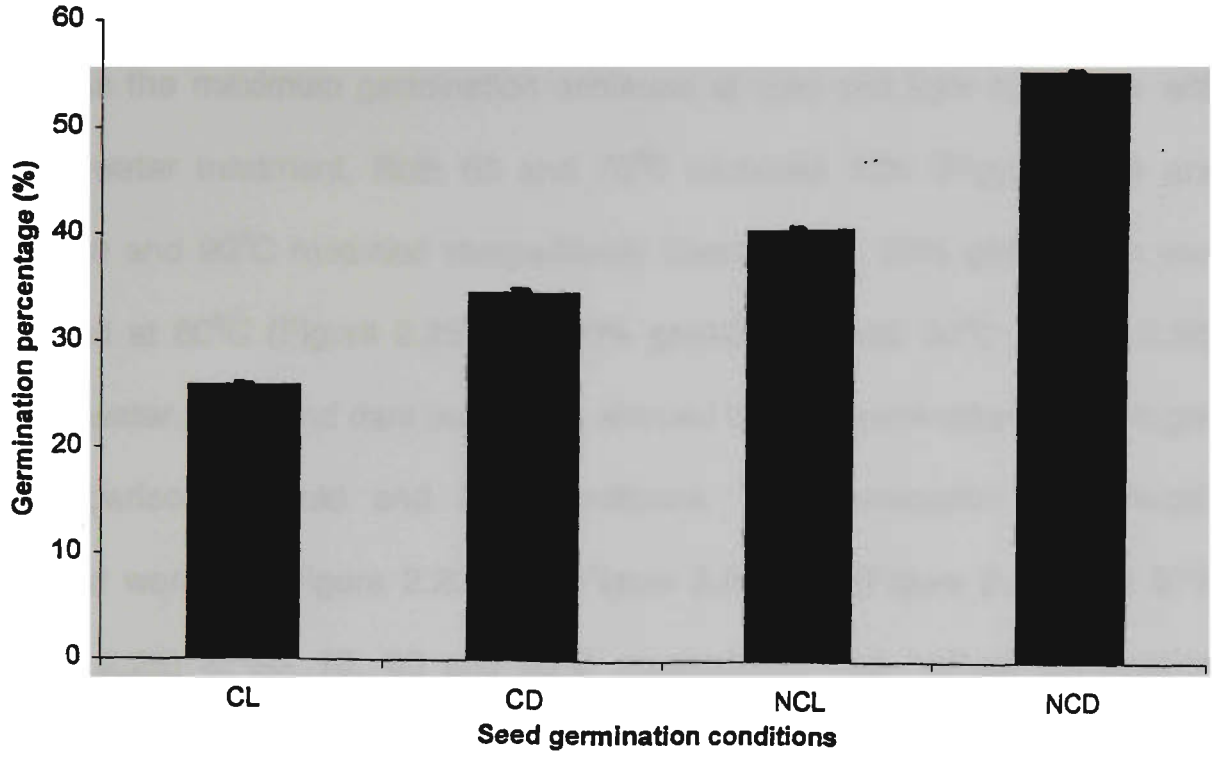

Figure 2.25 The effect of 80°C heated water and 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcatari*. Each experiment contained 25 replicates per experiment and was repeated four times.

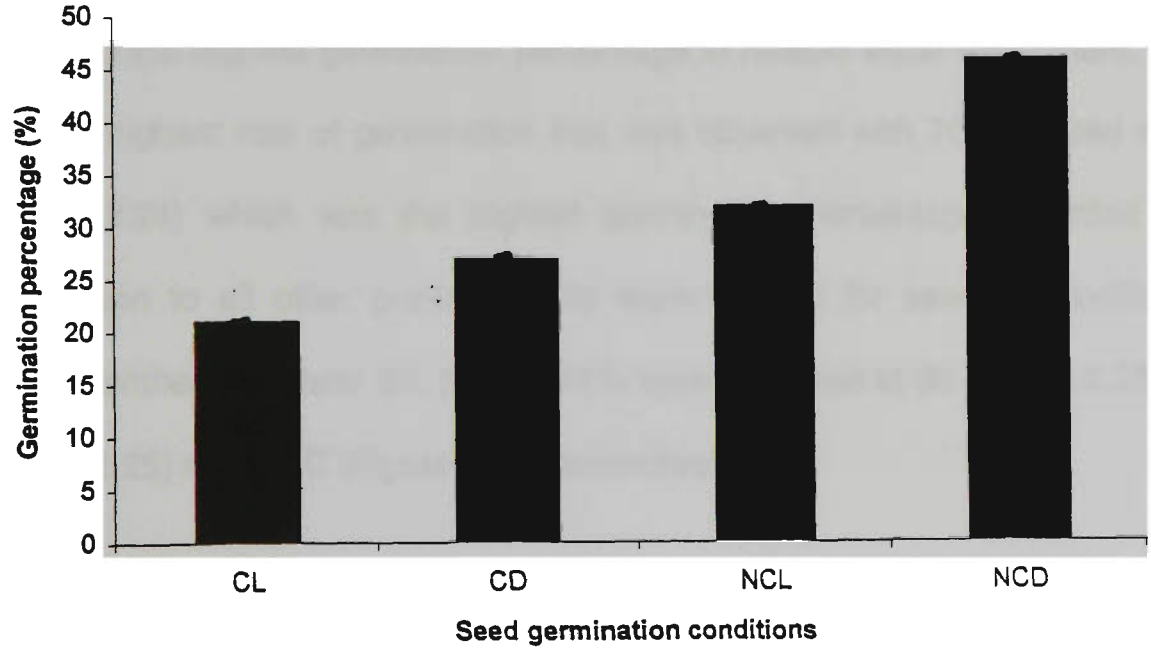

Figure 2.26 The effect of 90°C heated water and 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcatari*. Each experiment contained 25 replicates per experiment and was repeated four times.

40% was the maximum germination achieved at cold and light conditions with heated water treatment. Both 60 and 70^{0}C recorded 40% (Figures 2.23 and 2.24). 80 and 90^{0}C recorded comparitively lower values. 25% germination was observed at 80^{0}C (Figure 2.25) and 20% germination with 90^{0}C (Figure 2.26) heated water. Cold and dark conditions showed higher germination percentages in comparison to cold and light conditions. The germination percentages recorded were 50 (Figure 2.23), 55 (Figure 2.24), 35 (Figure 2.25) and 37% (Figure 2.26) at 60, 70, 80 and 90^{0}C respectively. The highest germination percentage was 75% at 70^{0}C (Figure 2.24) and the lowest was 35% with 90^{0}C (Figure 2.26) heated water. 60 and 80^{0}C water recorded 65 (Figure 2.23) and 40% (Figure 2.25) respectively for non-cold and light conditions. Similar to results obtained with all other pre-treatments non-cold and dark conditions produced the highest germination percentage in heated water experiment. 95% was the highest rate of germination that was observed with 70^{0}C heated water (Figure 2.24) which was the highest germination percentage recorded with comparison to all other pre-treatments experimented for seed germination in *Paraserianthes falcataria.* 80, 55 and 45% were observed at 60 (Figure 2.23), 80 (Figure 2.25) and 90^{0}C (Figure 2.26) respectively.

2.7.8 Sand Paper pre-treatment

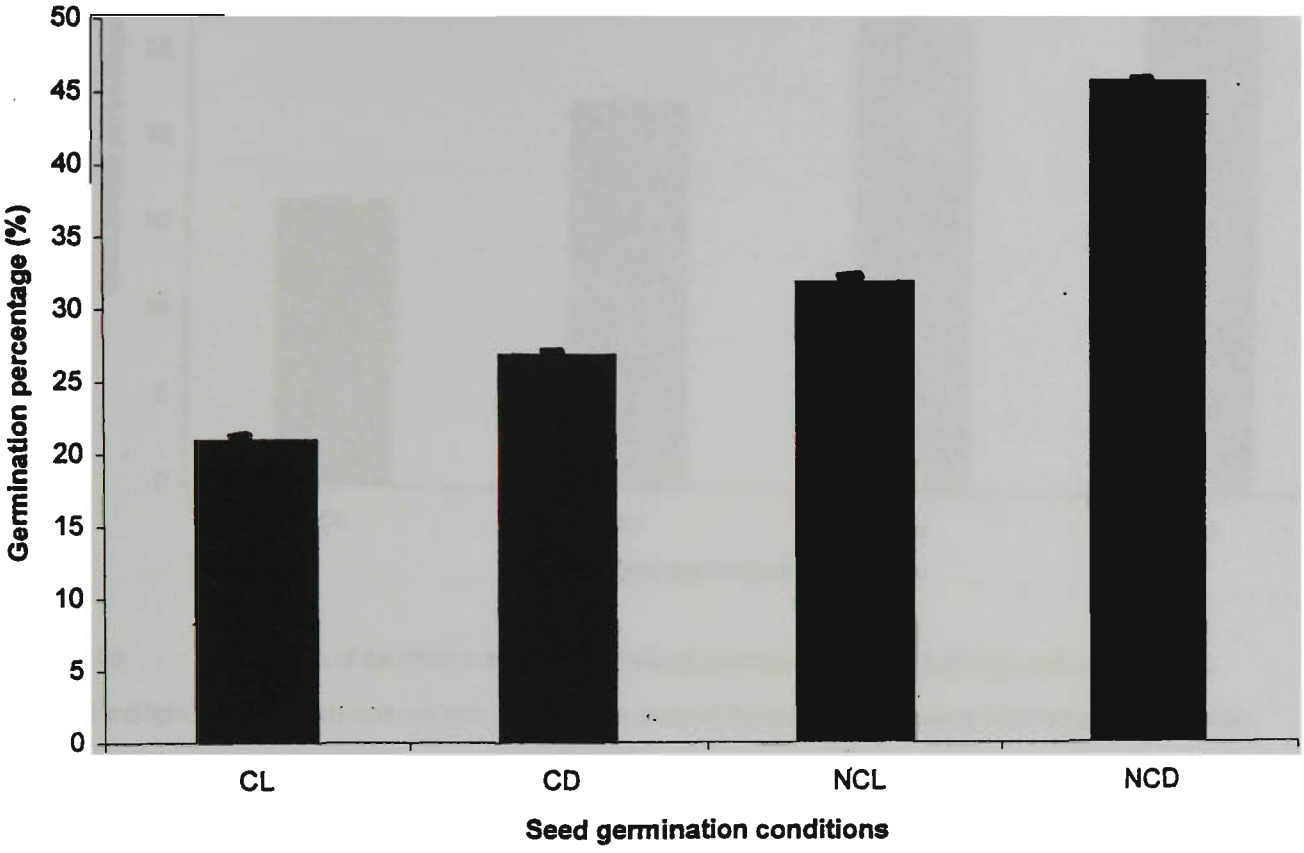

Figure 2.27 The effect of sand paper and 30% (v/v) single sterilisation with sodium hypochlorite pre-treatments in conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

20% germination was observed with sand paper pre- treatment under conditions of cold and light (Figure 2.27). A slightly higher percentage of germination was recorded with cold and dark conditions in comparison to cold and light conditions. 27% germination was observed with cold and dark conditions (Figure 2.27). Non-cold and light conditions produced 30% germination in the seeds of *Paraserianthes falcataria* (Figure 2.27). The highest percentage with sand paper pre-treatment under non-cold and dark conditions was 45% (Figure 2.27).

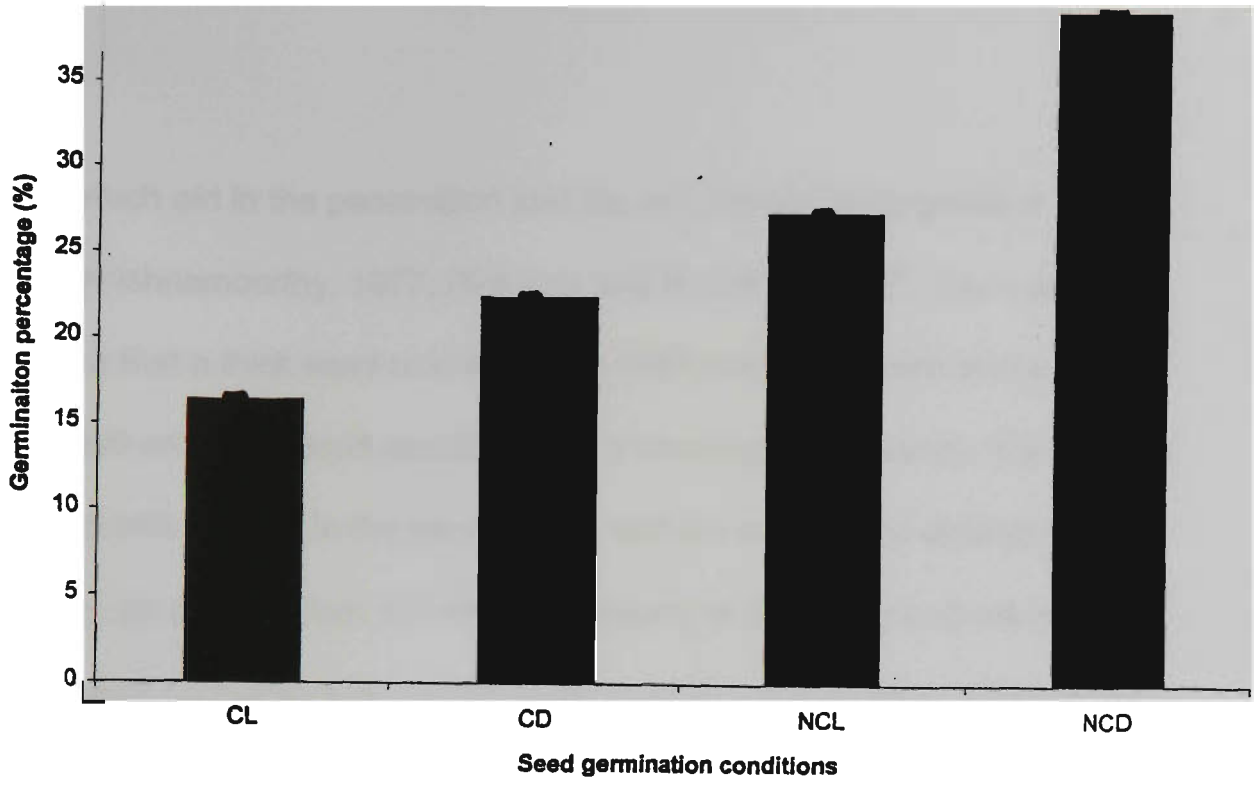

Figure 2.28 The mean of the effect of the four experimental conditions of cold and light (CL), cold and dark (CD), non-cold and light (NCL) and non-cold and dark (NCD) on the seeds of *Paraserianthes falcataria*. Each experiment contained 25 replicates per experiment and was repeated four times.

2.8 Discussion

The aim of this section of the study was to establish the optimum pre-treatment techniques for effective seed germination. Of all the techniques employed for pre-treatment, seeds treated with warm water at 70 ^{0}C and kept in conditions of non-cold and dark were found to give highest germination. Compared to all other pre-treatments the germination strike rate for warm water pre-treatment at 70 ^{0}C was 95% (Figure 2.24). This can be attributed to the fact that warm water weakens the seed coat to allow water to enter and the entry of water can be achieved via immersion in warm water which in effect ruptures the seed coat, while in nature temperature fluctuations and or mechanical breakage allow entry

of water which aid in the penetration and the concomitant emergence of the plumule (Krishnamoorthy, 1977; Richards and Beardsell, 1987). It is a common occurrence that a thick seed coat interferes with water absorption and embryo enlargement and as a result seeds require a breaking of dormancy. This may be associated with change in the seed coat or with the state of the embryo itself. Commonly, an embryo does not require breaking of dormancy and will develop after the seed coat is sufficiently damaged to allow water to enter (Richards and Beardsell, 1987). When a viable seed fails to germinate even in the presence of suitable conditions, it is said to exist in a state of dormancy (Krishnamoorthy, 1977). Exposing the seeds to varying conditions of seed pre-treatments, temperature, light or humidity can enhance breakage of dormancy especially in forestry species (Hartney, 1980).

Dormancy is not a result of the embryonic properties but is often due to mechanical constraint and supply of germination inhibitors by the seed coat. For example, species containing hard seeds which include the family Mimosaseae (Acacia and Albizia family), Fabaceae (pea family), Myrtaceae (Eucalyptus family) and Caesalpiniaceae (Cassia species) families have dormant seeds because their seed coats are impermeable to water posing mechanical constraints (Richards and Beardsell, 1987; Ridgeway and Kingsley, 1989). In this context, it can be mentioned that mechanical constraints related to hard seed coat were tried to be mitigated by the application of sand paper treatment, from which the germination rate obtained was fairly moderate (46%) when

subjected to non-cold and dark treatment (Figure 2.27). However, with chemical scarification using 40% (v/v) H_2SO_4 (Figure 2.20) the germination rate was more or less equivalent to mechanical scarification with sand paper, while scarification with 60 – 80% (v/v) H_2SO_4 (Figure 2.21 and 2.22) produced lower germination rates and the reason was attributed to the detrimental effects of H_2SO_4 on *Paraserianthes falcataria.* The findings reported by Palit, (1980) have shown that seeds subjected to mechanical scarification or treatment with H_2SO_4 resulted in 70 - 80% germination. It seems logical that this rate was obtained because of direct sowing in beds as well as sowing in polypots, whereas in the present study *in vitro* seed germination experiments were conducted on half strength MS media.

In some species the onset of germination is arrested by germination inhibiting substances such as abscisic acid (ABA), catechins or phenolics which have been found in both the seed coat and the outer layers of the embryo. Storage of seeds with such inhibitors has been known to cause true embryogenic dormancy after the movement of the substances from areas such as the testa and pericarp to the cotyledons and into the embryo (Richards and Beardsell, 1987).

Another aspect that can be postulated which aids in the breaking of dormancy when treated with warm water (70^0C) lies in the inactivation of abscisic acid (ABA). ABA is a single compound with a 6 - Carbon ring and number of side groups, which was first isolated in 1963 (Ridgeway and Kingsley, 1989;

Salisbury and Ross, 1992). It is a stress hormone that promotes changes in plant tissues when exposed to unfavourable conditions such as onset of winter, drought, etc. (Dunlap *et al.*, 1996; Redig *et al.*, 1996). It is also known to inhibit growth and induce dormancy (Steward and Krikorian, 1971). In a study carried out by Zeevaart and Boyer (1982) on *Xanthium strumarium*, it was observed that endogenous ABA present in the seed coat at high temperatures can be inactivated and hence, may lose some of its dormancy inhibiting characteristics. In the light of the above mentioned study it can be related that the inactivation of ABA at a high temperature of 70^{0}C may have led to the effective breakage of dormancy without being detrimental to the plant. However, as the temperature of the water was increased above 70^{0}C, a decrease was observed in the percentage of germination (Figures 2.23, 2.24, 2.25 and 2.26). In support of this phenomenon, a study conducted by Charomaini (1989) on *Gmelina arborea* in which a quick immersion of seeds in warm water maintained at 95^{0}C for 1, 5 and 10 minutes which led to the loss of viability and thereby resulted in poor germinaiton.

Seeds placed in the dark sprouted within two to three days whereas the seeds that were placed in light took longer (5 - 7 days) to initiate plumule and radicle extension. The seeds that were in the dark were healthier in appearance initially than the seeds placed in the light. But, the seeds in the dark had to be returned to conditions of light as soon as they had germinated. Longer periods affected the development of nodes and leaves, because the explants grew long stems

with very few nodes, which caused difficulties while subculturing. Therefore, periods of initial darkness were well received by the seeds and aided in faster germination, but establishment of seedling and further growth required exposure to light. Darkness triggers production of auxins that initiate the breaking of dormancy, thereby allowing seeds to germinate (Dunlap *et al.*, 1996; Redig *et al.*, 1996). In species such as *Allocasuarina, Regelia* and *Xanthorrhoea* light inhibits germination and a dark period is required. In the light of such a response system, it can be stated that *Paraserianthes falcataria,* being a tropical species the seeds did not require much exposure to light for the breakage of dormancy because in their natural habitat the seeds being embedded in the forest soil surface are restricted to positions protected from high light by the presence of a forest canopy (Bell *et al.*, 1995).

Seasonal fluctuations is also known to influence seed germination (Ridgeway and Kingsley, 1989; Raven *et al.*, 1992). In general, seeds of plants that endure cold winters will not germinate unless there is a period of low temperature, which is usually above freezing (Longkamp, 1987). For instance, incubation at 4^{0}C for 1 - 2 weeks may help for species requiring low temperatures as pre requisites for germination, otherwise germination may fail or may be much delayed, with the early growth of the seedling often-abnormal (Ridgeway and Kingsley, 1989). In trying to relate with the above contention a cold treatment strategy was adopted in the present study but, it was a observed that seeds subjected to non-cold conditions had a higher rate of germination (Figure 2.28) in comparison to seeds

exposed to cold treatments. This provides a conclusive evidence that seeds of *Paraserianthes falcataria* being tropical in origin (temperature range of 22 – 46^{0}C) did not require a cold phase to enhance their germination percentage for all pre-treatments. Another feasible explanation that can be sought is that mechanical fracturing of the seed coat inhibits the need for a lengthy cold treatment to break seed dormancy.

Studies showed that blue and red light of about 660 nm are highly effective in promoting germination in general, whereas light at 730 nm in the far red region inhibited the effect of red light (Raven *et al.,* 1992). The active chemical that responds to the changes in light is the pigment phytochrome, which exists in two forms depending on the wavelength of light shining on it (Raven *et al.,* 1992). At 660nm (red light) the pigment is in an active form which thus promotes seed germination, where as when the pigment is exposed to far red light (730nm) the pigment is altered to an inactive form which inhibits germination (Borthwick *et al.,* 1952). Large-seeded species were less sensitive to exposure to light compared to the smaller seeded species (Raven *et al.,* 1992). Seeds that were kept in cold and light, cold and dark and non-cold and light did not produce such high rates of germination as of non-cold and dark (Figure 2.28).

Exogenous application of hormones such as GA_3 has been very successful in breaking dormancy in woody plants like *Corylus avellana* (Bradbeer, 1988), *Eucalyptus pauciflora* (Barker *et al.,* 1977), *Ruellia humilis* (Buck, 1989) and

Melia azedarach (Domecq, 1988). Since gibberellins substitute for light and temperature dormancy breaking conditions in seeds that are placed in the dark by substituting for red light where this light stimulates germination (Anon, 1969). Hence, the use of exogenous gibberellic acid for 12 h and double sterilisation complemented with conditions of non-cold and dark produced the second highest (71%) percentage of germination (Figure 2.16) in relation to warm water treatment at 70^{0}C which had the highest (95%) rate of germination (Figure 2.24). It was also observed that in *Paraserianthes falcataria* seed treated with GA_3, once germinated attained longer lengths rapidly in comparison to seeds subjected to other pre-treatments. This phenomenon can be attributed to the fact that GA_3 stimulates the conversion of storage polymers into sucrose first and then into mobile amino acids or amides which can be readily translocated via the phloem into and throughout the young roots and shoots to be utilised as a nutrient source for the growing seedling (Salisbury and Ross, 1992).

Double sterilisation was more effective in breaking dormancy than single sterilisation which is apparent from the results obtained (Figures 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9). The incubation of seeds at 37^{0}C for 4 hours encouraged the growth of microorganisms that if left untreated would interfere with the normal growth of the explants. Thus by permitting them to grow prior to the germination of the young seedlings reduces the level of contamination in future experiments. Double sterilisation was more effective than single sterilisation owing to the fact that sterilising the seeds for the second time would eliminate the microbial

contaminants more effectively (Blomstedt *et al.*, 1991). Moreover, double sterilisation weakens the seed coat considerably making it less resistant to the force exerted by the developing hypocotyl allowing it to rapture easily in the process. Studies by Barker *et al.* (1977) and Le Roux and Van Staden (1991a), have shown that tolerance of seeds to sterilisation depends on size and thickness of the seed coats. For instance, *Eucalyptus citriodora* seeds are small and thin walled and can be sterilised in 2% (v/v) NaOCl for 20 minutes, whereas *Eucalyptus marginata* seeds, which are larger and have thicker seed coats can tolerate 5% NaOCl (v/v) for 60 to 90 minutes.

Sucrose is abundant in plants and metabolically a continuous and essential source of energy in the form of carbohydrates which is required for growth. Since, it acts as an energy source in photosynthetic cells and is readily translocated in the phloem to the growing tissues as an essential nutrient, a reduction in the sucrose concentration (< 2% (w/v)) in tissue culture situations may be detrimental (Blomstedt *et al.*, 1991; Duhoux and Davies, 1985). Damiano *et al.* (1985) have established that the greater the difference in the concentration (10 - 40%) of sucrose, the greater the difference in the growth rate with the optimum achieved at a high sucrose concentration. Mohammed and Vidaver (1988) founded that reducing the concentration of sucrose in tissue culture may be detrimental to growth which was evidenced in the present study where 1 and 2%(w/v) sucrose concentrations of sucrose (Figure 2.10, 2.11) produced lower germination percentages compared to 30% (w/v) sucrose (Figure 2.12).

2.9 Summary

In conclusion in all treatments non-cold and dark conditions produced the highest percentages of growth compared to the other three conditions of cold and light, cold and dark and non-cold and dark. Amongst the various pre-treatments, warm water at 70^{0}C produced the highest percentage of germination. Gibberellic acid pre-treatment with double sterilisation produced the second highest group of seed germinates. Non- gibberellic acid, single sterilisation, concentrated sulphuric acid and sandpaper were not very beneficial in seed germination. Abrasion with sand paper was very laborious and time consuming as compared to the other pre-treatments and did not produce significant results. Therefore these pre-treatments can well be omitted in the future.

The optimum concentration for sterilising agent (sodium hypochlorite) was identified as 30% (v/v) for 30 minutes and the optimum for carbon source in the media was 30% (w/v) sucrose.

Therefore, pre-treatment of seeds with 70^{0}C warm water, followed by sterilisation with 30% (v/v) sodium hypochlorite for 30 minutes and planted in half strength MS basal media with 30% (w/v) sucrose is the best pre-treatment for germination of *Paraserianthes falcataria* seeds.

CHAPTER 3

SHOOT INITIATION

3.1 Introduction

Shoot regeneration can occur through one of many pathways and it is necessary to understand the origin of the shoots to determine their future potential. The stimulation of pre-existing meristems present in the axils of the leaves results in the arisal of shoots. This arisal can be achieved by the incorporation of a growth regulator in the media, which stops the growth of the apical meristem and stimulates lateral shoot growth. Such shoots are the preferred materials for micropropagation due to the unlikely chance of genetic variants of off-types. On the other hand the explant can produce differentiated cell masses called callus which can then be induced to undergo embryogenesis or adventitious bud formation (Barlass, 1983;1991). Investigation of embryogenesis lay beyond the parameters of this present study.

It is important to remember that success of plantlet formation results from the interplay of the explant, medium and culture conditions (Chen, 1978). It is good to determine if other species of the same genus have been successfully cultured. The experimental approach will depend on the material available that is seed, greenhouse grown or field grown material. On some occasions one test will give

the indications required, but it may be necessary to run further parallel screening tests using two or more formulations and two different explants and for a even longer period in culture. (Thorpe, 1983; Thorpe and Patel, 1984).

Systematic sequential testing can be carried out to test one after the other, such as (a) the optimum age of the explant; (b) any pre-treatments (eg., cold period incubation in antioxidant) needed; (c) any manipulations needed on the explant for optimum culture results (eg., trimming of cotyledons, orientation in culture) (d) level of sucrose (e) level of and necessity of individual vitamins (f) effects of amino acids (eg., glutamate, asparagine, tyrosine)and other organic addenda (eg., adenine sulfate); (g) effects of mixed phytohormones (eg.. BAP, 2iP) and (h) reinvestigation of the phytohormones level resulting from the tests (Thorpe and Patel, 1984).

The second phase will involve the development into shoots and multiplication. This is usually achieved in a medium lacking phytohormones, including the testing of several other factors such as optimum length of time in phytohormones in the first phase for subsequent shoot development, level of sucrose needed for low concentrations of phytohormones during later stages of shoot development, determining if any other mineral salt formulation is better than the one used in the first phase, frequency of reculture and effect of subdivision on development and multiplication rates (Thorpe and Patel, 1984). Some of the factors stated above were not considered relevant to the type of research undertaken in this

project and therefore were not experimented with.

Using the systematic approach it would be possible to achieve plantlet formation in a number of woody plants, both softwood and hardwood. The use of micropropagation techniques allows rapid multiplication of clonal stock, giving rise to increased financial returns in the short term (De Fossard and De Fossard, 1988; Le Roux and Van Staden, 1991a). The ability to produce plants with specific characteristics, such as the micropropagation of *Eucalyptus grandis* for use in the pulp industry or other important timber species such as *Eucalyptus gunni, Eucalyptus pauciflora, Eucalyptus delegatensis and Eucalyptus nitens* is one of the reasons that in the past few years investigations of media composed of, carbohydrate source and exogenous hormones have stepped up a level (Barlass, 1983; Le Roux and Van Staden, 1991b). Tissue culture is a promising technique for mass production of large number of superior trees by regenerating plants from single meristems in desired quantity. One hundred million shoots were obtained from a single shoot within a year in *Eucalyptus* species through clonal propagation (Barlass, 1983; Blomstedt *et al.,* 1991; Le Roux and Staden, 1991b).

Most of the information on tissue culture propagation of plants has been obtained from experiments with herbaceous, but not woody species (Murashige, 1974; Barlass, 1983). The forest trees of *Paraserianthes falcataria* can be difficult to breed and select due to the long generation period and the change of growth rate and morphology with time (Hartney, 1980).

The culturing of explants gained from pre existing aseptic cultures is an integral part of the micropropagation seen in this chapter. The use of already existing cultures removes the need for direct sterilisation of plant material, which can quite often damage tissues. Also, external sterilisation fails to remove endogenous microbial infections (Le Roux and Van Staden, 1991a). Cultures of *Eucalyptus* have been gained from scion, epicormic and young shoots on mature trees as well from epicormic shoots on *Eucalyptus grandis* branches (Ikemori, 1987). Lignotuber and floral bud cultures can also be gained (Le Roux and Van Staden, 1991a). It has been found that for the micropropagation of *Eucalyptus* the use of adventitious or axillary shoots formed on explants gained from seedlings (nodal explants) and others are highly desirable for regenerative purposes (Le Roux and Van Staden, 1991a). Therefore, axillary shoots were used as starting material for this study. Shoot regeneration and multiplication are vital for *in vitro* selection, somaclonal variation and genetic engineering techniques aimed at plant improvement (Subbiah and Minocha, 1990).

Choice of inoculum: The choice of inoculum or the explant is extremely important in the manipulation of organ formation (Thorpe and Patel, 1984). The behaviour of the explants in culture is influenced by many factors, there is also a possibility of the heterozygote seed possessing unwanted, recessive characteristics not apparent in the parent plants (Murashige, 1974). The factors of the organ that is to serve as the tissue source are the physiological and

ontogenic age of the explant, the season in which the explants are obtained, the size of the explant and the overall quality of the plant from which the explants are obtained. In some cases, the pre- treatments of explant sources eg., chilling of bulbs or spraying of trees with cytokinins may be a requirement for successful organ formation in culture. Some of the variables may be easy to control but others are more difficult and require experimentation. *Eucalyptus sideroxylon* plants hailing from poorly rooted explants form calluses much more readily than those from well rooted explants; 40 % of shoots on plantlets from poorly rooting explants had calluses after four weeks while only 20 % of others did (Cheng *et al.*, 1992), suggesting that not only are root and callus formation related to the hormone and sucrose concentrations of culture media, but also to the biochemical status of individual plants (Cheng *et al.*, 1992). It is thus important to understand various characteristics of the intended plant material (Thorpe and Patel, 1984). The classical methods of vegetative reproduction in many woody species of trees have proven negative (De Fossard and De Fossard, 1988). However, tissue culture of several woody species has been reported. This technique is based on the plant's ability to regenerate tissues and parts from cell or organ culture. It ensures a superior plant may be reported endlessly without variation (Le Roux and Van Staden, 1991b). Tissue culture therefore is a promising technique and it may prove widely applicable to *Albizia* species and related leguminous trees (Anon, 1979).

Sterilisation of explants: Sterilisation of explants is very important. The explant should be thoroughly washed in running tap water with or without detergent, followed by a quick dip (30 - 60 seconds) in 70 % (v/v) ethanol and rinsed with sterile water. The explant is then stirred for 10-20 minutes in diluted laundry bleach (final concentration 0.5 - 1.0% (v/v) NaOCl) containing a wetting agent (eg., Tween 20 or Tween 80) and finally rinsed several times with sterile water under aseptic conditions. H_2O_2 is also an effective sterilant (Thorpe and Patel, 1984). Some explants require more extensive sterilising, eg., using a gentle vacuum during the bleach treatment. Using a two step procedure in which the surface sterilised material was placed on media lacking phytohormones or vitamins for 24 hours, followed by treatment with a fungicide and resterilization before planting on complete culture media (Jones *et al.*, 1977). If seedling parts are used as explants, the surface sterilised seed is often germinated aseptically on an agar medium containing 1 - 2 % (v/v) sucrose (Thorpe and Patel, 1984).

Fungal and bacterial contamination often occur within 5-10 days of culture. Visual contaminated seeds can be discarded on sight but the absence of visual contamination cannot mean that the explant is pathogen free. During reculture, bits of residue tissue can be diced and placed in sterile culture containing Bacto nutrient broth for a period of 10 days to determine the pathogen status of the explant (Anderson, 1980). Difficult to decontaminate material should be grown in media containing antibiotics and fungicides for a short period of time before transfer to media to check for contaminations. It is important to check for

contaminants, because contaminated cultures would give rise to infected tissues and cultures. In order to avoid the propagation of infected plant material seeds were used as starting material for this project.

3.2 Objectives

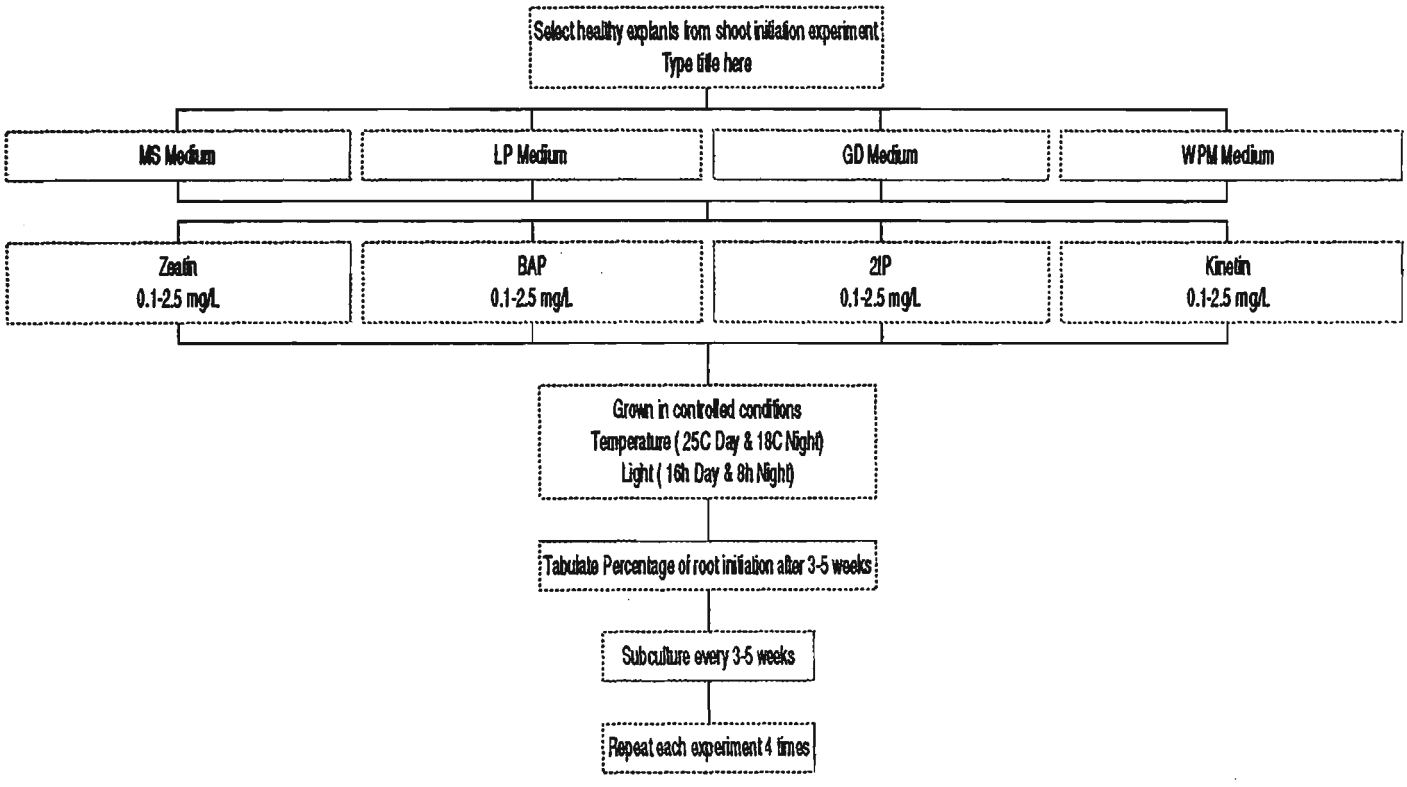

Figure 3.1. Overall experimental design for determination of shoot initiation in *Paraserianthes falcataria.*

The main aim of this series of experiments was to establish shoot initiation and multiplication techniques for *Paraserianthes falcataria.* This was achieved by investigating shoot culture initiation, maintenance and multiplication with commonly used media such as MS (Murashige and Skoog, 1962), LP (Le Poivre,

1977), GD (Gresshoff and Doy, 1972) and WPM (Woody plant media, Lloyd and McCown, 1980). Cytokinins namely Zeatin, BAP (Benzylamino purine), 2iP (2-isopentenyl adenine) and Kinetin were investigated for optimum concentration for shoot initiation (Figure 3.1). Finally the appropriate combination of media and cytokinin for maximum shoot initiation was established.

3.3 Growth hormones for shoot initiation

The micropropagation of explants have to be carried out on a sterile media containing constituents such as inorganic macro and micronutrients, carbon and energy sources, vitamins, nitrogen and phytohormones (auxins and cytokinins) which are required parameters for successful growth. The type and amount of these constituents will affect the growth of the explant (Escalante and Langville, 1995; Thorpe, 1982). The hormones present in a media can influence how healthy the shoots are growing. A large number of species respond to a suitable auxin - cytokinin balance but in some cases, a mixture of two cytokinins or two auxins has proven superior to a single cytokinin or auxin. Other phytohormones, including gibberellins and abscisic acid and metabolites such as phenolic acids added to the medium have shown to play a role in organ formation (Chandler and Thorpe, 1982). Auxins and cytokinins are phytohormones that are required for plant growth. In addition to the inorganic macro and micronutrients, carbon and energy sources, vitamins, reduced nitrogen are discussed under seed media. The concentration and ratio of the phytohormones often control the

pattern of differentiation in culture (Skoog and Miller, 1957). Natural as well as synthetic compounds are used (Thorpe and Patel, 1984). Skoog and Miller (1957) have showed that shoot and root initiation is regulated by the interaction between the two hormones auxin and cytokinin. A relatively high concentration of auxin favours root initiation but represses shoot formation whereas high concentrations of cytokinin produces better shoot than root initiation (Murashige, 1977). The successful induction of shoots and roots can therefore be achieved by manipulating hormone concentrations and this varies among plant species. The presence of auxins IAA (Indole acetic acid), IBA (Indole butyric acid) and NAA (Napthalene acetic acid) in low concentrations promotes the growth of *Eucalyptus nova-anglica* as opposed to inducing buds onto fresh induction media (1 mgL^{-1} Zeatin, 0.01 mgL^{-1} IBA) allows rapid multiplication of the species at hand (Evans, 1984; Mehra- Palta, 1982).

In 1956, an active substance that induced cell division or cytokinesis was isolated from herring sperm and called cytokinin. Cytokinins are synthesised in roots from which they move upwards in the xylem and pass into the leaves (Raven *et al.*, 1992). Higher levels of endogenous cytokinins have been detected in root tips of flowering plants than non-flowering plants (Zhang *et al.*, 1995). Cytokinins have an intriguing structure that promotes cell division and differentiation in intact plants (Raven *et al.*, 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992). Cytokinins are required as an active ingredient of plant tissue culture as they aid in mitosis (Table 3.1) and organogenesis from

interaction with auxins ((Raven *et al.*, 1992; Redig *et al.*,1996; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992)

Table 3.1 Table outlining some of the interactions of cytokinins during various aspects of plant growth (Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992)

Physiological activity	Action of cytokinin
Growth of seedlings into mature plant	Cell Division and differentiation, organogenesis
Apical Dominance	Promotes lateral bud development
Leaf Abscission	Inhibits

Cytokinins: In 1963, the first naturally occurring cytokinin, zeatin (6-(4-hydroxyl-3-methyl but-2-enyl amino purine)(Figure 3.2) was identified from corn (Raven *et al.*, 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992). Apart from naturally occurring cytokinins such as purine and adenine derivatives there are synthetic compounds such as kinetin (6-furfuryl amino purine) (Figure 3.5), Benzyl amino purine (BAP) (Figure 3.3) and 2- Isopentenyl adenine (2iP) (Figure 3.4) that have cytokinin activity. kinetin and BAP are commonly used cytokinins with 2iP and zeatin used less frequently mainly because of their higher cost. 75% of the species forming shoots containing kinetin and BAP in a concentration of 0.05 - 4.0 μM (Evans *et al*, 1981). Cytokinin such as zeatin was isolated from seed kernels and substances similar to kinetin in physiological and

morphological effects have been found in coconut milk, in immature caryopses of *Zea mays* and in immature fruits of *Aesculus hipocasanum*, banana and apple (Akyeampong *et al.*, 1995). Cytokinins such as kinetin, zeatin, 2iP and Benzyl amino purine (BAP) are required for initiation of shoots (Le Roux and Van Staden, 1991b).

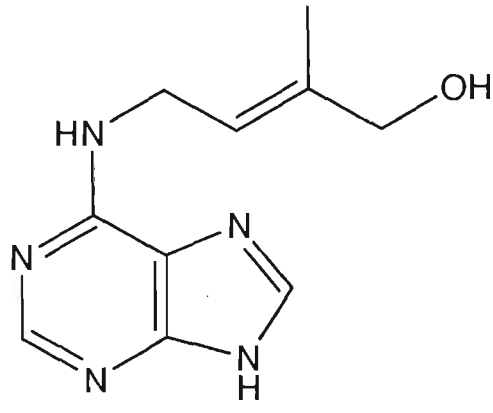

Figure 3.2 Structure of Zeatin (Raven *et al.*, 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

On similar lines addition of kinetin, BAP, 2iP or zeatin have been used for initiation of shots in various plant species. Some of which are listed in Table 3.2.

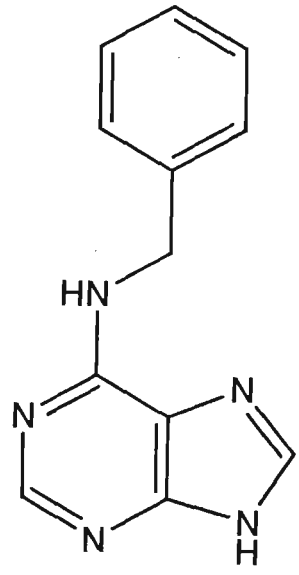

Figure 3.3 Structure of BAP (Raven *et al.,* 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

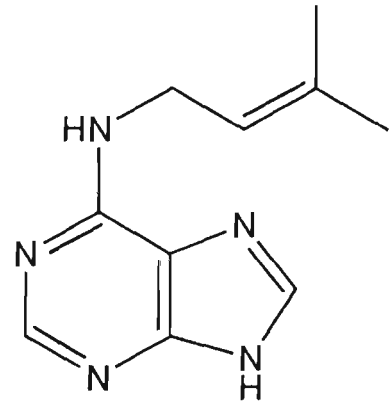

Figure 3.4 Structure of 2iP(Raven *et al.,* 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

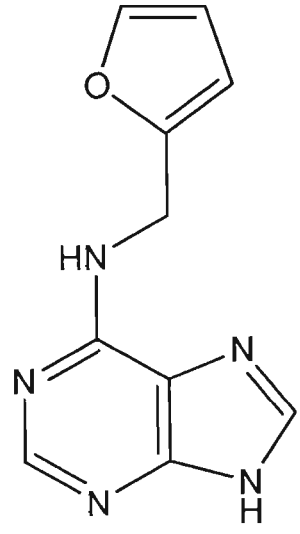

Figure 3.5 Structure of kinetin (Raven *et al.*, 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

Other phytohormones , including gibberellins and abscisic acid and metabolites such as phenolic acids added to the medium have been shown to play a role in organ formation (Chandler and Thorpe, 1982). The plant hormones induce or enhance specific growth phenomena. The site of hormone synthesis is different from its place of action and they are transported before they can exert their effects (Table 1.5).

Table 3.2. Summary of cytokinins used in shoot initiation of certain plant species.

Cytokinin	Species	Author
Hormone free	*Lillium japonicum*	Mizuguchi and Ohkawa, 1994
Zeatin	*Eucalyptus grandis*	Furze and Cresswell, 1985
		Warrag *et al.*, 1990
		Coleman and Ernst, 1989
	Eucalyptus regnans	Blomstedt *et al.*, 1991
	Eucalyptus globulus	Blomstedt *et al.*, 1991
		Mascarenhas *et al.*, 1982
	Eucalyptus nitens	Blomstedt *et al.*, 1991
	Eucalyptus marginata	Bennett and McComb, 1982
BAP	*Eucalyptus tereticornis*	Ilahi and Jamal, 1987
	Acacia mangium	Ahmad, 1991
		Wong and Haines, 1992
		Darus, 1991; 1992
		Galiana *et al.*, 1991
		Meyer and Van Staden, 1987
	Acacia auriculiformis	Darus, 1991; 1992
		Meyer and Van Staden, 1987
	Leucaena leucocephala	Dhawan and Bhojwani, 1985; 1987
	Eucalyptus globulus	Trindade *et al.*, 1990
2iP	*Hibiscus cannabinus*	Cristofari *et al.*,1988
	Eucalyptus grandis	Sita *et al.*, 1986
Kinetin	*Acacia mangium*	Ahmad, 1991
	Eucalyptus novoanglica	Mehra-Palta, 1982
	Eucalyptus viminalis	Mehra-Palta, 1982
	Eucalyptus globulus	Mascarenhas *et al.*, 1982

3.4 Media

The major constituents for successful growth and organogenesis are inorganic macro and micro nutrients, carbon and energy sources, vitamins, reduced nitrogen and phytohormones. These five classes are sufficient for most plant species, but in some cases natural complexes such as hydrolysed protein preparation, brewer's byproducts, endosperm fluids, fruit pulp and juice, animal byproducts and coconut milk are also added to the medium (Thorpe and Patel, 1984).

Inorganic macro and micronutrients: The following salts such as N (Nitrogen), K (Potassium), P (Phosphorus), Ca (Calcium), S (Sulphur) and Mg (Magnesium) are required in millimole quantities and collectively called macronutrients. The optimum concentration of each nutrient for achieving maximum growth varies from species to species. A nutrient media should contain atleast 25 and up to 60 mM inorganic nitrogen. Nitrate is commonly used in the range of 25-40 mM. Cells may grow on just nitrate but sometimes the use of ammonia or reduced nitrogen can prove effective. The amount of ammonium varies between 2 and 20 mM with an optimum concentration of 2 - 8 mM, wherein amounts exceeding 8 mM may result in reduced growth. Cells can be grown on ammonium as the sole nitrogen source provided citrate, succinate, malate or another TCA cycle acid is present at 10 mM. Other nitrogen sources such as urea, glutamine or casein hydrolysate are also applied. A concentration

of 20 mM or higher is required for potassium, which is usually supplied as a nitrate or a chloride. Sodium cannot be used as a substitute, although plants can tolerate high concentrations of chloride and sodium and have no apparent effect on growth rate. The optimum concentrations of P, Mg, Ca and S for cell growth vary from 1 – 3 mM under conditions where other requirements are satisfied (Gamborg *et al.,* 1968, 1976; Gamborg and Shyluk, 1981).

The essential micronutrients required in micromolar concentrations include Fe (Iron), Mn (Manganese), Zn (Zinc), B (Boron), Cu (Copper) and Mo (Molybdenum). Iron and sometimes Zinc are supplied as the chelate with versene (ethylenediaminetetraacetic acid). Co (Cobalt) can also be added. Iodine is also included in several media but is not a necessity (Gamborg and Shyluk, 1981).

The most widely used nutrients are those of White (1943) and Heller (1953). Since, 1960 Murashige and Skoog's (MS) high salt formulation (1962) or its derivatives such as B5 (Gamborg *et al.*, 1968) and Schenk and Hildebrandt (SH) (1972) is being widely used. The major differences are the relative form and amount of nitrogen, plus the amounts of some of the microelements (Gamborg *et al.*, 1976).

Carbon and energy sources: Sucrose or glucose is added to the media to meet the energy requirement (Thorpe *et al.*, 1991). This is usually fulfilled by

sucrose of 2 - 4 % (w/v), sometimes sucrose can be replaced by glucose (Thorpe and Patel, 1984; Gamborg and Shyluk, 1981). Fructose can also be used but is less suitable. Lactose, maltose, galactose and starch are other carbohydrates that have been investigated but, are generally inferior as carbon sources. Species of Rosaceae and cells of apple have used sorbitol as their energy source. A concentration of sucrose is generally 2 - 3% (w/v). The inclusion of myo-inositol in some media is also prevalent, although there is no requirement. 100 mgL^{-1} improves cell growth (Gamborg and Shyluk, 1981).

Vitamins: Vitamins are required for growth, development and for micropropagation, some vitamins may become limiting. Vitamins such as thiamine, nicotinic acid and pyridoxine are required to aid the growth and differentiation of numerous tissues in culture (Thorpe *et al.*, 1991). Thiamine is the most commonly used vitamin followed by nicotinic acid and pyridoxine (Gamborg and Shyluk, 1981; Thorpe and Patel, 1984). Studies have shown that there is no requirement for thiamine (Ohira *et al.*, 1976). Panthothenate and biotin are used in some media, but these vitamins are not considered growth-limiting factors. Folate, choline, chloride, riboflavin, p-amino- benzoic acid and ascorbic acid would be desirable to grow cells in if used at very low concentrations (Gamborg and Shyluk, 1981).

Reduced nitrogen: Organic nitrogen is often added during micropropagation but may be beneficial during subculture and organised

development. Casein hydrolysate (0.02 - 0.1 % (w/v)) is a frequent non- specific organic nitrogen source, whereas glutamate, asparagine, tyrosine and adenine are the most frequently used specific reduced nitrogen additives (Thorpe and Patel, 1984).

Phytohormones: Phytohormones play an active part in shoot and root initiation and have been discussed under the relevant topics of shoot (Section 3.3) and root initiation (Section 4.3) hormones.

Considering the above requirements of media the microcuttings were inserted in sterile conditions into four different media namely; MS (Murashige and Skoog, 1962) (Table 3.4); LP (Le Poivre, 1977) (Table 3.5), GD (Gresshoff and Doy, 1972) (Table 3.6) and WPM (Woody Plant media, 1980)(Table 3.7). The major differences of these media lie in the amount and form of nitrogen and the relative amounts of some of the microelements (Thorpe, 1982). The substances found in the media are required for shoot and root formation. These include inorganic salt mixture and organic substances. Among the organic substances are carbohydrates (30 gL^{-1}), vitamins, amino acids and growth regulators such as auxins (root growth hormones) and cytokinins (shoot growth regulators). MS, LP, GD and WPM have been successfully used in woody species for shoot initiation experiments. A brief list of certain plant species experimented on each media is listed in Table 3.3.

Table 3.3. Summary of the media experimented with certain woody species.

Media	Species	Author
MS	*Acacia mangium*	Galiana *et al.*, 1991
		Gan and Liang, 1992
		Ahmad, 1991
	Acacia auriculiformis	Darus, 1992
	Hibiscus cannabinus	Cristofari *et al.*, 1988
	Eucalyptus sideroxylon	Cheng *et al.*, 1992
	Eucalyptus tereticornis	Rao, 1988
		Das and Mitra, 1990
	Eucalyptus gunnii	Curir *et al.*, 1985; 1986; 1990
	Eucalyptus stuartiana	Curir *et al.*, 1985; 1986; 1990
	Eucalyptus saligna	Le Roux & Van Staden, 1991a; b
	Melia azedarach	Domecq, 1988
	Eucalyptus curvifolia	Texier and Faucher, 1986
	Eucalyptus camaldulensis	Kumar and Ayyappan, 1987
		Kumar and Raman, 1989
	Eucalyptus macarthurii	Le Roux & Van Staden, 1991a; b
LP	*Eucalyptus gunnii*	Curir *et al.*, 1985; 1986; 1990
	Eucalyptus stuartiana	Curir *et al.*, 1985; 1986; 1990
	Eucalyptus curvifolia	Texier and Faucher, 1986
GD	*Eucalyptus gunnii*	Curir *et al.*, 1986
	Eucalyptus stuartiana	Curir *et al.*, 1986
	Eucalyptus novoanglica	Mehra Palta, 1982
WPM	*Eucalyptus tereticornis*	Subbiah and Minocha, 1990
	Eucalyptus regnans	Blomstedt *et al.*, 1991

MS media

Table 3.4 Constituents of Murashige and Skoog Basal Medium (1962) for the preparation of 1 litre stock solutions.

	Ingredients	Formulae	gL^{-1} (100x)
Major salts	Ammonium Nitrate	NH_4NO_3	165
	Potassium Nitrate	KNO_3	190
	Magnesium sulphate	$MgSO_4.7H_2O$	37
	Potassium Hydrogen Sulphate	KH_2PO_4	17
	Calcium chloride	$CaCl_2.6H_2O$	44
Minor salts	Iron Sodium EDTA	FeNaEDTA	3.67
	Cobalt chloride	$CoCl_2\,6H_2O$	0.0025
	Copper sulphate	$CUSO_4.H_2O.$	0.0025
	Sodium Molybdate	$NaMoO_4.H_2O$	0.025
	Boric acid	H_3BO_3	0.62
	Potassium Iodide	KI	0.083
	Manganese sulphate	$MnSO_4.4H_2O$	2.23
	zinc sulphate	$ZnSO_4.7H_2O$	0.86
Vitamins	Inositol		10
	Nicotinic acid		0.05
	Thiamine HCl		0.01
	Pyridoxine HCl		0.05
	Glycine		0.2

LP Media

Table 3.5 Constituents of LP media (Le Poivre, 1977) for the preparation of 2 litre stock solutions

	Ingredient	Formulae	gL^{-1} (50x)
Major salts	Potassium Nitrate	KNO_3	72
	Calcium Nitrate	$Ca(NO_3)_2.4H_2O$	48
	Ammonium Nitrate	NH_4NO_3	16
	Magnesium Sulphate	$MgSO_4.7H_2O$	14.4
	Potassium Hydrogen Phosphate	KH_2PO_4	10.8
Minor salts and vitamins	Zinc Sulphate	$ZnSO_4.7H_2O$	8.6
	Boric acid	H_3BO_3	6.2
	Manganese Sulphate	$MnSO_4.4H_2O$	20
	Copper Sulphate	$CuSO_4.5H_2O$	0.25
	Potassium Iodide	KI	0.08
	Sodium Molybdate	$Na_2MoO_4.2H_2O$	0.25
	Cobalt Chloride	$CoCl_2.6H_2O$	0.025
		Thiamine HCl	0.4

GD Media

Table 3.6 Constituents of GD media (Gresshoff and and Doy, 1972)for the preparation of 1 litre stock solutions

	Ingredient	Formulae	gL^{-1} (50x)
Major salts	Ammonium Sulphate	$(NH_4)_2SO_4$	4
	Magnesium Sulphate	$MgSO_4.7H_2O$	5
	Potassium Nitrate	KNO_3	20
	Potassium Chloride	KCl	6
	Sodium Hydrogen Phosphate	$NaH_2PO_4.2H_2O$	2
	Disodium Hydrogen Phosphate	Na_2HPO_4 or	0.6
		$Na_2HPO_4.12H_2O$	1.5
Minor salts	Manganese Sulphate	$MnSO_4$ $4H_2O$	2
	Boric acid	H_3BO_3	0.5
	Zinc Sulphate	$ZnSO_4$ $7H_2O$	0.1
	Potassium iodide	KI	0.1
	Copper Sulphate	$CuSO_4$ $5H_2O$	0.02
	Sodium Molybdate	Na_2MoO_4 $2H_2O$	0.02
	Cobalt Chloride	$CoCl_2$ $6H_2O$	0.02
Vitamins	Thiamine HCl		0.5
	Nicotinic acid		0.5
	Pyridoxine HCl		0.05

WPM Media

Table 3.7. Constituents of WPM (Lloyd and McCown, 1980) for the preparation of 1 litre stock solutions

	Ingredient	Formulae	gL^{-1} (100x)
Major salts	Magnesium sulphate	$MgSO_4\ 7H_2O$	15
	Potassium sulphate	K_2SO_4	57
	Potassium hydrogen phosphate	KH_2PO_4	15
	Calcium chloride	$CaCl_2\ 2H_2O$	6.5
	Ammonium nitrate	NH_4NO_3	50
	Calcium nitrate	$Ca(NO_3)_2\ 4H_2O$	23
Minor salts	Boric acid	H_3BO_3	1.0
	Manganese sulphate	$MnSO_4\ H_2O$	1.32
	Zinc sulphate	$ZnSO_4\ 7H_2O$	0.3
	Copper sulphate	$CuSO_4\ 5H_2O$	0.01
	Sodium molybdate	$Na_2MoO_4\ 2H_2O$	0.01
	Iron EDTA	FeEDTA	0.5
Vitamins	Inositol		5.55
	Thiamine HCl		0.03
	Pyridine HCl		0.02
	Nicotinic acid		0.04
	Glycine		0.27

3.5 Materials and Methods

3.5.1 Preparation of shoot initiation media

The procedure for the preparation of MS (Murashige and Skoog, 1962) has been described under seed germination methodology and techniques (Section 2.5.2). This media however was full strength (Table 3.4) unlike that used in seed germination. LP, GD and WPM media were prepared using the contents listed under Tables 3.5, 3.6 and 3.7. Concentrations of the various cytokinins such as zeatin, BAP, 2iP and kinetin (Cytokinins were purchased from Sigma, U.S.A) ranging from 0.1 to 2.5 mgL^{-1} were added to each of the media alongwith 0.5 mgL^{-1} IBA and pH adjusted to 5.6 by adding 0.1 M KOH and HCl. Since, zeatin could be inactivated by autoclaving due to its thermolabile properties therefore, cytokinins were filter sterilised. High temperature can denature proteins and cause poor root and shoot growth, therefore extra precaution was taken during the preparation of the various concentrations of the hormones.

3.5.2 Selection of germinated explants

Nodal explants from seed germination experiments that had developed 3 to 4 internodes or that which have reached a height of 3 cms or more were used for shoot initiation studies and deemed as shoots per explant. Callus growth was observed in few explants after seed germination experiments, however, these

explants were utilised as long as they fulfilled the height and nodal requirements. Any plants with visible signs of necrosis such as yellowing leaves or wilted stems were discarded.

Plants were aseptically dissected at the internodes which contained lateral buds large enough to exist as explants into 1cm long segments. Two to three leaves were left on the explant and the rest of them removed to curtail excessive transpiration. 25 bisected nodal explants were transferred to MS hormone free media was deemed as control. Similarly, 25 explants each were planted in 0.1 0.25, 0.5, 0.75,1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 mgL^{-1}. Zeatin supplemented MS media. Similar concentration ranges were carried out with BAP, 2iP and kinetin for MS media. Similarly all four hormones with above concentrations were supplemented for LP, GD and WPM. To minimise contamination and maintain sterility of the explants contact with the sides of the petridish in which they were subcultured or into the jars they were being transplanted were avoided. The sealed containers were then placed under controlled conditions as underlined under Table 2.8 (Section 2.5).

At 3-5 weeks intervals the explants were subcultured again. Healthy explants were transferred into their respective media and hormone concentrations in a manner similar to the one outlined above. Likewise, four subsequent trials were conducted for each hormone free and cytokinin supplemented media.

3.5.3 Multiplication studies

Established cultures were planted on shoot multiplication media as listed under 3.5.1. Shoots with elongated stems and at least two well-developed leaves were taken as microcuttings (around 1cm tall) were cultured vertically on the four different media supplemented with various concentrations of the cytokinins.

There were 25 replicates per concentration per hormone for each media. The explants were observed at regular intervals and results tabulated. After 4 weeks, the explants that had reached the required height of 3 cms or more or containing 3 to 4 internodes were subcultured again into the four types of media (MS, LP, GD and WPM) and hormones (Zeatin, BAP, 2iP and Kinetin) as listed. Shoot initiation experiments were repeated four times for each media and hormone type.

3.6 Statistical Analysis

Repeated Measures Design and Duncan Multiple Range Test was performed using SAS (Version '95) package. Significant levels between each Cytokinin was determined using Paired samples Statistics and Paired samples Correlations. Confidence Interval (range) was calculated using the formula the statistical formula Mean +/- 1.96 x s.e, where, 1.96 is the decimal representation for both

sides of the curve of a normal distribution for a 95% confidence interval and s.e is the standard error. Confidence intervals are calculated at 95% for experimental purposes with a 5% risk involved in the experiments.

3.7 Results

Shoot growth was compared between the respective media (MS, LP, GD and WPM) supplemented with cytokinins (Zeatin, BAP, 2iP and Kinetin) over a range of increasing concentrations as well as the controls (hormone free) for each media. The highest length of shoot obtained for each concentration for all four media is listed in Plate 3.1, 3.2 and 3.3.

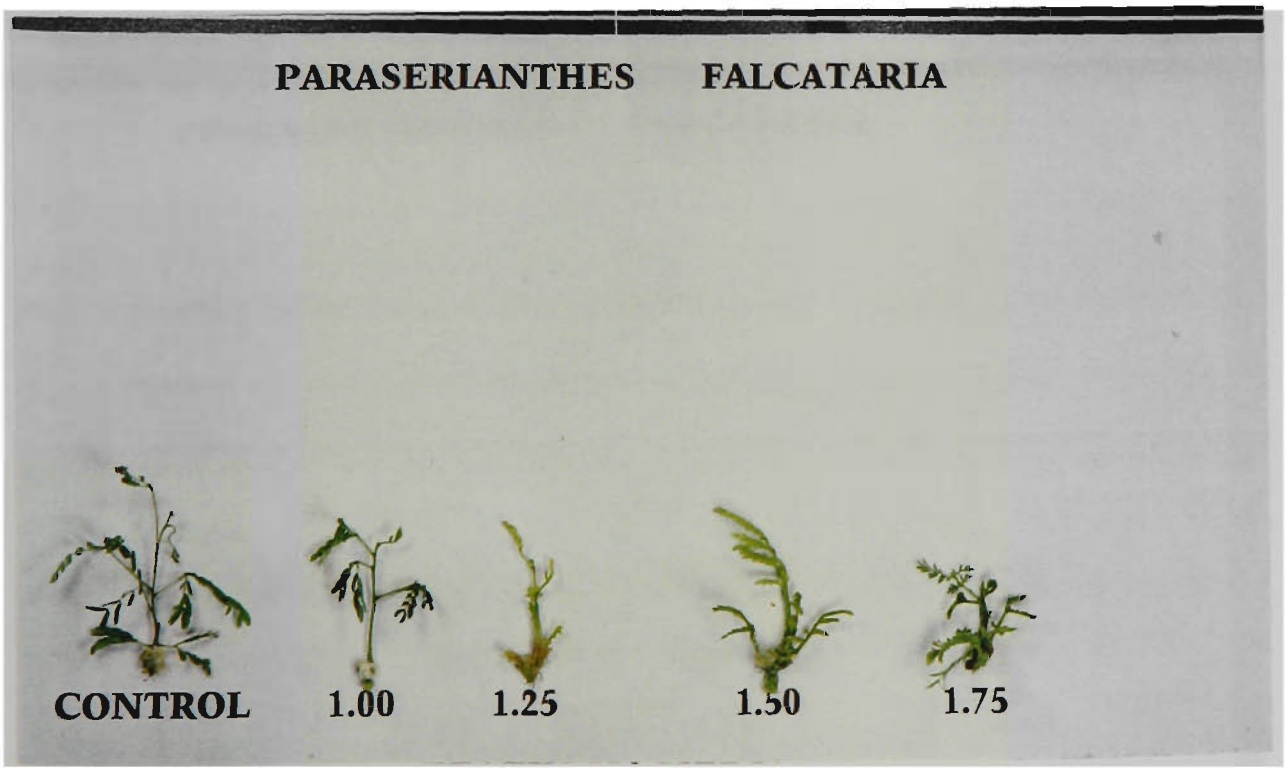

Plate 3.1 Highest length of shoot obtained for concentrations 0.1 to 0.75 mgL^{-1} for all four media and all four cytokinins.

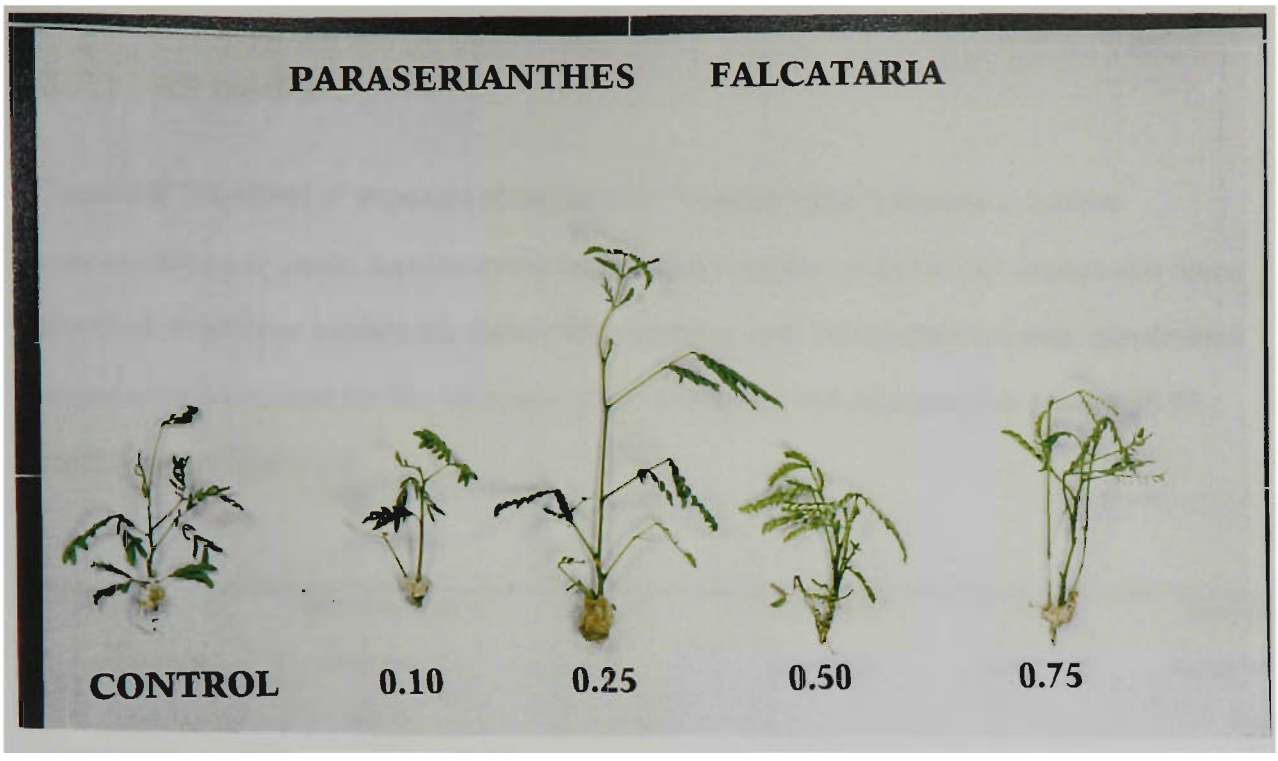

Plate 3.2 Highest length of shoot obtained for concentrations 1.0 to 1.75 mgL^{-1} for all four media and all four cytokinins.

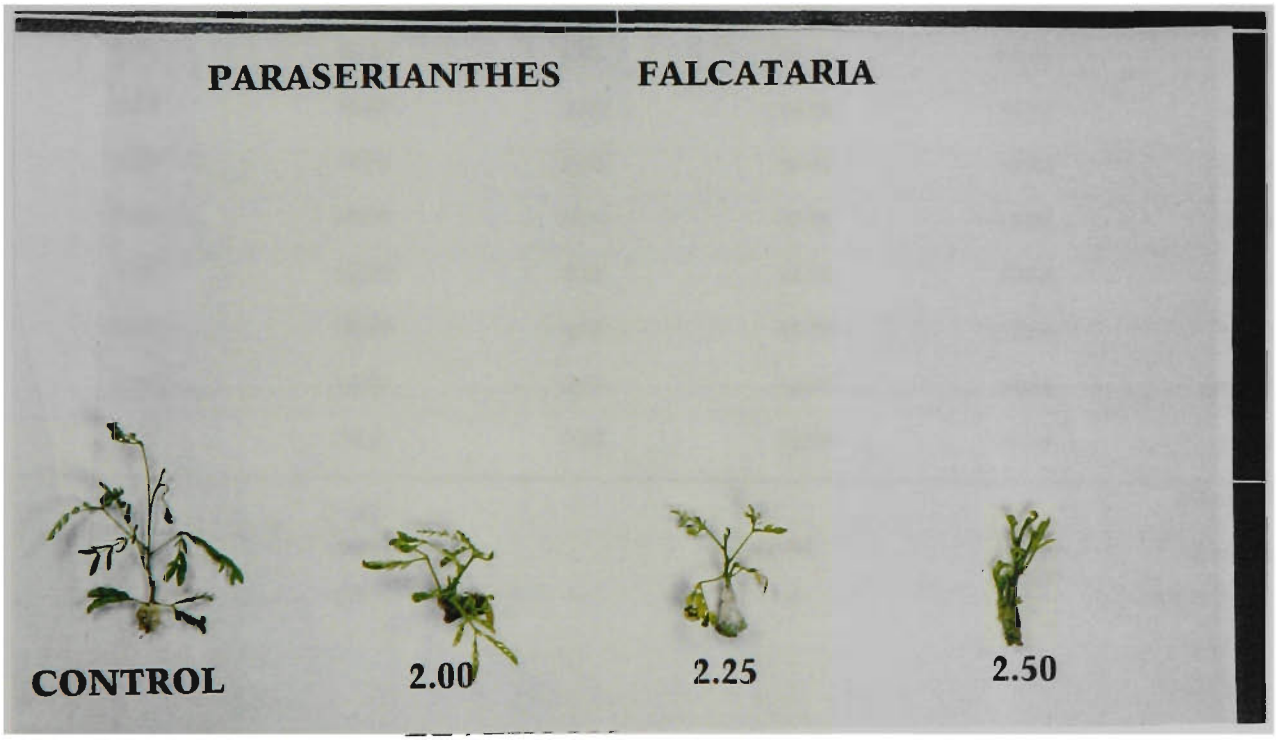

Plate 3.3 Highest length of shoot obtained for concentrations 2.0 to 2.5 mgL^{-1} for all four media and all four cytokinins.

3.7.1 MS media

Table 3.8 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of zeatin supplemented in MS; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from MS media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Interval Upper Limit	Lower Limit	Mean length of shoots per explant (cms)
0.00	10.00	0.03	10.06	9.94	13.50
0.10	18.10	0.03	18.16	18.04	22.10
0.25	19.40	0.03	19.46	19.34	23.40
0.50	21.20	0.01	21.22	21.18	25.60
0.75	18.10	0.02	18.14	18.06	21.90
1.00	16.80	0.02	16.84	16.76	19.40
1.25	16.70	0.03	16.76	16.64	19.40
1.50	16.00	0.03	16.06	15.94	20.00
1.75	15.90	0.02	15.94	15.86	18.00
2.00	15.70	0.03	15.76	15.64	18.20
2.25	14.60	0.03	14.66	14.54	17.10
2.50	13.6	0.03	13.66	13.54	17.10
					Mean = 19.64
					s.e. = 0.01

Table 3.9 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of BAP supplemented in MS; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from MS media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Interval Upper Limit	Lower Limit	Mean length of shoots per explant (cms)
0.00	10.00	0.03	10.06	9.94	13.50
0.10	19.60	0.02	19.64	19.56	23.20
0.25	18.20	0.02	18.24	18.16	22.20
0.50	17.00	0.03	17.06	16.94	21.50
0.75	17.00	0.03	17.06	16.94	21.40
1.00	16.50	0.02	16.54	16.46	19.70
1.25	15.50	0.01	15.52	15.48	18.40
1.50	14.50	0.02	14.54	14.46	17.20
1.75	14.40	0.02	14.44	14.36	17.20
2.00	14.30	0.03	14.36	14.24	16.90
2.25	13.40	0.03	13.46	13.34	15.00
2.50	12.20	0.02	12.24	12.16	14.90
					Mean = 18.43
					s.e. = 0.02

Table 3.10 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of 2iP supplemented in MS; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from MS media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	10.00	0.03	10.06	9.94	13.50
0.10	18.80	0.03	18.86	18.74	22.40
0.25	17.90	0.02	17.94	17.86	21.50
0.50	17.20	0.03	17.26	17.14	21.50
0.75	16.40	0.03	16.46	16.34	21.40
1.00	15.60	0.02	15.64	15.56	20.60
1.25	13.60	0.01	13.62	13.58	16.50
1.50	13.10	0.03	13.16	13.04	13.50
1.75	12.40	0.03	12.46	12.34	15.90
2.00	12.40	0.03	12.46	12.34	15.40
2.25	11.30	0.02	11.34	11.26	14.70
2.50	11.10	0.01	11.12	11.08	14.60
					Mean = 17.63
					s.e. = 0.01

Table 3.11 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of Kinetin supplemented in MS; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from MS media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	10.00	0.03	10.06	9.94	13.50
0.10	17.80	0.02	17.84	17.76	21.60
0.25	17.60	0.01	17.62	17.58	21.60
0.50	16.40	0.03	16.46	16.34	20.50
0.75	15.50	0.03	15.56	15.44	19.40
1.00	13.90	0.03	13.96	13.84	18.60
1.25	13.20	0.03	13.26	13.14	16.50
1.50	12.70	0.02	12.74	12.66	15.90
1.75	11.00	0.01	11.02	10.98	14.60
2.00	10.90	0.03	10.96	10.84	14.50
2.25	10.80	0.03	10.86	10.74	14.50
2.50	10.50	0.03	10.53	10.47	14.50
					Mean = 17.14
					s.e. = 0.01

The control for MS had a confidence interval of 10.06 to 9.94 for the mean number of shoots per explant (Tables 3.8, 3.9, 3.10 and 3.11). Mean length of shoots per explant for hormone free treatment was 13.50 cms. The highest mean number of shoots per explant was obtained at 0.5 mgL^{-1} zeatin (Table 3.8) which was evidenced in the confidence interval of 21.22 to 21.18 number of shoots per explant (Table 3.8). However, with increasing zeatin concentrations a marked decline in the mean number of shoots per explant became apparent. The least mean

number of shoots per explant was observed at the concentration of 2.5 mgL^{-1} with a confidence interval of 13.66 to 13.54 (Table 3.8) which was the highest concentration of zeatin utilised. Cultures were much healthier and were easily distinguishable with elongated shoots. Mean length of explants for zeatin treatment in MS media was 17.10 to 25.60 cms. In MS supplemented with BAP the highest mean number of explants was obtained at the concentration of 0.1 mgL^{-1} (Table 3.9) within the confidence interval of 19.64 to 19.56 number of shoots per explant (Table 3.9). Similar to the case of zeatin, there was a decrease in the mean number of shoots per explants with increasing concentrations for BAP. The least mean number of explants was observed at the concentration of 2.5 mgL^{-1} with a confidence interval of 12.24 to 12.16 (Table 3.9). Length of explants varied between 14.90 to 23.20 cms per explant. There was a decrease in the length of the explants with increase in BAP concentrations. Similar to shoot initiation results obtained with BAP, 2iP showed an optimum at the concentration of 0.10 mgL^{-1}. Mean number of shoots per explants ranged between the confidence intervals of 18.86 to 18.74 (Table 3.10). A marked decline in the mean number of shoots per explant was noticed with increasing cytokinin concentrations (Table 3.10). The least mean number and mean length (14.60 to 22.40 cms) of explants was evidenced at the highest concentration of 2.5 mgL^{-1} (Table 3.10). The highest mean number of shoots per explant was obtained at 0.1 mgL^{-1} kinetin (Table 3.11) which was evidenced in the confidence interval of 17.84 to 17.76 number of shoots per explant (Table 3.11). However, with increasing kinetin concentrations a marked decline in the mean number of shoots per explant became apparent. The least mean number of shoots per explant was observed at the concentration of 2.5 mgL^{-1} with a confidence interval of 10.53 to 10.47 (Table 3.11). Mean length of explants varied between 14.50 and 21.60 cms.

3.7.2 LP Media

Table 3.12The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of Zeatin supplemented in LP; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from LP media from seed germination Experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment

Concentration of cytokinin (mgL^{-1})	Mean number of Shoots per Explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	8.00	0.01	8.02	7.98	11.20
0.10	17.90	0.02	17.96	17.84	21.50
0.25	18.60	0.02	18.66	18.54	22.40
0.50	18.80	0.03	18.86	18.74	22.50
0.75	17.00	0.03	17.06	16.94	21.90
1.00	16.30	0.02	16.34	16.26	20.50
1.25	16.30	0.01	16.32	16.28	20.50
1.50	15.30	0.02	15.36	15.24	19.40
1.75	13.20	0.02	13.26	13.14	17.50
2.00	12.20	0.03	12.26	12.14	17.50
2.25	12.10	0.03	12.16	12.04	17.40
2.50	12.10	0.02	12.14	12.06	17.30
					Mean = 19.63
					s.e. = 0.02

Table 3.13The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of BAP supplemented in LP; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from LP media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	8.00	0.01	8.02	7.98	11.20
0.10	16.00	0.02	16.06	15.94	19.20
0.25	16.90	0.02	16.96	16.84	19.50
0.50	17.20	0.03	17.26	17.14	20.60
0.75	15.90	0.01	15.96	15.84	16.80
1.00	15.30	0.02	15.34	15.26	16.80
1.25	14.30	0.02	14.36	14.24	15.70
1.50	13.30	0.03	13.36	13.24	15.70
1.75	12.50	0.03	12.52	12.48	15.50
2.00	12.20	0.02	12.26	12.14	15.50
2.25	12.00	0.03	12.06	11.94	15.40
2.50	12.10	0.02	12.16	12.04	15.40
					Mean = 16.44
					s.e. = 0.02

Table 3.14 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of 2iP supplemented in LP; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from LP media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	8.00	0.03	8.02	7.98	11.20
0.10	16.70	0.03	16.74	16.66	19.40
0.25	15.70	0.03	15.74	15.66	18.80
0.50	13.70	0.03	13.74	13.66	16.50
0.75	13.60	0.02	13.62	13.58	16.50
1.00	13.20	0.01	13.26	13.14	16.30
1.25	13.20	0.03	13.24	13.16	16.30
1.50	12.90	0.02	12.96	12.84	15.90
1.75	12.10	0.03	12.14	12.06	15.40
2.00	12.10	0.03	12.14	12.06	15.30
2.25	12.00	0.02	12.02	11.98	15.20
2.50	12.00	0.01	12.02	11.98	15.20
					Mean = 16.00
					s.e. = 0.03

Table 3.15 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of kinetin supplemented in LP; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from LP media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	8.00	0.03	8.04	7.96	11.20
0.10	15.90	0.02	15.92	15.88	17.90
0.25	14.70	0.01	14.76	14.64	16.50
0.50	14.20	0.03	14.24	14.16	16.30
0.75	14.70	0.03	14.76	14.64	15.90
1.00	12.90	0.03	12.94	12.86	13.20
1.25	12.80	0.03	12.84	12.76	13.20
1.50	12.70	0.02	12.72	12.68	13.00
1.75	12.60	0.01	12.66	12.54	12.90
2.00	11.90	0.03	11.94	11.86	12.50
2.25	11.00	0.03	11.06	10.94	12.30
2.50	11.00	0.03	11.04	10.96	12.10
					Mean = 13.92
					s.e. = 0.03

The control for LP had a confidence interval of 8.02 to 7.98 for the mean number of shoots per explant (Tables 3.12, 3.13, 3.14 and 3.15). Mean length of explants was 11.20 cms. The highest mean number of shoots per explant was obtained at 0.5 mgL^{-1} zeatin (Table 3.12) which was evidenced in the confidence interval of 18.86 to 18.74, number of shoots per explant (Table 3.12). However, with increasing zeatin concentrations a marked decline in the mean number of shoots per explant became apparent. The least mean number of shoots per explant was

observed at the concentration of 2.0 to 2.5 mgL^{-1} with a confidence interval of 12.26 to 12.06 (Table 3.12) which was the highest concentration of zeatin utilised. Mean length of shoots was between 17.30 and 21.50 cms. In MS supplemented with BAP the highest mean number of explants was obtained at the concentration of 0.5 mgL^{-1} (Table 3.13) within the confidence interval of 17.26 to 17.14 number of shoots per explant (Table 3.13). Similar to the case of zeatin, there was a decrease in the mean number of shoots per explants with increasing concentrations for BAP as well. The least mean number of explants was observed at the concentration of 2.0 to 2.5 mgL^{-1} with a confidence interval of 12.26 to 12.04 (Table 3.13). The average length of the explants was between 15.40 and 20.60 cms. Similar to shoot initiation results obtained with BAP, 2iP showed an optimum at the concentration of 0.10 mgL^{-1}. Mean number of shoots per explants ranged between the confidence intervals of 16.74 to 16.66 (Table 3.14). A marked decline in the mean number of shoots per explant was noticed with increasing cytokinin concentrations (Table 3.14). The least mean number (12) of explants was evidenced at the highest concentration of 2.25 to 2.5 mgL^{-1} (Table 3.14). Length of the explants was between 15.20 and 19.40 cms. The highest mean number of shoots per explant was obtained at 0.1 mgL^{-1} kinetin (Table 3.15) which was evidenced in the confidence interval of 15.94 to 15.88 number of shoots per explant (Table 3.15). However, with increasing kinetin concentrations a marked decline in the mean number of shoots per explant became apparent. The least mean number of shoots per explant was observed at the concentration of 2.25 to 2.5 mgL^{-1} with a confidence interval of 11.06 to 10.96 (Table 3.15). Mean length of the shoots was in the range of 12.10 to 17.90.

3.7.3 GD Media

Table 3.16 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of zeatin supplemented in GD; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from GD media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of Shoots per Explant	Standard error	Confidence Interval Upper Limit	Lower Limit	Mean length of shoots per explant (cms)
0.00	6.00	0.02	6.04	5.96	9.50
0.10	12.80	0.01	12.82	12.78	15.50
0.25	13.90	0.03	13.96	13.84	16.50
0.50	13.40	0.02	13.44	13.36	16.70
0.75	13.40	0.02	13.44	13.36	16.80
1.00	12.30	0.01	12.32	12.28	15.90
1.25	12.30	0.03	12.36	12.24	15.40
1.50	11.30	0.02	11.34	11.26	14.90
1.75	11.20	0.01	11.22	11.18	14.70
2.00	10.90	0.03	10.96	10.84	14.50
2.25	10.00	0.02	10.04	9.96	13.50
2.50	10.00	0.02	10.04	9.96	13.40
					Mean = 14.78
					s.e = 0.02

Table 3.17 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of BAP supplemented in GD; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from GD media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Interval Upper Limit	Lower Limit	Mean length of shoots per explant (cms)
0.00	6.00	0.03	6.06	5.94	9.50
0.10	15.40	0.02	15.44	15.36	18.20
0.25	14.40	0.03	14.46	14.34	17.60
0.50	14.30	0.02	14.34	14.26	17.50
0.75	14.30	0.01	14.32	14.28	17.40
1.00	14.20	0.03	14.26	14.14	17.40
1.25	14.10	0.02	14.14	14.06	17.30
1.50	13.00	0.02	13.04	12.96	16.80
1.75	12.60	0.01	12.62	12.58	15.30
2.00	12.20	0.03	12.26	12.14	15.20
2.25	11.90	0.02	11.94	11.86	15.10
2.50	11.70	0.03	11.76	11.64	15.10
					Mean = 16.03
					s.e. = 0.02

Table 3.18 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of 2iP supplemented in GD; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from GD media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	6.00	0.02	6.04	5.96	9.50
0.10	13.90	0.01	13.92	13.88	16.70
0.25	14.00	0.03	14.06	13.94	17.50
0.50	13.90	0.02	13.94	13.86	16.80
0.75	12.80	0.03	12.86	12.74	15.70
1.00	12.70	0.02	12.74	12.66	15.50
1.25	12.40	0.02	12.44	12.36	15.70
1.50	12.40	0.01	12.42	12.38	15.50
1.75	11.30	0.03	11.36	11.24	14.80
2.00	11.10	0.02	11.14	11.06	14.70
2.25	10.90	0.03	10.96	10.84	14.50
2.50	10.80	0.02	10.84	10.76	14.20
					Mean = 15.09
					s.e. = 0.02

Table 3.19 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of kinetin supplemented in GD; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from GD media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard Error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	6.00	0.02	6.04	5.96	9.50
0.10	14.40	0.01	14.42	14.38	17.30
0.25	14.10	0.03	14.16	14.04	17.20
0.50	13.90	0.02	13.94	13.86	16.50
0.75	13.90	0.03	13.96	13.84	16.20
1.00	13.70	0.02	13.74	13.66	15.90
1.25	12.50	0.02	12.54	12.46	15.40
1.50	12.30	0.01	12.32	12.28	15.20
1.75	11.90	0.03	11.96	11.84	15.20
2.00	11.80	0.02	11.84	11.76	15.20
2.25	11.80	0.03	11.86	11.74	15.10
2.50	11.70	0.02	11.74	11.66	15.10
					Mean = 15.32
					s.e. = 0.01

The control for GD had a confidence interval of 6.04 to 5.96 for the mean number of shoots per explant (Table 3.16, 3.17, 3.18 and 3.19). Average length of the shoots was 9.50 cms. The highest mean number of shoots per explant was obtained at 0.25 mgL^{-1} zeatin (Table 3.16) which was evidenced in the confidence interval of 13.96 to 13.84 number of shoots per explant (Table 3.16).

However, with increasing zeatin concentrations a marked decline in the mean number of shoots per explant became apparent. The least mean number of shoots per explant was observed at the concentration of 2.25 to 2.5 mgL^{-1} with a confidence interval of 10.04 to 9.96 (Table 3.16) which was the highest concentration of zeatin utilised. Length of the explants was between 13.40 and 16.80 cms. In MS supplemented with BAP the highest mean number of explants was obtained at the concentration of 0.1mgL^{-1}(Table 3.17) within the confidence interval of 15.44 to 15.36 number of shoots per explant (Table 3.17). Similar to the case of zeatin, there was a decrease in the mean number of shoots per explants with increasing concentrations for BAP as well. The least mean number of explants was observed at the concentration of 2.5mgL^{-1} with a confidence interval of 11.76 to 11.64 (Table 3.17). Mean length of the explants was between 15.10 and 18.20 cms. Similar to shoot initiation results obtained with BAP, 2iP showed an optimum at the concentration of 0.25 to 0.5 mgL^{-1}. Mean nurnber of shoots per explants ranged between the confidence intervals of 14.06 to 13.86 (Table 3.18). A marked decline in the mean number of shoots per explant was noticed with increasing cytokinin concentrations (Table 3.18). The least mean number of explants was evidenced at the highest concentration of 2.25 to 2.5 mgL^{-1} with a confidence interval of 10.96 to 10.76 (Table 3.18). 14.20 to 16.80 cms was the range of the mean length of the explants.The highest mean number of shoots per explant was obtained at 0.1 mgL^{-1} kinetin (Table 3.19) which was evidenced in the confidence interval of 14.42 to 14.38 number of shoots per explant (Table 3.19). However, with increasing kinetin concentrations a marked

decline in the mean number of shoots per explant became apparent. The least mean number of shoots per explant was observed at the concentration of 1.75 to 2.25mgL^{-1} with a confidence interval of 11.96 to 11.66 (Table 3.19). Mean length of the shoots ranged between 15.10 and 17.30 cms.

3.7.4 WPM Media

Table 3.20 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of zeatin supplemented in WPM; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from WPM media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	7.00	0.02	7.04	6.96	10.90
0.10	18.30	0.01	18.32	18.28	23.40
0.25	19.40	0.03	19.46	19.34	24.10
0.50	19.30	0.03	19.36	19.24	24.20
0.75	18.60	0.02	18.64	18.56	23.00
1.00	17.50	0.02	17.54	17.46	22.50
1.25	16.70	0.03	16.76	16.64	21.80
1.50	15.30	0.02	15.34	15.26	20.50
1.75	14.90	0.02	14.94	14.86	20.20
2.00	13.70	0.01	13.72	13.68	19.70
2.25	12.80	0.02	12.84	12.76	18.60
2.50	12.70	0.03	12.76	12.64	18.50
					Mean = 20.62
					s.e. = 0.01

Table 3.21 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of BAP supplemented in WPM; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from WPM media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Interval Upper Limit	Lower Limit	Mean length of shoots per explant (cms)
0.00	7.00	0.02	7.04	6.96	10.90
0.10	17.10	0.03	17.16	17.04	20.50
0.25	17.10	0.02	17.14	17.06	20.40
0.50	17.40	0.02	17.44	17.36	20.40
0.75	16.80	0.01	16.82	16.78	19.60
1.00	16.30	0.02	16.34	16.26	19.40
1.25	12.90	0.03	12.96	12.84	17.90
1.50	12.60	0.02	12.64	12.56	16.50
1.75	12.40	0.02	12.44	12.36	16.40
2.00	12.20	0.03	12.26	12.14	15.70
2.25	12.10	0.02	12.14	12.06	14.70
2.50	11.90	0.02	11.94	11.86	13.20
					Mean = 17.13
					s.e = 0.02

Table 3.22 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of 2iP supplemented in WPM; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from WPM media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Interval Upper Limit	Lower Limit	Mean length of shoots per explant (cms)
0.00	7.00	0.02	7.04	6.96	10.90
0.10	16.20	0.01	16.22	16.18	19.60
0.25	16.30	0.03	16.36	16.24	19.40
0.50	16.10	0.03	16.16	16.04	17.90
0.75	15.90	0.02	15.94	15.86	16.50
1.00	14.70	0.02	14.74	14.66	16.40
1.25	14.20	0.03	14.26	14.14	16.40
1.50	14.20	0.02	14.24	14.16	15.70
1.75	14.10	0.02	14.14	14.06	14.70
2.00	13.90	0.01	13.92	13.88	12.20
2.25	13.00	0.03	13.06	12.94	12.10
2.50	12.90	0.02	12.94	12.86	11.90
					Mean = 15.31
					s.e. = 0.01

Table 3.23 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of kinetin supplemented in WPM; Mean number of shoots per explant and mean length of shoots per explant are shown. Microcuttings from WPM media from seed germination experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration Of cytokinin (mgL^{-1})	Mean number of shoots per explant	Standard error	Confidence Upper Limit	Interval Lower Limit	Mean length of shoots per explant (cms)
0.00	7.00	0.03	7.06	6.94	10.90
0.10	16.10	0.02	16.14	16.06	21.80
0.25	15.50	0.02	15.54	15.46	20.50
0.50	14.40	0.01	14.42	14.38	20.20
0.75	13.40	0.03	13.46	13.34	19.70
1.00	12.90	0.02	12.94	12.86	18.60
1.25	12.90	0.02	12.94	12.86	18.50
1.50	12.80	0.03	12.86	12.74	16.50
1.75	12.70	0.02	12.74	12.66	16.40
2.00	12.70	0.02	12.74	12.66	16.40
2.25	12.50	0.01	12.52	12.48	15.70
2.50	12.40	0.02	12.44	12.36	14.70
					Mean = 17.49
					s.e. = 0.01

The control for MS had a confidence interval of 7.04 to 6.96 for the mean number of shoots per explant (Table 3.20, 3.21, 3.22 and 3.23). Mean length of the explants was 10.90 cms. The highest mean number of shoots per explant was obtained at 0.25 to 0.5 mgL^{-1} zeatin (Table 3.20) which was evidenced in the confidence interval of 19.46 to 19.24 number of shoots per explant (Table

3.20). However, with increasing zeatin concentrations a marked decline in the mean number of shoots per explant became apparent. The least mean number of shoots per explant was observed at the concentration of 2.25 to 2.5mgL^{-1} with a confidence interval of 12.84 to 12.64 (Table 3.20) which was the highest concentration of zeatin utilised. Average length of the shoots was between 18.50 and 24.20 cms. In MS supplemented with BAP the highest mean number of explants was obtained at the concentration of 0.5mgL^{-1}(Table 3.21) within the confidence interval of 17.44 to 17.36 number of shoots per explant (Table 3.21). Similar to the case of zeatin, there was a decrease in the mean number of shoots per explants with increasing concentrations for BAP as well. The least mean number of explants was observed at the concentration of 2.5mgL^{-1} with a confidence interval of 11.94 to 11.86 (Table 3.21). Length of the shoots varied between 13.20 and 20.50 cms. Similar to shoot initiation results obtained with BAP, 2iP showed an optimum at the concentration of 0.25 mgL^{-1}. Mean number of shoots per explants ranged between the confidence intervals of 16.36 to 16.24 (Table 3.22). A marked decline in the mean number of shoots per explant was noticed with increasing cytokinin concentrations (Table 3.22). The least mean number of explants was evidenced at the highest concentration of 2.25 to 2.5 mgL^{-1} (Table 3.22) with a confidence interval of 13.06 to 12.86. Mean length of the explant was between 11.90 and 19.60 cms. The highest mean number of shoots per explant was obtained at 0.1 mgL^{-1} kinetin (Table 3.23) which was evidenced in the confidence interval of 16.14 to 16.06 number of shoots per explant (Table 3.23). However, with increasing kinetin concentrations a marked

decline in the mean number of shoots per explant became apparent. The least mean number of shoots per explant was observed at the concentration of 2.5mgL^{-1} with a confidence interval of 12.44 to 12.36 (Table 3.23). Range of the mean length of the explants was between 14.70 and 21.80 cms.

Table 3.24 Repeated measures analysis and Duncan Grouping (Analysis of variance).

Media	Hormone	Pr > F	Mean	Duncan Grouping
MS	Control	0.0001	10.0000	A
LP		0.0001	8.0200	B
GD		0.0001	6.0400	D
WPM		0.0001	7.0400	C
MS	Zeatin	0.0001	16.3417	A
LP		0.0001	14.8167	C
GD		0.0001	11.4583	D
WPM		0.0001	15.5167	B
MS	BAP	0.0001	15.2167	A
LP		0.0001	13.8083	B
GD		0.0001	12.8417	C
WPM		0.0001	13.8167	B
MS	2iP	0.0001	14.1500	A
LP		0.0001	12.9333	B
GD		0.0001	11.8500	C
WPM		0.0001	14.0417	A

MS	Kinetin	0.0809	13.3583	A
LP		0.0809	12.7000	A, B
GD		0.0809	12.3333	B
WPM		0.0809	12.9417	A, B
MS	Zeatin	0.0001	16.3417	A
	BAP	0.0001	15.2167	B
	2iP	0.0001	14.1500	C
	Kinetin	0.0001	13.3583	D
			59.0667	
LP	Zeatin	0.0001	14.8167	A
	BAP	0.0001	13.8083	B
	2iP	0.0001	12.9333	C
	Kinetin	0.0001	12.7000	C
			54.2583	
GD	Zeatin	0.0001	11.4583	D
	BAP	0.0001	12.8417	A
	2iP	0.0001	11.8500	C
	Kinetin	0.0001	12.3333	B
			48.4833	
WPM	Zeatin	0.0001	15.5167	A
	BAP	0.0001	13.8167	B
	2iP	0.0001	14.0417	B
	Kinetin	0.0001	12.9417	C
			56.3167	

Pr = Significance probability F = Degrees of freedom

A, B, C and D = Duncan Grouping system. Means with the same letter are not significantly different.

3.8 Discussion

The aim of this study was to establish the optimum combination and concentration of hormone and media concentrations for shoot initiation in *Paraserianthes falcataria* in comparison to hormone free (control) media. Shoot formations were observed from all of the nodal explants that were subcultured in the various media supplemented with cytokinins in the concentration range of 0.1 to 2.5 mgL^{-1} as well as in hormone free media. Since it is a documented fact that a large number of species respond to a suitable auxin - cytokinin balance (Chandler and Thorpe, 1982), in the present study also a similar strategy was implemented. 0.5 mgL^{-1} IBA was added as a root initiating auxin in addition to the various concentration ranges of cytokinins.

From the results obtained in the present study, it was apparent that MS media produced statistically higher number of shoots (Mean = 59.0667) in comparison to the other three media namely LP (Mean = 54.2583), GD (Mean = 48.4833) and WPM (Mean = 56.3167) (Table 3.24), in the establishment of mean number of shoots per explant. The present phenomenon can be attributed to the fact that all the hormones tested were more adaptable to MS media. It is also evident from Duncan's Multiple Range Test that the mean of the number of shoots per explant for the range of concentrations of all four cytokinins in a single media were highest in MS media (Mean = 59.0667) with the statistical difference of P> 0.0001 between each media (Table 3.24). This proves that MS media is most

suitable for shoot initiation in *Paraserianthes falcataria* in comparison to LP, GD and WPM. It can be said that MS media has been widely used in many forestry species and has been proven adaptable due to the nitrogen content by a wide range of species. Similarly from a study conducted by Curir *et al.*(1985, 1986, 1990) on *Eucalyptus gunnii* and *Eucalyptus stuartiana* it was observed that the highest proliferation rate was also observed with MS media in comparison to GD and LP (Blomstedt *et al.*, 1991) .However, in comparison to LP (Mean = 54.2583) and GD (Mean = 48.4833), WPM (Mean = 56.3167) (Table 3.24) proved to be more optimal for shoot initiation than the other two. And thus can be concluded that MS media and to a certain extent WPM can be successfully utilised for shoot initiation in *Paraserianthes falcataria*.

Cytokinins are plant growth hormones involved in cell growth and differentiation. Their action is based on specific interactions with kinases that regulate plant cell cycle progression (Redig *et al.*,1996). Phytohormones like auxins and cytokinins are also involved in several stages of plant growth and development such as cell elongation, cell division, tissue differentiation and apical dominance (Costacurta and Vanderleyden, 1995), moreover they are also known to play critical roles in coordinating plant growth and development (Takahashi *et al.*,1993). Cytokinin is also a major limiting factor which controls *in vitro* shoot multiplication (Trindade *et al.*, 1990). A relatively high ratio of cytokinin to auxin favours shoot formation and the reverse favours root formation (Raven *et al,* 1992; Thorpe and Patel 1984).

Curir *et al.*(1985, 1986, 1990), on a study conducted on *Eucalyptus gunnii* and

Eucalyptus stuartiana also established that Kinetin and Zeatin used in combination with the auxin IBA (0.2 mgL^{-1}) resulted in a high proliferation of shoots in MS media. Further, Klimaszewska and Keller (1985), Negrutiu *et al.*(1978), Pence and Caruso (1984), Profumo *et al.* (1985), Sangwan *et al.* (1976) and Van den Ende (1984) have supported this contention from their work in tobacco tissue cultures that cytokinin alone or a high ratio of exogenous cytokinin to exogenous auxin will have a synergistic effect in stimulating a better adventitious shoot formation. Plant hormones are rarely known to act alone and for most processess at organ level, many of these regulators interact to produce the final effect (Gaspar *et al.,* 1996). In this context, it can be stated that this study also coincided with the above findings where the highest shoot initiation was recorded with exogenous cytokinin (zeatin, BAP, 2iP and kinetin) and auxin (0.5 mgL^{-1} IBA) interaction against hormone free media (Table 3.24).

The influences of exogenously applied auxin / cytokinin responses in plant cell culture system especially on root and shoot initiation and the morphogenesis of plant tissue have been studied for the past four decades (Reynolds, 1987, Skoog and Miller, 1957). In this light, the effect produced by various ranges of concentrations of zeatin, BAP, 2iP and kinetin (cytokinin) used in combination with 0.5 mgL^{-1} IBA (auxin) were investigated for shoot initiation responses in *Paraserianthes falcataria.*

Zeatin is commonly used in tissue culture manipulations since it is more active than other synthetic cytokinins such as kinetin and BAP although they are less susceptible to degradative enzymes (Mok *et al.*, 1987). In the present study, it became apparent that shoot initiation tends to follow a certain trend where proliferation was optimal at zeatin concentrations between 0.25 and 0.5 mgL^{-1} for all four media (Tables 3.8, 3.12, 3.16, 3.20). While at higher concentrations there was a remarkable decline in the mean number of shoots produced – a common occurrence in all four cytokinins in all four media. In a similar study conducted by Mok *et al.* (1987) zeatin induced the highest microshoot production in *Rhododendron spp* at moderate levels of 50 μM while at higher concentrations the proliferation rate was poor. This can be attributed to the fact that high concentrations of hormones can kill the shoot tips or produce a small brown mass of callus that dies soon after formation, thereby restricting the growth of adventitious shoots (Warrag *et al.,* 1990). However, zeatin's (A$ 300 - 400 for 10 mg) expense makes its use impractical and can be effectively substituted where less expensive cytokinins are available (Mok *et al.* 1987).

Although zeatin may be equally or more effective (Anderson, 1980; Ecenomou, 1982; Ecenomou and Read, 1980a, b; Lloyd and McCown,1980; McCown and Lloyd 1983), 2iP was tested because it is less expensive and can be determined as a potential substitute for zeatin in *Paraserianthes falcataria.* With comparison to zeatin and BAP, the results obtained with the addition of 2iP was relatively lower. It can be postulated in *Paraserianthes falcataria* that the sole usage of 2iP

without the addition of any other cytokinin will not have a significant effect on shoot initiation. Studies carried out by Rumary and Thorpe (1984) on White and Black Spruce showed that the maximum synergistic effect of 2iP was achieved when blended with BAP. Although not investigated in the present study, other studies on woody species have revealed that combination of cytokinin/cytokinin have proven to be more beneficial in the manipulation of shoot initiation as observed by McClean *et al.* (1992) with *Hibiscus cannabinus* (BAP and kinetin) and by Sita *et al.* (1986) in *Eucalyptus grandis* (kinetin, BAP and 2iP).

Barker *et al.* (1977); De Fossard *et al.* (1977); Hartney and Barker (1980); Hartney (1981) and McComb and Bennett (1986) have proposed the use of BAP for micropropagation in most woody species as a suitable exogenous cytokinin. In this context, a study conducted by Mascarenhas *et al.* (1982) and Trindade *et al.* (1990) on *Eucalyptus globulus* showed that higher rates of shoot multiplication were achieved by the addition of BAP to the media in comparison to kinetin and shooting tended to be more vigorous with BAP than with kinetin. This observation of Trindade *et al.* (1990) was held true in the present study where it was observed that BAP in the moderate concentrations of 0.1 to 0.5 mgL^{-1} produced the optimum mean number of shoots per explant with a confidence interval ranging from 19.64 to 15.36 (Table 3.9, 3.13, 3.17, 3.21) while in the case of kinetin the optimum was achieved at the concentration of 0.1 mgL^{-1} with a confidence interval of 17.84 to 14.38 (Table 3.11, 3.15, 3.19, 3.23). Further studies conducted by Ahmad (1991) on nodal explants of *Acacia*

mangium also evidenced that 0.5 mgL^{-1} BAP was the best combination to induce a higher shoot multiplication with an average of 25.4 shoots per explant.

According to Voesenek *et al.* (1996), adaptative responses in *Rumex* species stimulated shoot elongation was linked to the changes in the concentrations of hormones involved. On similar lines in the current study an increasing concentration gradient of cytokinin was administered to the subcultured explants in order to establish a concentration at which the shoot initiation was optimum. It was revealed that there were different optima for the four cytokinins that were tested in the four different media (Tables 3.8 to 3.23)

Manipulation of the concentrations gives a clear insight into the optimum concentration that can be prevalently used for each media type. Both low and high concentrations of hormones were investigated. However, the results were not favourable towards higher concentrations of all four hormones since the growth rates were very poor, with few of the explants failing to even initiate shoots. Moreover, callus formation was extensive at higher concentrations. Blomstedt *et al.* (1991) have found that BAP was toxic in concentrations of 2 mgL^{-1} and above in *Eucalyptus regnans.* Concentrations of 1.75 mgL^{-1} or more produced bushy cultures with fewer developed internodes which caused a great threat to further subculturing as the explants could not be divided and this did not contribute a great deal towards micropropagation and regeneration as was the case in the present study as well. At this (1.75 mgL^{-1}) concentration bushy

cultures were produced and at lower concentrations cultures were poorly elongated (Blomstedt *et al.,* 1991).

Studies conducted by Trindade *et al.* (1990) found in *Eucalyptus globulus* that absence of exogenous cytokinin in the culture media almost 75% of the shoots died after the second subculture, while media that contained exogenous cytokinins stimulated shoot formation. This phenomenon was common in the present study as well in which hormone free media had the lowest confidence interval (10.06 to 5.96).

3.9 Summary

Amongst the four media and cytokinins that were investigated MS media supplemented exogenously with zeatin, between the concentration ranges of 0.1 to 0.5 mgL^{-1} produced the highest mean number of shoots per explant. However, it should be emphasised that hormones do not appear to be the only controlling factors and that there are probably complex interactions between endogenous and exogenous hormones and such other factors. Hence it cannot be altogether relevant to cite that the results obtained were in compliance with the strategy adopted, since endogenous hormones may have a certain influence on the growth cycle of *Paraserianthes falcataria.* However, this concept of investigation is beyond the scope of the present study. Owing to the dearth of relevant literature and the complexity of the investigation on the endogenous hormones,

certain aspects of the present concepts remain controversial.

CHAPTER 4

ROOT INITIATION AND PLANT ACCLIMATION

4.1 Introduction

The induction and production of roots on shoots grown *in vitro* is imperative for large-scale commercial micropropagation of screened *Eucalyptus* strain (for commercial purposes) (Cheng *et al.*, 1992; Gaspar and Coumans, 1987). Micropropagation involves four basic stages. First, the growth of shoots *in vitro* from pre-existing meristems in the shoot tip or from axillary buds is induced. The shoots need to be maintained and proliferated through a series of subcultures and then the individual shoots are induced to initiate individual adventitious roots. Last stage involves acclimation of the plantlets to free-living conditions (Hutchinson *et al.*, 1992).

Many tree species that successfully developed roots and shoots are not suited for large scale clonal propagation because the mortality rates on transfer to soil are too high (Thorpe and Patel, 1984). This makes it too expensive and the rate of multiplication is too slow. Root initiation and acclimation is therefore an important part of tissue culture technology and cannot be neglected.

The selection of explants is of utmost importance, as this will influence the ability

to regenerate whole plants from subsequent cultures; selection of young actively growing explants provides a more easily regenerated product. The rooting ability of explants taken from older regions of *Eucalyptus* trees have shown reduced capabilities of regenerating from micropropagation (Le Roux and Van Staden, 1991a). *Eucalyptus regnans* does not root easily from mature cuttings (Blomstedt *et al.,* 1991). Therefore, the selection of explant material is vital for root initiation in *Paraserianthes falcataria.*

The stage at which plants are transferred into the soil involves the rooting of shoot cuttings, hardening of plants to impart some tolerance to mixture stress (low humidity etc.), conferring of a degree of resistance to certain pathogens and conversion of plants from the heterotropic to the autotrophic state (Murashige, 1974).

The rooting of shoot obtained through micropropagation is most difficult part of the propagation process, especially in woody species (Murashige, 1974). This is because the hormonal requirements for root initiation in cell cultures are generally wider than those for shoot formation (Chandler *et al.,* 1989). The establishment of an effective root system on shoots grown *in vitro* is however essential for largescale micropropagation of falcata species for commercial purposes.

Special physiological needs also need to be determined and these vary among species. They may include light intensity, temperature and humidity. Light

intensity is very important at his stage and it needs to be increased. This is because plants are not any more provided with carbohydrates and they rely on photosynthesis for their production. Higher temperatures are also required for root formation. It was suggested that higher temperature was essential for the formation of cambia and the lower temperature for the differentiation of the cambia into root primordia (Thorpe and Patel, 1984). Some plants could not survive in the soil without being exposed to a temperature of 20^{0}C for a period of 4-6 weeks prior to the transfer to soil. Plants that come from an ideal environment which is rich in nutrients and sugars and sterile need to be rinsed in fungicide before they can be planted into the soil to avoid fungal contamination. Different types of soil can be used and the examples of these are peat : perlite (3:1); perlite : vermiculite (1:1) or peat : perlite :coarse sand (1:1:1) (Blomstedt *et al.*, 1991; Poissonnier *et al.*, 1984).

4.2 Objectives

The main aim of this chapter was to optimise root initiating systems and maximise the transfer of root induced shoots to field sites in *Paraserianthes falcataria*. This was achieved by inducing root generating systems in *Paraserianthes falcataria* using the various media and root initiating hormones (auxins) with different concentrations to effectively optimise roots in the established shoot cultures (Figure 4.1).

The media that were used were MS (Murashige and Skoog, 1962) (Table 3.4), LP (Le Poivre, 1977) (Table 3.5), GD (Gresshoff and Doy, 1972) (Table 3.6), and WPM (Woody plant media, Lloyd and McCown, 1980) (Table 3.7) supplemented with 30 gL^{-1} Carbon source (sucrose), 3.2 gL^{-1} phytagel and auxins namely, IAA (Indole Acetic acid), IBA (Indole Butyric Acid) and NAA (Naphthalene Acetic acid) (concentration range of 0.1 to 2.5 mgL^{-1}). Explants were grown in temperature conditions of 25^{0}C day with 16hour photoperiod and 18^{0}C night with 8h dark period. The optimum concentration for each media and auxin was established.

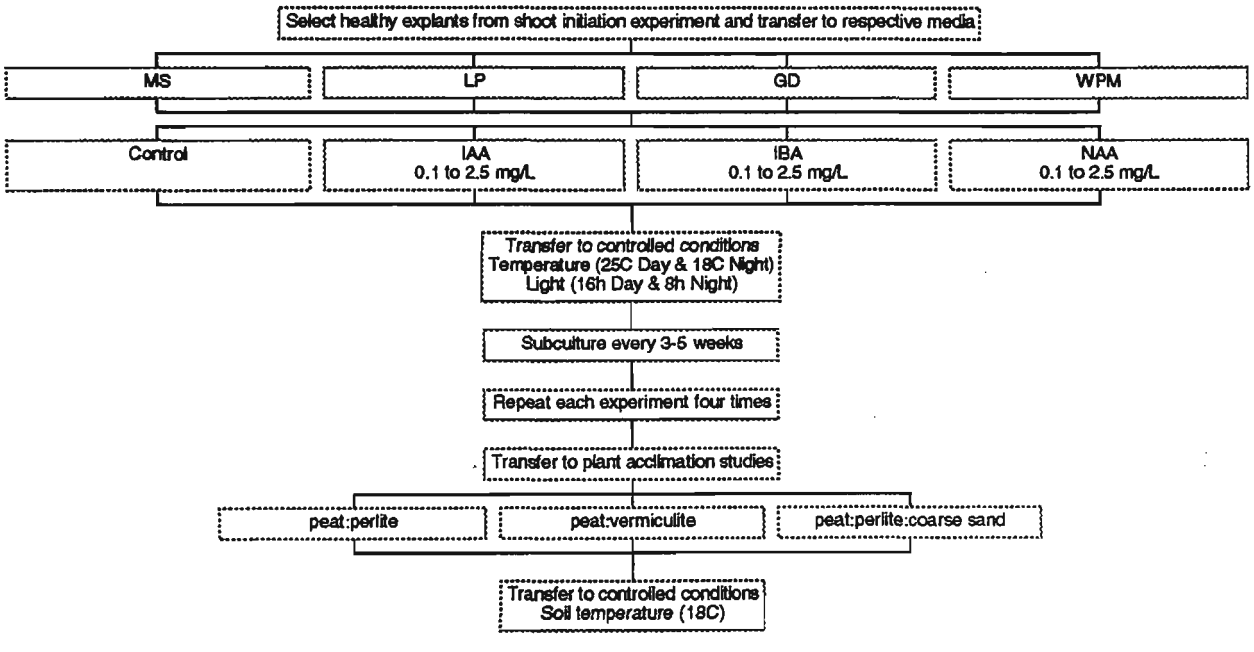

Figure 4.1 Overall experimental design for determination of root initiation in *Paraserianthes falcataria*.

Rooted explants were subcultured at regular intervals of 3-5 weeks and results

tabulated. After four (12-20 weeks) subsequent subcultures rooted explants were tested for acclimation and establishment. Rooted explants were grown in soil types peat : perlite (3:1); perlite : vermiculite (1:1) and peat : perlite : coarse sand (1:1:1) were investigated. The established plants were assessed after 3-5 weeks.

4.3 Growth Hormones

Among the phytohormones, auxins enhance root initiation and cytokinins enhance shoot initiation (De and Basu, 1996). The most frequently used auxins are 2,4-dichloro-phenoxy acetic acid (2,4,D), Indoleacetic acid (IAA), Napthaleneacetic acid (NAA) and Indolebutyric acid (IBA). Auxins such as IAA and NAA were used in concentrations of 0.06 - 27.0 mgL^{-1} in 75% of plant species. Skoog and Miller, (1957) have shown that shoot and root initiation is regulated by the interaction between the two hormones, auxin and cytokinin. A relatively high concentration of auxin favours root initiation but represses shoot formation whereas high concentrations of cytokinin have the opposite effect (Murashige, 1974). The successful induction of shoots and roots can therefore be achieved by manipulation of hormone concentrations. The optimum concentration varies from species to species.

Auxins are formed in young growing organs, such as opening buds and are transported away from tips of shoots towards the base of the plant. Levels of

plant hormones are usually higher in the root tips than elsewhere in the roots (Zhang *et al.,* 1995). Here, they stimulate the cell to elongate and to divide (Raven *et al.,* 1992). Auxins are the primary plant hormones that are responsible for the initiation of adventitious roots in many species including *Rumex* (Voesenek *et al.,* 1996). Auxins, natural or artificially applied are also involved in actions such as cell differentiation, a role in cell division, in fruit development and in leaf fall (abscission) (Table 4.1).

Table 4.1 Table outlining some of the interactions between plant hormones during various aspects of plant growth.

Physiological activity	Action of auxin
Fruit development	Promotes cambial activity
Apical Dominance	Vascular tissue differentiation
Abscission	Inhibits

Auxins such as IBA (Indole butyric acid) and NAA (Naphthalene acetic acid) are not naturally occurring compounds but they promote root formation in cuttings better than naturally occurring compounds such as IAA (Indole acetic acid) (Murashige, 1974).

Auxins such as indolebutyric acid (IBA) (Figure 4.3), Indoleacetic acid (IAA) (Figure 4.2) and Naphthalene acetic acid (NAA) (Figure 4.4) are needed for root

initiation and development. The presence of auxins IAA, IBA and NAA in low concentrations (0.01 mgL^{-1} IBA) promotes the growth of *Eucalyptus* species (Mehra- Palta, 1982). IBA is the most commonly used auxin for the rooting of woody microcuttings (Bennett and McComb, 1986; Blomstedt *et al.,* 1991;Burger, 1987; Franclet and Boulay, 1982; McComb and Bennett, 1986). Auxin used also influences the amount of callus produced and this callusing can be reduced by the amount of auxin that is added (Blomstedt *et al.,* 1991; Cheng *et al.,* 1992).The auxins used in root initiation experiments of certain plant species are listed in the Table 4.2.

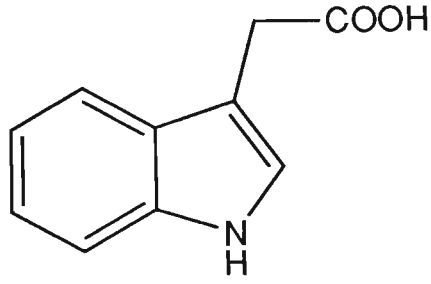

Figure 4.2 Structure of IAA (Raven *et al.,* 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

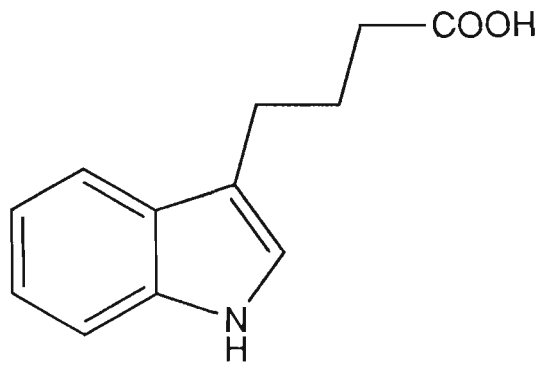

Figure 4.3 Structure of IBA(Raven *et al.*, 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

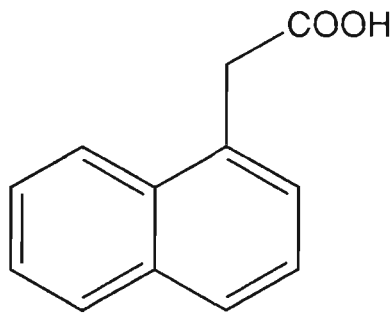

Figure 4.4 Structure of NAA (Raven *et al.*, 1992; Ridgeway and Kingsley, 1989; Salisbury and Ross, 1992).

Table 4.2 Summary of root initiation techniques conducted in certain woody species.

Treatment	Species	Author
IAA	*Eucalyptus grandis*	Sita *et al.*, 1986
	Eucalyptus robusta	Yamaguchi *et al.*,1986a & b
	Eucalyptus tereticornis	Rao, 1988
	Chamaelaucium uncinatum	Damiano *et al.*, 1989
	Genista monosperma	Damiano *et al.*, 1989
	Eucalyptus globulus	Trindade *et al.*, 1990
	Acacia mangium	Galiana *et al.*,1991
	Acacia saligna	Barakat and Lakany, 1992
IBA	*Eucalyptus tereticornis*	Rao, 1988
		Das and Mitra, 1990
	Eucalyptus globulus	Mascarenhas *et al.*, 1982
		Trindade *et al.*, 1990
	Eucalyptus grandis	Warrag *et al.*, 1990
	Eucalyptus spp	Mehra-Palta, 1982
		Le Roux and Staden, 1991
	Eucalyptus regnans	Blomstedt *et al.*, 1991
	Acacia mangium	Darus, 1991
		Gallana *et al.*, 1991
	Gmelina arborea	Tang and Srivastava, 1985
		Hamsari and Srivastava, 1985
		Sandum *et al.*, 1986
	Eucalyptus sideroxylon	Burger, 1987
		Cheng *et al.*, 1992
	Acacia saligna	Barakat and Lakany, 1992
	Melia azedarach	Domecq,1988
	Eucalyptus robusta	Yamaguchi *et al.*, 1986a; b

	Eucalyptus gunnii	Curir *et al.*, 1986
	Eucalyptus stuartiana	Curir *et al.*, 1986
	Eucalyptus ficifolia	Gorst *et al.*, 1983
	Eucalyptus botyroides	Zhang *et al.*, 1982
	Eucalyptus marginata	Bennett and McComb, 1982
	Eucalyptus globulus	Trindade *et al.*, 1990
	Eucalyptus sideroxylon	Burger, 1987
		Cheng *et al.*, 1992
	Gmelina arborea	Hamsari and Srivastava,1985
		Tang and Srivastava, 1985
		Sandum *et al.*, 1986
	Hibiscus cannabinus	McLean *et al.*, 1992
NAA	*Eucalyptus robusta*	Yamaguchi *et al.*, 1986 a; b
	Eucalyptus parvifolia	Texier and Faucher, 1986
	Eucalyptus gunnii	Boulay, 1987
	Eucalyptus tereticornis	Rao, 1988
	Eucalyptus camaldulensis	Kumar and Ayyappan, 1987
		Kumar and Raman, 1989
	Eucalyptus regnans	Blomstedt *et al.*, 1991
	Rice	Whitney, 1996
Hormone free	*Acacia mangium*	Darus, 1991
	Rye	Whitney, 1996
	Hibiscus cannabinus	McLean *et al., 1992*
	Wheat	Whitney, 1996

4.4 Methods and Techniques

4.4.1 Preparation of media

Full strength MS (Murashige and Skoog, 1962), micro and macro salts (Table 3.4) supplemented with 30 gL^{-1} sucrose (established in seed germination experiment, Table. 2.8), 3.2 gL^{-1} phytagel, 0.5 mgL^{-1} zeatin and pH 5.6 was prepared. The media was sterilised by autoclaving at 1.05 kg cm^{-2} at 120^0C for 20 minutes. IAA, IBA and NAA were filter sterilised and added in the concentrations of 0.1,0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 mgL^{-1}. Auxin concentrations were selected on the basis of the experiments conducted by the various authors as listed in Table 4.2 and the most suitable range of concentrations were selected. The same procedure was followed with the preparation of the other three media, LP (Le Poivre, 1977) (Table 3.5), GD (Gresshoff and Doy, 1972) (Table 3.6) and WPM (Lloyd and McCown, 1980) (Table 3.7). All media used contained full strength micro and macro salts and vitamins, chemicals for media were purchased from Sigma, U.S.A.

4.4.2 Selection of shoot material

Well grown, healthy shoots acquired from shoot initiation studies were used for this experiment. Shoots with elongated stems from the mainstem or elongated nodal shoots from the distal end of the principal shoot axis with at least two well

developed leaves were taken as microcuttings (1 cm tall). Studies by Cheng *et al.* (1992), have shown that distal end microcuttings root are better in *Eucalyptus sideroxylon* than the proximal ends. Axillary bud development and root development is usually most frequent in nodal explants at the basal end of seedlings, particularly with foliated explants (De Fossard *et al.*, 1974). Therefore, the distal ends were used for root initiation and the proximal ends were used as blocks for the formation of more axillary buds. Any additional leaves were removed before planting. The plant tissue was cut in sterile laminar flow cabinet to avoid contamination and the microcuttings were cultured vertically on the four different media supplemented with various concentrations of the auxins. There were 25 replicates per concentration of auxin per media. Shoots (5 per container) were planted in glass containers containing the prepared solidified media. The subcultured explants were placed in growth chambers as per conditions listed under Table 2.8.

4.4.3 Maintenance of cultures

The explants were checked every week for any signs of contamination. Contaminated explants were promptly removed and replaced with new explants to maintain the number of 25 replicates per treatment. After 3-5 weeks the rooting percentage, length of the root and number of roots produced per explant of the cultures were calculated for each concentration of auxin and media tested, before the second subculture. The length of the roots was measured *ex vitro* and

tabulated. Explants that had developed roots (0.5 cm or more) were removed for further subculturing. The roots were subcultured *ex vitro* and placed in the same initiation media with the same concentration of auxin and media for further establishment. Each treatment was repeated four times with 25 replicates each time. The same conditions of temperature and light were maintained throughout the experiment.

4.4.4 Plant acclimation and establishment

Successful regenerated plants were transferred to polypots (3 inches in diameter) for establishment studies. 20 rooted microcuttings were used for each treatment Rooted microcuttings were carefully washed free of adhering gelrite under running tap water. After submergence in Benlate (1 gL^{-1}) plants were blotted and packed in loosely packed autoclaved peat : perlite (3:1), perlite : vermiculite (1:1) and peat : perlite : coarse Sand (1:1:1). The plants were then placed in the fungicide solution of " Mancozeb plus" (1gL^{-1}) for 2 to 3 minutes. The fungicide was washed off the plant by immersing it in distilled water. Few Osmocote pellets were placed per well. The peat pots were placed onto the bottom of the heated tray to increase the ambient temperature and covered with the lid. Plants were grown under artificial light at 16 hour photoperiod and watered.

4.5 Statistical Analysis

Rooting percentages were calculated based on biostatistical analysis of Zar (1984) and ANOVA (Systat, version '95) software. Repeated Measures design and Duncan Multiple Range Test was performed using SAS (Version '95) package. Significant levels between each cytokinin was determined using Paired Samples Statistics and Paired Samples Correlations. Confidence intervals were calculated on similar lines as in shoot initiation (section 3.6).

4.6 Results

Roots became visible on microcuttings 7-14 days after transfer to rooting medium. 3-5 weeks *in vitro* rooting period was sufficient for most explants to produce roots. Microcuttings obtained through axillary bud formations from shoot initiation experiments showed different rooting responses depending on the medium used for initiation.. Based on the amount of basal callus formed explants could be divided into three categories: Explants with no basal callus formation, Explants with Very little basal callus formation and explants with excessive basal callus formation. Excessive callus formation in the media supplemented with high concentrations of auxins inhibited the development of roots in certain explants. The other explants with no basal callus and very little basal callus performed better than the explants with excessive basal callus. The explants

with excessive basal callus eventually died and had to be replaced with new microcuttings. The explants that survived were statistically analysed. Plates 4.1, 4.2 and 4.3 show culture that had successfully acclimated into polypots.

Plate 4.1 Successful acclimated cultures of *Paraserianthes falcataria* after root initiation experiments

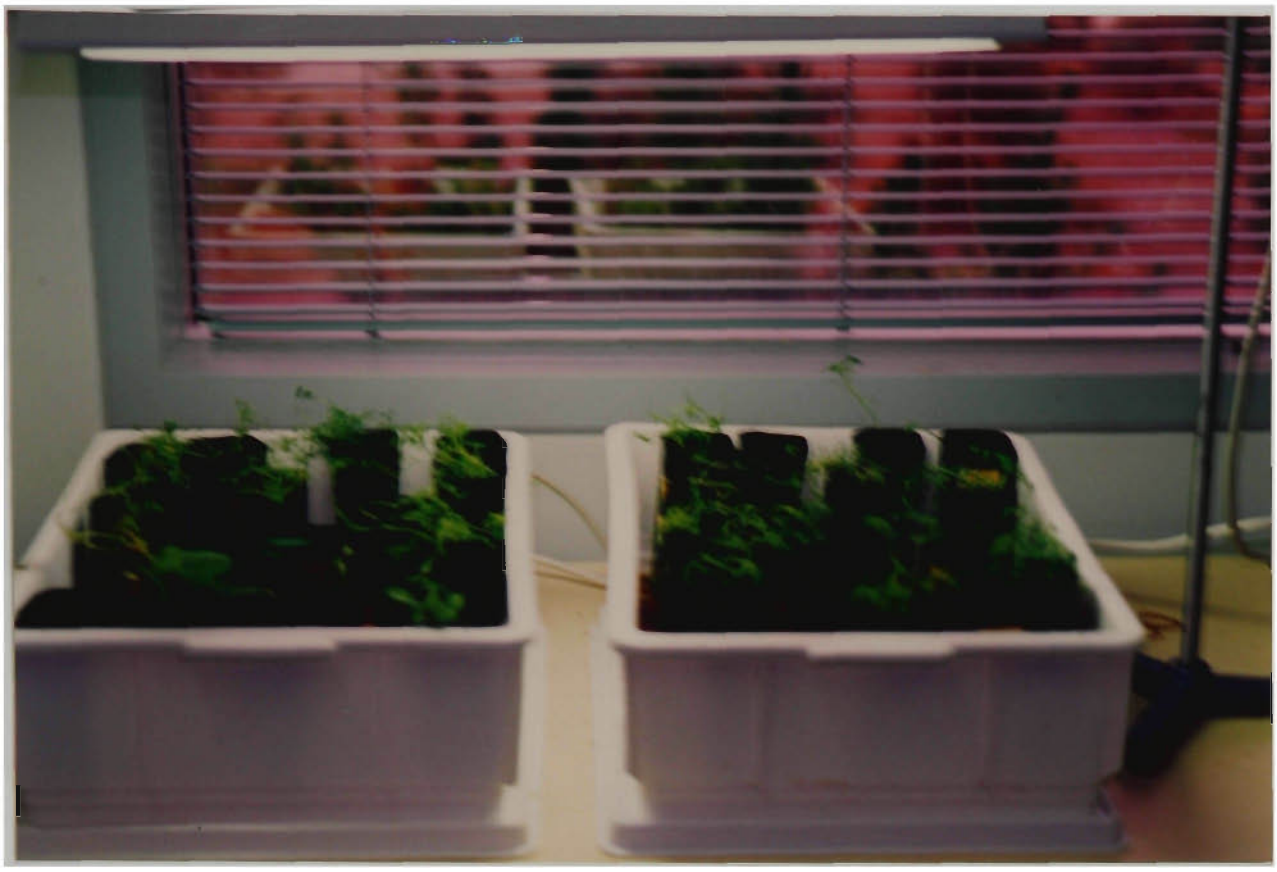

Plate 4.2 Acclimated cultures of *Paraserianthes falcataria* after 6 weeks in polypots.

Plate 4.3 Individual healthy cultures of *Paraserianthes falcataria* (6 week old).

4.6.1 MS media

Table 4.3 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of IAA supplemented in MS; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from MS Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of Roots per explant (cms)
0.00	3	0.03	3.06	2.94	0.5	0.7
0.10	9	0.02	9.04	8.96	1.5	1.2
0.25	10	0.02	10.04	9.96	1.5	1.15
0.50	8	0.01	8.02	7.98	1.3	1.1
0.75	7	0.02	7.04	6.96	1.1	1.01
1.00	5	0.03	5.06	4.94	0.7	1
1.25	4	0.03	4.06	3.94	0.6	0.95
1.50	3	0.02	3.04	2.96	0.5	0.91
1.75	2	0.02	2.04	1.96	0.3	0.8
2.00	2	0.01	2.02	1.98	0.3	0.69
2.25	2	0.02	2.04	1.96	0.3	0.67
2.50	1	0.03	1.06	0.94	0.3	0.67
				Mean =	0.74	0.9
				s.e. =	0.02	0.01

Table 4.4 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of IBA supplemented in MS; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from MS Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of Roots per explant (cms)
0.00	3	0.02	3.04	2.96	0.5	0.7
0.10	14	0.02	14.04	13.96	1.9	1.6
0.25	13	0.01	13.02	12.98	1.7	1.5
0.50	13	0.03	13.06	12.94	1.5	1.47
0.75	11	0.02	11.04	10.96	1.3	1.3
1.00	7	0.02	7.04	6.96	1.2	1.21
1.25	6	0.01	6.02	5.98	1.1	1
1.50	4	0.03	4.06	3.94	0.9	0.8
1.75	3	0.02	3.04	2.96	0.5	0.8
2.00	3	0.02	3.04	2.96	0.3	0.7
2.25	2	0.01	2.02	1.98	0.3	0.7
2.50	2	0.02	2.04	1.96	0.3	0.7
				Mean =	0.96	1.04
				s.e. =	0.02	0.03

Table 4.5 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of NAA supplemented in MS; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from MS Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of Roots per explant (cms)
0.00	3	0.02	3.04	2.96	0.5	0.7
0.10	11	0.01	11.02	10.98	1.4	1.11
0.25	12	0.02	12.04	11.96	1.5	1.1
0.50	11	0.03	11.06	10.94	1.1	1
0.75	8	0.02	8.04	7.96	1.1	0.2
1.00	6	0.02	6.04	5.96	0.7	0.9
1.25	5	0.03	5.06	4.94	0.6	0.92
1.50	3	0.02	3.04	2.96	0.5	0.8
1.75	3	0.02	3.04	2.96	0.4	0.8
2.00	2	0.01	2.02	1.98	0.4	0.71.
2.25	2	0.02	2.04	1.96	0.3	0.71
2.50	2	0.03	2.06	1.94	0.3	0.69
				Mean =	0.73	0.74
				s.e. =	0.01	0.03

Hormone free treatment did not produce a high rooting percentage (3%) in MS (Tables 4.3, 4.4 and 4.5). The number of roots per explant were 0.5 while the mean length per explant was 0.7 (Tables 4.3, 4.4 and 4.5). The maximum rooting percentage observed in IAA supplemented MS media was 10% (Table 4.3). The minimum was 1% (Table 4.3). A decrease in the rooting percentage was

observed as the concentration of the auxin was increased with the highest concentrations giving the least roots (Table 4.3). Mean number of roots per explant ranged between 1.5 to 0.3 (Table 4.3), while the mean length per explant was between 1.2 and 0.67 cms (Table 4.3). IBA produced optimum results in MS media compared to IAA and NAA (Tables 4.3, 4.4 and 4.5). With increasing concentrations of IBA a decrease in rooting percentage was observed (Table 4.4). The optimum root initiation percentage (14%) was obtained at 0.10 mgL^{-1} IBA (Table 4.4). Mean number of roots per explant and mean length of roots per explant ranged between 1.9 to 0.3 and 1.6 to 0.7 cms respectively (Table 4.4). NAA showed better rooting percentages than IAA, however the percentages were not as high as those observed with IBA (Tables 4.3, 4.4 and 4.5). Observations similar to IAA and IBA wherein high concentrations producing lesser rooting percentages was observed with NAA as well (Table 4.5). Mean number of roots per explants was between 1.4 to 0.3 and mean length of roots per explant ranged between 1.11 to 0.69 cms (Table 4.5).

4.6.2 LP Media

Table 4.6 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of IAA supplemented in LP; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from LP Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of Roots per explant	Mean length of roots per explant (cms)
0.00	2	0.03	2.06	1.94	0.9	0.5
0.10	9	0.02	9.04	8.96	1.7	1.01
0.25	10	0.03	10.06	9.94	1.6	1.02
0.50	9	0.02	9.04	8.96	1.5	1.01
0.75	7	0.02	7.04	6.96	1.5	0.95
1.00	6	0.01	6.02	5.98	1.2	0.93
1.25	4	0.02	4.04	3.96	1.1	0.78
1.50	4	0.02	4.04	3.96	0.9	0.75
1.75	3	0.02	3.04	2.96	0.9	0.63
2.00	2	0.01	2.02	1.98	0.7	0.58
2.25	2	0.02	2.04	1.96	0.6	0.55
2.50	1	0.03	1.06	0.94	0.5	0.4
				Mean =	13.1	0.76
				s.e. =	0.02	0.02

Table 4.7 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of IBA supplemented in LP; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from LP Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of Roots per explant (cms)
0.00	2	0.03	2.06	1.94	0.9	0.5
0.10	12	0.02	12.04	11.96	1.4	1.31
0.25	11	0.02	11.04	10.96	1.4	1.22
0.50	10	0.01	10.02	9.98	1.3	1.32
0.75	9	0.02	9.04	8.96	1.2	1.21
1.00	5	0.03	5.06	4.94	1.2	1.1
1.25	4	0.02	4.04	3.96	1.1	0.99
1.50	3	0.01	3.02	2.98	0.8	0.86
1.75	3	0.02	3.04	2.96	0.6	0.79
2.00	2	0.03	2.06	1.94	0.3	0.75
2.25	2	0.02	2.04	1.96	0.3	0.69
2.50	2	0.02	2.04	1.96	0.3	0.68
				Mean =	0.90	0.95
				s.e. =	0.01	0.03

Table 4.8 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of NAA supplemented in LP; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from LP Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of Roots per explant	Mean length of roots per explant (cms)
0.00	2	0.02	2.04	1.96	0.9	0.5
0.10	10	0.03	10.06	9.94	1.5	1.01
0.25	11	0.02	11.04	10.96	1.4	1.01
0.50	10	0.02	10.04	9.96	1.2	0.95
0.75	9	0.02	9.04	8.96	1.1	0.92
1.00	7	0.03	7.06	6.94	0.9	0.83
1.25	4	0.02	4.04	3.96	0.9	0.78
1.50	3	0.03	3.06	2.94	0.9	0.75
1.75	2	0.02	2.04	1.96	0.8	0.65
2.00	2	0.02	2.04	1.96	0.8	0.57
2.25	1	0.01	1.02	0.98	0.6	0.57
2.50	1	0.02	1.04	0.96	0.6	0.49
				Mean =	0.97	0.75
				s.e. =	0.02	0.02

Hormone free LP media recorded 2% rooting percentage for *Paraserianthes falcataria* (Tables 4.6, 4.7 and 4.8). 0.9 to 0.5 mean roots per explant and 1.01 to 0.4 cms mean length of roots were observed with control treatment (Tables 4.6, 4.7 and 4.8). Rooting percentages of 10 to 1% (Table 4.6) were recorded with IAA supplemented LP media. In general, rooting percentages decreased as the

concentration of IAA was increased. Mean number of explants per root were between 1.7 to 0.5 and mean length of roots per explant were between 1.01 and 0.4 cms (Table 4.6). IBA showed better rooting percentages than both IAA and NAA (Tables 4.6, 4.7 and 4.8). 12 to 2% rooting were observed with 1.4 to 0.3 mean number of roots per explant and 1.31 to 0.68 mean length of roots per explant (Table 4.7). 11 to 1% rooting was observed in LP media supplemented with NAA (Table 4.8). 1.5 to 0.6 mean number of roots and 1.01 and 0.49 cms mean length of roots per explant were recorded (Table 4.8).

4.6.3 GD Media

Table 4.9 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of IAA supplemented in GD; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from GD Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of roots per explant (cms)
0.00	2	0.02	2.04	1.96	0.5	0.49
0.10	9	0.03	9.06	8.94	1.1	1
0.25	9	0.02	9.04	8.96	1.1	1
0.50	8	0.02	8.04	7.96	1.1	0.86
0.75	7	0.02	7.04	6.96	0.9	0.85
1.00	5	0.03	5.06	4.94	0.9	0.62
1.25	4	0.02	4.04	3.96	0.9	0.62
1.50	3	0.02	3.04	2.96	0.8	0.54
1.75	2	0.03	2.06	1.94	0.8	0.49
2.00	1	0.02	1.04	0.96	0.7	0.49
2.25	1	0.02	1.04	0.96	0.6	0.38
2.50	1	0.03	1.06	0.94	0.6	0.37
				Mean =	0.83	0.64
				s.e. =	0.02	0.03

Table 4.10 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of IBA supplemented in GD; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from GD Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of roots per explant (cms)
0.00	2	0.01	2.02	1.98	0.5	0.49
0.10	10	0.02	10.04	9.96	1.1	1.1
0.25	11	0.03	11.06	10.94	1.1	1.22
0.50	8	0.02	8.04	7.96	1.1	1.11
0.75	6	0.02	6.04	5.96	1.1	1.05
1.00	4	0.03	4.06	3.94	0.9	0.96
1.25	4	0.03	4.06	3.94	0.9	0.92
1.50	3	0.02	3.04	2.96	0.8	0.9
1.75	3	0.02	3.04	2.96	0.6	0.52
2.00	2	0.03	2.06	1.94	0.6	0.52
2.25	2	0.02	2.04	1.96	0.6	0.48
2.50	2	0.02	2.04	1.96	0.6	0.48
				Mean =	0.83	0.81
				s.e. =	0.01	0.02

Table 4.11 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of NAA supplemented in GD; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from GD Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of Roots per explant (cms)
0.00	2	0.02	2.04	1.96	0.5	0.49
0.10	9	0.03	9.06	8.94	1.1	0.95
0.25	10	0.03	10.06	9.94	1.1	0.95
0.50	8	0.02	8.04	7.96	1.1	0.86
0.75	6	0.02	6.04	5.96	1.1	0.86
1.00	4	0.03	4.06	3.94	0.9	0.83
1.25	3	0.02	3.04	2.96	0.9	0.76
1.50	2	0.03	2.06	1.94	0.8	0.69
1.75	2	0.02	2.04	1.96	0.8	0.61
2.00	1	0.02	1.04	0.96	0.6	0.49
2.25	1	0.03	1.06	0.94	0.6	0.35
2.50	1	0.02	1.04	0.96	0.6	0.35
				Mean =	0.84	0.68
				s.e. =	0.02	0.03

Hormone free media recorded 2% with 0.5 mean number of roots per explant and 0.49 cms mean length of roots per explant (Tables 4.9, 4.10 and 4.11). 9% was the highest rooting percentage obtained with IAA supplemented GD media, while 1% was the lowest (Table 4.9). 1.1 to 0.6 and 1 to 0.37 cms were mean number of explants per root and mean length of roots per explant respectively

(Table 4.9). IBA recorded a highest of 11% and a lowest of 2% rooting percentage (Table 4.10). Mean number of roots ranged between 1.1 to 0.6 per explant and mean length of explants were 1.1 to 0.48 cms per root (Table 4.10). 10% was the highest rooting percentage achieved with NAA (Table 4.11). Mean number of roots per explant ranged between 1.1 to 0.6 and mean length of roots ranged between 0.95 to 0.35 cms per explant (Table 4.11).

4.6.4 WPM Media

Table 4.12 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of IAA supplemented in WPM; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from WPM Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of roots per explant (cms)
0.00	4	0.02	4.16	3.84	1.1	0.9
0.10	13	0.03	13.76	12.24	1.5	1.31
0.25	14	0.02	14.55	13.45	1.4	1.25
0.50	13	0.02	13.51	12.49	1.4	1.2
0.75	12	0.01	12.24	11.76	1.3	1.1
1.00	10	0.02	10.39	9.61	1.2	1
1.25	9	0.03	9.53	8.47	1.1	0.91
1.50	7	0.02	7.27	6.73	1.1	0.91
1.75	5	0.02	5.20	4.80	1.1	0.82
2.00	5	0.01	5.10	4.90	1.1	0.73
2.25	4	0.02	4.16	3.84	1.1	0.73
2.50	3	0.02	3.04	2.96	1.1	0.73
				Mean =	1.21	0.97
				s.e. =	0.03	0.02

Table 4.13 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of IBA supplemented in WPM; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from WPM Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of roots per explant (cms)
0.00	4	0.03	4.24	3.76	5	0.4
0.10	20	0.02	20.78	19.22	15.7	1.5
0.25	21	0.02	21.82	20.18	15.6	1.6
0.50	19	0.01	19.37	18.63	16	1.4
0.75	17	0.02	17.67	16.33	15	1.2
1.00	13	0.02	13.51	12.49	14	1.1
1.25	9	0.02	9.35	8.65	13.9	0.8
1.50	7	0.02	7.27	6.73	12	0.7
1.75	5	0.03	5.29	4.71	11	0.6
2.00	4	0.02	4.16	3.84	9	0.5
2.25	3	0.02	3.12	2.88	8	0.3
2.50	3	0.01	3.06	2.94	8	0.3
				Mean =	11.93	0.87
				s.e. =	0.02	0.02

Table 4.14 The effect of exposure of explants of *Paraserianthes falcataria to* various concentrations of NAA supplemented in WPM; Percentage rooting is shown, mean number of roots per explant and mean length of explants. Microcuttings from WPM Shoot initiation experiment were used for this treatment. The experiment was repeated four times with 25 replicates per treatment.

Concentration of auxin (mgL^{-1})	Rooting percentage of explants (%)	Standard error	Confidence Upper	Interval Lower	Mean number of roots per explant	Mean length of roots per explant (cms)
0.00	4	0.03	4.24	3.76	1.1	0.92
0.10	15	0.02	15.59	14.41	1.2	1.21
0.25	16	0.02	16.63	15.37	1.2	1.31
0.50	14	0.01	14.27	13.73	1.2	1.25
0.75	12	0.02	12.47	11.53	1.2	1.15
1.00	11	0.02	11.43	10.57	1.1	1.14
1.25	8	0.02	8.31	7.69	1.1	0.92
1.50	7	0.03	7.41	6.59	0.9	0.87
1.75	6	0.02	6.24	5.76	0.9	0.79
2.00	5	0.02	5.20	4.80	0.7	0.79
2.25	3	0.01	3.06	2.94	0.7	0.75
2.50	3	0.02	3.12	2.88	0.7	0.75
				Mean =	1	0.99
				s.e. =	0.01	0.02

Hormone free WPM media recorded 4% rooting percentage, which was the highest percentage observed in all four media (Tables 4.3 to 4.14). Mean number of roots and mean length per root per explant were 1.1 and 0.9 cms respectively (Table 4.12, 4.13 and 4.14). 14% rooting was observed with IAA supplemented WPM media (Table 4.12) which was the highest rooting

percentage obtained in comparison to IAA supplemented MS, LP and GD media (Tables 4.3, 4.6, 4.9 and 4.12). 1.5 to 1.1 were the ranges of mean number of roots per explant while 1.31 to 0.73 cms were the ranges of the mean length of roots per explant. The highest rooting percentage in comparison to all four media and all three auxins was observed with IBA supplemented WPM media. Rooting percentages ranged between 21 to 3% (Table 4.13). 15.7 to 8 were the mean number of roots per explant while the mean length of the explants ranged between 1.5 to 0.3 cms (Table 4.13). 16% rooting was recorded with NAA (Table 4.14). Mean number of roots and mean length of roots per explant ranged between 1.2 to 0.7 and 1.21 to 0.75 cms respectively (Table 4.14).

Table 4.15 Repeated Measures analysis and Duncan Grouping (Analysis of variance procedure)

Media	Hormone	Pr > F	Mean	Duncan Grouping
MS	Control	0.0001	3.0000	B
LP		0.0001	2.0000	C
GD		0.0001	2.0000	C
WPM		0.0001	4.0000	A
MS	IAA	0.0001	4.6667	B
LP		0.0001	4.9167	B
GD		0.0001	4.3333	B
WPM		0.0001	8.2500	A

MS	IBA	0.0001	6.7500	B
LP		0.0001	5.4167	B, C
GD		0.0001	4.7500	C
WPM		0.0001	10.4167	A
MS	NAA	0.0001	6.0833	B
LP		0.0001	5.1667	C
GD		0.0001	4.0833	D
WPM		0.0001	8.6667	A
MS	IAA	0.0011	4.6667	B
	IBA	0.0011	6.7500	A
	NAA	0.0011	6.0833	A
			17.50000	
LP	IAA	0.2691	4.9167	A
	IBA	0.2691	5.4167	A
	NAA	0.2691	5.1667	A
			15.5001	
GD	IAA	0.0103	4.3333	B
	IBA	0.0103	4.7500	A
	NAA	0.0103	4.0833	B
			13.1666	
WPM	IAA	0.0126	8.2500	B
	IBA	0.0126	10.4167	A
	NAA	0.0126	8.6667	B
			27.3334	

Pr = Significance probability F= Degrees of freedom

A, B, C, D = Duncan grouping system. Means with the same letter are not significantly different.

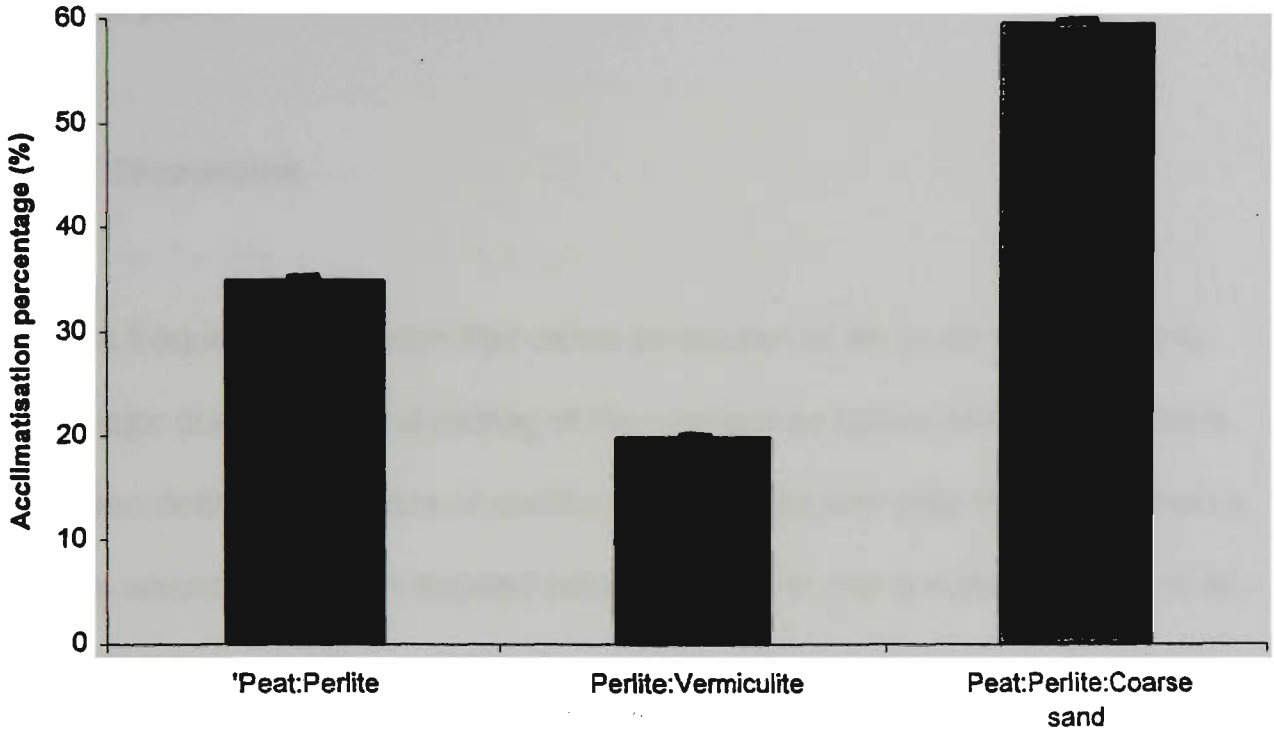

Figure 4.2 Plant acclimation results for the various soil pre-treatments of Peat: Perlite, Perlite: Vermiculite and Peat: Perlite: Coarse Sand. Each treatment consisted of 20 explants attained from root initiation experiments.

4.6.5 Plant acclimation

Among the 20 explants that were planted for each soil type about 35% survived for peat: perlite mixture, 25% for perlite:vermiculite and 60% for peat:perlite: coarse sand mixture (Figure 4.2). The cuttings seemed to be weaker during the time of acclimation. This period is rather important and the survival of transferred plants depend on it. During acclimation roots need to alter their structure i.e. they need to grow new root hairs that could have been damaged during the transfer or during washing to remove adhering gelrite. New root hairs increase the surface area through which better uptake of water and nutrients from the soil

can take place.

4.7 Discussion

It was a frequent observation that callus production at the basal end proved to be a major drawback in the rooting of *Paraserianthes falcataria* explants. Callus has been defined as a mass of undifferentiated polyploid cells that forms when a plant is wounded or when isolated cells are grown in tissue culture (Raven *et al.*, 1992; Salisbury and Ross, 1992). Amongst the explants that were cultured for root initiation studies three types of callus formation was observed and were categorised as explants with no callus, explants with very less callusing and explants with excessive callusing. In the present study, it was observed that at high concentrations of auxins the explants with excessive callusing had very low survival rates. Similarly findings by Cheng *et al.* (1992) observed similar callus retarding root growth in *Eucalyptus sideroxylon* explants, with excessive callus being formed with high concentrations of IBA (5 mgL^{-1}).

In trying to relate the behaviour of explants with excessive callus formation and retarded root growth it can be stated that plants without the presence of a callus possess a better connection between the vascular system of the plant and the medium, also, they are better adapted to conditions of drought and stress and therefore can survive better when transferred to soil (Martin, 1985). This explains why the explants that did not develop a basal callus had a better

survival rate in the present study. Studies by Cheng *et al.* (1992) have also supported this phenomenon where plantlets without callus had a significantly ($p>0.05$) higher survival rate than plants with callus. Another explanation can be related to the fact that in whole plants the energy and the carbon sources are derived from photosynthesis while in contrast for callus grown in cultures these factors are supplied externally (Dracup, 1991). Therefore, this explains why explants that developed very little basal callus were able to initiate roots and survive since the callus did not deplete the energy and carbon sources from being translocated into the vascular system (Martin, 1985). This study however concentrated only on organ culture and therefore detailed studies were not carried out on the type of callus.

From the results it was observed that hormone free treatment was not beneficial to rooting of *Paraserianthes falcataria*. It was essential to supplement root initiation auxins like IAA, IBA and NAA for this investigation. Rooting percentage in all four media for hormone free treatment was extremely low in comparison to media supplemented with low to moderate concentrations (0.1 to 1.5 mgL^{-1}) of auxins (Tables 4.15). Callus formation was also limited to the categories of very low and friable in nature or no callus formation at all. In spite of the absence of callus poor rooting percentages were observed in all four media. This can be attributed to the non-availability of auxins that promote cell division and differentiation of root tissues (Raven *et al.,* 1992). Similarly, only sporadic rooting was reported in *Eucalyptus regnans* in hormone free MS and WPM

media (Blomstedt *et al.*, 1991) and with the case of *Acacia mangium*, only 12 - 15% rooting was observed against 65 - 75% rooting with 0.5 ppm IBA supplemented in MS (Darus, 1991, 1992).

Of the three hormones that were employed in the investigation of root initiation in *Paraserianthes falcataria* IBA proved to be highly effective. The optimum for rooting percentage for IBA was observed in WPM (Table 4.13) at the concentration range of 0.1 to 0.25 mgL^{-1} with a confidence interval of 21.82 to 19.22. The optimum concentration for maximum expression of IBA as a root initiating hormone was between the concentrations of 0.1 to 0.25 mgL^{-1} in all four media. A wide number of publications are available on the action of exogenous IBA and its analogs on root production to support the above findings like studies conducted by Darus (1991; 1992) and Galiana *et al.* (1991) on *Acacia mangium* using 0.5 ppm IBA and Cheng *et al.* (1992) on *Eucalyptus sideroxylon* with 0.1 mgL^{-1} IBA. The overall performance of IBA (Mean = 10.4167) was deemed the best in WPM in comparison to the other three media namely MS (Mean= 6.7500), LP (Mean = 5.4167) and GD (Mean = 4.7500) (Table 4.15). Similar findings have been reported by Blomstedt *et al.* (1991) in *Eucalypt* species using IBA. However there have been other findings where MS has proved to be a better media for root initiation. But, in which case WPM was not experimented with to make a statistical comparison between the two. Further studies conducted on woody species by Bennett and McComb (1982); Blomstedt *et al.* (1991); Franclet and Boulay (1982), McComb and Bennett (1986) and Burger

(1987) also comply with our findings regarding the feasibility of IBA on the effective physiology of rooting.

For IAA the optimum rooting percentage was observed with WPM at the concentration ranges of 0.1 to 0.5 mgL^{-1} with a confidence interval of 14.55 to 12.24. Overall, the optimum concentrations in all four media were observed between the concentrations of 0.1 to 0.5 mgL^{-1} (Tables 4.3 to 4.14). Similar to the reaction of IBA, IAA showed a higher rooting percentage in WPM (Mean = 8.2500) against the percentages of MS (Mean = 4.6667), LP (Mean = 4.9167) and GD (Mean = 4.3333) (Table 4.15). NAA reached its optimum at lower concentrations in *Paraserianthes falcataria*. The optimum concentration for NAA was achieved in WPM at the concentration range of 0.1 to 0.5 mgL^{-1} with a confidence interval of 21.82 to 18.63 (Table 4.14). The optimum concentration range for all four media was obtained between the concentratrions of 0.1 to 0.5 mgL^{-1} (Table 4.3 to 4.14). NAA showed a higher rooting percentage in WPM (Mean = 8.6667) in comparison to the other three media of MS (Mean = 6.0833), LP (Mean = 5.1667) and GD (Mean = 4.0833) (Table 4.15). Similar to findings in IBA, higher concentrations of IAA and NAA were not beneficial for root initiation. Others have similar findings wherein IBA has proved to be better at inducing roots in comparison to IAA and NAA (Rao, 1988; Cheng *et al.*, 1992;Trindade *et al.*, 1990; Sandum *et al.*, 1989; Tang and Srivastava, 1988; Hamsari and Srivastava, 1988; Galiana *et al.*, 1991; Barakat and Lakany, 1992) .

Better rooting percentages were observed for all three auxins on WPM (Table 4.15) than on MS. LP or GD. WPM therefore supports initiation of roots better than MS, LP or GD in the present study. In the case of *Paraserianthes falcataria*, MS was second best to WPM, with statistically significant ($p>0.0001$) values (Table 4.15). Considering LP and GD, LP has performed better with mature tree explants of *Eucalyptus parvifolia* rather than juvenile explants according to Texier and Faucher (1986). This fact can be emphasised with studies by Curir *et al.* (1986) on *Eucalyptus sideroxylon* that have shown that the shoot initiation hormones used prior to root initiation can play a part in adventitious root formation. Kinetin and zeatin may induce a physiological state, which may increase the sensitivity of the explants to the rooting medium, but this effect may not be produced by BAP. Therefore, it can be stated that the supplementation of $0.5mgL^{-1}$ zeatin to the root induction medium may have aided in rooting, but the effect of the preceding cytokinins used in shoot initiation would have been responsible for the lack of root multiplication. To further this argument another reason may be the age of the explant used, the explants used in this study were juvenile or 4-12 weeks old. Studies by Galiana *et al.* (1991) in *Acacia mangium* and Texier and Faucher (1986) in *Eucalyptus parvifolia* have shown better rooting percentages (10-20% more) for mature explants (6months to 10 years old) than juvenile ones.

According to the growth hypothesis, auxin action promotion of root cell elongation is due partially to the stimulation of hydrogen ions (H^+) efflux

from the cytoplasm into the cell wall. Acidification of the wall is thought to enhance wall loosening and allow rapid growth (Rayle and Cleland, 1977). There is now considerable evidence that acid efflux plays a role in auxin stimulation of cell elongation in roots. This proposal is strengthened by the finding that root growth is strongly stimulated by acid pH (Edwards and Scott, 1974; Evans, 1976). Addition of moderate concentration of IAA and IBA stimulate the H^+ efflux thereby promoting root growth while on the other hand increase in the concentration of IAA and IBA will inhibit H^+ efflux from the cytoplasm into the cell wall and therefore arrest root initiation (Evans and Mulkey, 1982).

After the initiation of both the root and the shoot system the next phase of the experiment dealt with the acclimation of the *in vitro* plants in order to harden them so that they would survive the transfer to the greenhouse and then finally to the field. The hardening and the establishing of these regenerated plants are of crucial importance both for agricultural and economic value as well as a hallmark of successful plant propagation through tissue culture. Further, this micropropagation system of successful plant acclimation has potential as a system for mass production of ramets for plantations or for the establishment of clonal hedges from which propagation by cuttings can be obtained (Le Roux and Van Staden, 1991a). The plants were subjected to intermittent misting for 2-3 weeks by providing bottom heat and daily watering which according to Boulay (1987) is important for the maintenance of high relative humidity. Moreover, root initiation is also stimulated more than the shoots by heating the substrate, (Le

Roux and Van Staden *et al.,* 1991a). Results from plant acclimation studies showed that the soil combination of Peat : Perlite : Coarse sand (1:1:1) (Figure 4.2) was found to be most suitable for plant regeneration in *Paraserianthes falcataria.*

These young *in vitro* grown plantlets are often sensitive to attack from pathogens and sprays with fungicides and insecticides have been recommended, (Le Roux and Van Staden *et al.,* 1991a) however the plants can be immersed in fungicides like Mancozeb Plus (zinc) or Benlate (1 gm/L) for a brief period of two to three minutes to control fungal contamination. Although the nutrient media and distilled water was sterilised by autoclaving at 120^{0}C for 20 minutes as well as the instruments and the transfer area restricted within a laminar flow cabinet still the plants obtained from the tissue culture cannot be assumed to be disease free nor pathogen free. The peat box can also contain pathogens that may harm the plants although the peat was steam sterilised at 60^{0}C. The agar has to be thoroughly washed away which is the nutrient media of many microbes.

4.8 Summary

WPM supplemented with 0.1 to 0.25 mgL^{-1} IBA proved to be the most beneficial exogenous auxin treatment for *Paraserianthes falcataria.* Rooting percentages in MS, LP and GD media were comparitively low compared to WPM. IAA and NAA were not effective root initiators in comparison to IBA in all four media screened

in the present study. Hormone free media recorded the lowest rooting percentage. Thus it can be concluded that exogenous administration of auxins is important, but the optimal concentration varies from species to species as shown in other forestry studies. The best results for plant acclimation was achieved using soil combinations of Peat : Perlite : Coarse sand mixtures in the ratio of 1:1:1.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

Forestry is an important worldwide industry enjoying the privileges of being one of the main export earner for many countries including Australia and New Zealand. Aforestation and revegetation of forest land therefore are main issues that aid in the conservation of natural resources and the environment which strive to maintain the balance between demand and production (Le Roux and Van Staden, 1991a). Loss of productive stock can therefore disadvantage this industry causing great delays and losses. This disadvantage can be prevented by the induction of fast growing forest species that would diminish the lengthy growth cycle attributed to such plantations. Forest molecular biology and tissue culture technologies could play an important role in the choice of genotypes for successful and rapid establishment of these agroforestry practices (Klopfenstein and Kerl, 1995).

One of the traditional uses of tissue culture was in the diagnosis of diseases. Most recently, however, the use of tissue culture methodology in the discipline of somatic cell genetics has opened new areas of investigation in the study of genetics (Teasdale, 1995). One great advantage of tissue culture as a research tool is that it permits control of the cellular environment in a fashion, allowing the behaviour of cells to be both explored and manipulated (Skolmen, 1986;

Sommer and Caldas, 1981). The developments in tissue culture have made possible enormous gains in the understanding of the genetic process and have permitted application of the techniques of molecular biology (Teasdale, 1995). Plants typically exhibit morphological and metabolic changes in response to perturbation to their normal environment. Many of these changes are believed to be of adaptive significance which makes the organism more fit in the altered environment (Singh *et al.,* 1995). The sessile nature of plants has forced them to adopt a flexible developmental program that can be coordinated by environmental cues. It is evident that plant growth regulators are potential candidates to transduce these environmental signals. A crucial question is whether biochemical and physiological events are triggered by a change in the plant growth regulators levels, or whether they act to coordinate the progression of a developmental sequence after it has been initiated (Roberts and Hooley, 1988). Hussey (1980) has made the novel suggestion that plant growth regulators would be able to alter the pattern of cell differentiation in root and shoot meristems in a predefined way. This can be correlated to the results obtained from this study of *Paraserianthes falcataria* wherein the introduction of auxins and cytokinins to micropropagated explants have largely influenced the growth and development of the subcultures.

Warm water at 70^{0}C was observed to produce the highest seed germination percentage (Figure 2.24) coupled with conditions of non-cold and dark. In fact, warm water treatment may enhance the seed germination of tropical species

according to Longkamp (1987). Similarly seeds grown in non phototrophic conditions and non-cold produced a higher percentage of growth than those placed under cold and light, cold and dark, and non-cold and light conditions. Similar studies by Longkamp (1987) have shown that since it is a tropical species lower temperatures were not expected to be required for growth. Discussing seed germination the most important limiting factor was dormancy. Dormancy can be considered to begin at an early stage in seed development, for immature embryos can 'germinate' when isolated from the seeds but are prevented from doing so *in vivo*. Commonly this phase of development terminates concurrently with seed maturation and the mature seeds will germinate when provided with a suitable environment (Roberts and Hooley, 1988). This important phase is a common occurrence in most plant species and can prove to be a rather difficult pattern to break and vary from species to species. Therefore, a suitable environment is a main prerequisite. This principle was experimented with in seed germination experiments wherein the dormancy of the seeds proved to be a hindrance even though the seeds were in viable condition. In spite of treating the seeds to various pre-treatments as listed under Chapter 2.5 only certain conditions were seen to effectively penetrate the seed coat and facilitate germination of the seed.

Light proved to be another contributing factor to effective seed germination. In certain species, germination is promoted by exposure to light. Small seeds usually germinate in loose, unpacked soil and near the surface where light is

present (Ridgeway and Kingsley, 1989). However in the present study non-phototropic conditions especially in the initial stages of growth proved to be highly beneficial for hypocotyl initiation. This may be due to the fact that light does not seem to play a role in the response of cotyledonous tissues to Gibberellin. In internode segments that were excised from 45-day-old *Avena sativa, Oryza sativa* and *Triticum aestivum* plants light had no effect on the elongation response in the explants (Kaufman *et al.,* 1968; Roesel and Haber, 1964; Soni and Kaufman, 1972). Sterilisation was another limiting factor observed in the present study. This is supported by studies by Barker *et al.* (1982) and Le Roux and Van Staden (1991a), have shown that tolerance of seeds to sterilisation depends on size and thickness of the seed coats. For instance, *Eucalyptus citriodora* seeds are small and thin walled and can be sterilised in 2% NaOCl (v/v) for 20 minutes, whereas *Eucalyptus marginata* seeds, which are larger and have thicker seed coats can tolerate 5% NaOCl (v/v) for 60 to 90 minutes. This can explain why the single sterilisation techniques of high concentrations of bleach (40 and 50% (v/v)) failed to produce better germiantion percentages than 10, 20 and 30% (v/v) sodium hypochlorite.

Gibberellins are endogenous plant hormones that can also play an important role in elongation of plant cells and can increase or sometimes inhibit the rate of cell division (Le Roux and Van Staden, 1991b; Ridgeway and Kingsley, 1989). Gibberellins are factors that may inhibit or enhance growth of an explant and should be considered as a major limiting factor. This is because they act on the

living cells (aleurone layer) surrounding the food reserves (endosperm). This action induces the aleurone cells to produce enzymes that break down starch to sugars and release tryptophan from the protein of the endosperm. The tryptophan migrates to the coleoptile tip and is transferred into IAA, which in turn moves to the growth zone and weakens the cell walls, thus permitting water uptake (Ridgeway and Kingsley, 1989). One of the morphological site of action of gibberellin is the embryo. It is not known at this stage whether the gibberellin treatment was more beneficial because of the alternation of plant hormone activity or the seeds were subsequently weakened by the action of sodium hypochlorite allowing the growing embryo to expand and break through more easily. Gibberellin promote embryo growth by stimulating the synthesis of the hydrolytic enzymes which break down the starch reserves into amino acids and sugars making them available for plant growth. (Raven *et al.,* 1992). Gibberellic acid also aids as an activator in red beans (Kai *et al.,* 1995) and was also seen to increase plant growth, stem diameter and shoot succulence while also inducing leaf hyponasty and vegetative bud break of apices in Lemonwood (Dwyer *et al.,* 1995). However the hypothesis that gibberellin (growth promoter) and ABA (growth inhibitor) may regulate protein synthesis by turning on or turning off genes has been suggested but not proved (Anon 1969). Thus in conclusion it can be emphasised that higher concentration of gibberellin (50 mgL^{-1}) help in breaking the dormancy but other optimal factors such as light and temperature also has to be taken into consideration which may combine to aid in the breaking of dormancy.

Seed pre-treatments such as non-Gibberellic acid and double sterilisation (Chapter 2.7.2), gibberellic acid and single sterilisation (Chapter 2.7.4), Concentrated Sulphuric acid (Chapter 2.7.6) and Sand Paper treatment (Chapter 2.7.8) did not produce high results as compared to warm water treatment. The poor results may be due to the fact that the thick seed coats of *Paraserianthes falcataria* pose a major restriction to the embryo growth. The rate of germination was therefore, less than 50% for all these pre-treatments which was caused by inefficient damage to the seed coat. Even though the viability of the seeds were tested prior to pre-treatment experiments, the failure to germinate well may be due to various enzymes stored within the seeds that inhibit germination (Raven *et al.,* 1992). In addition, seeds can contain hormones such as ethylene, which further inhibit the germination of seeds. Considering the nature of the percentages produced by direct sowing and sucrose pre-treatments 3% (w/v) sucrose produced the best growth. Studies by Thorpe (1982) established that sucrose provides both a carbon source as well as osmoticum. Therefore, providing a suitable stage for the development of the growing embryo.

Summarising the findings for shoot initiation in *Paraserianthes falcataria* zeatin supplemented in MS media between concentration ranges of 0.1 to 0.5 mgL^{-1} produced the highest mean number of explants. It can be concluded that the formation of phytohormones is closely connected with the formation of natural growth inhibitors in plant tissues. Such an interrelation becomes possible

through their common precursors are a starting point for the metabolic pathways of the synthesis of these growth hormones in the plant. The stem growth and formation of stem buds depend strongly on the activity of phytohormones (Kefeli, 1978).

Cytokinins constitute a group of factors regulating the growth of stem and axial organs. The application of cytokinin intensifies the synthesis of proteins, nucleic acids, lipids, starch and other compounds. In early studies, the synthetic kinetin was the main compound used for growth of lateral buds. Prolonged elongation of the bud could be caused only by the addition of an auxin or a gibberellin to it. Another cytokinin BAP sometimes causes substantially more elongation than kinetin but, its effects have been studied with only a few species. In the present study, even though four different types of cytokinins were investigated, the results produced were varied. Zeatin by far was found to be the best cytokinin for shoot initiation in *Paraserianthes falcataria.* BAP was second best, followed by 2iP and kinetin. BAP and zeatin dramatically enhance elongation for at least two weeks, whereas 2iP and kinetin promote only short term growth. The short answer is that no concise statement can yet be made which delimits the substances that stimulate cell division in cells. In some cases, single substances may suffice, more usually substances interact synergistically and in different composite systems outwardly to bring about the effects of growth. Investigators who have attributed the full growth stimulus solely to single substances did so because the assay system used was inherently delimited in its range of

responses (Steward and Krikorian,1971). Another reason may be that the closely related hormones zeatin and 2iP cause such different effects is unknown but is speculated that 2iP is only weakly active because it is slowly hydroxylated to the much more active zeatin in the buds. Uncertainty still exists about the relative importance of cytokinins and other hormones and nutritional factors in controlling lateral bud development (Salisbury and Ross, 1992).

According to Blomstedt *et al.* (1991) *Eucalyptus globulus* and *Eucalyptus nitens* grew much better in zeatin. Zeatin has also been reported to improve shoot elongation in *in vitro* cultures of *Eucalyptus* (Warrag *et al.,* 1989); and Poplar (Coleman and Ernst 1989). Bennett and McComb (1982) have also used zeatin to induce adventitious shoot regeneration in *Eucalyptus marginata,* which supports the findings of the present study. Mehra-Palta (1982) contradicted the findings of Mascarenhas *et al.* (1982) and Trindade *et al.* (1990) in which he found that kinetin was a suitable cytokinin for adventitious shoot formation for seedlings of *Eucalyptus nova-anglica* and *Eucalyptus viminalis* and hence can be concluded that effect of cytokinins vary from species to species.

Higher concentrations as well as continuous application of cytokinins (Kim *et al,* 1985; Thorpe, 1983) either during induction or during the maintenance of cell cultures are inhibitory (Negrutiu *et al.*, 1978). This was true in the case of the present study in which continuous exposure to cytokinins over 3-5 weeks intervals with four subsequent subcultures may have been responsible for the

low mean number of shoots produced per explant (mean number between 21 to 10). Studies demonstrated that cyokinins and auxins regulate the growth of lateral buds. Auxins inhibit the process of lateral growth and cytokinin neutralizing the inhibitory effect of auxin, by directly affecting differentiation of xylem vessels of a breaking bud. On investigating the effect of cytokinin, auxin and gibberellin in different combinations on the growth of isolated cotyledons found that although each phytohormone stimulates the growth of isolated cotyledons, the maximum effect was obtained when a combination of all three groups of phytohormones were used. The analysis of the concentration curves describing the effect of the phytohormones permitted the authors to come to the conclusion that none of the phytohormnes can totally be replaced by another. Thus, each phytohormone displays its own specific properties in plant growth regulation and a combination of all three are required for the growth of an explant (Kim *et al.,* 1985; Negrutiu *et al.,* 1978; Thorpe, 1983).

Root initiation and plant acclimation are major factors to consider in the micropropagation of woody species. In the present study explants adapted well to root initiation studies but formation of roots was a lengthy process. Rooting percentages were highest with WPM supplemented with 0.1 to 0.25 mgL^{-1} IBA. Auxins play a prominent role in the synthesizing activity of cells and are among components necessary for the formation and regeneration of explants. Cells and tissues enriched with growth regulating substances attract water and nutrients, this is probably the reason for intensified growth of the cells and tissues, while

promoting the inflow of nutrients to certain parts of a plant, growth regulators thereby favour their transport from other parts (Maksimov, 1941). Exogenous auxin can be considered to have two major effects,the initiation and/or enhancement of root organogenesis and as an inhibitor of cytokinin-induced shoot initiation (Carswell and Locy, 1984; Chylah and Tran Thanh Van, 1975; Coleman and Greyson, 1977; Cross *et al.,* 1985; Haddon and Northcote, 1976; Profumo *et al.,* 1985; Rucker, 1982). Another major application of exogenous auxin is in the rooting of shoot obtained through micropropagation, though in some woody plants this is the most difficult phase (Hussey, 1980; Murashige, 1974). The hormonal requirements for root organogenesis are generally higher than shoot organogenesis. In the present study it was observed that the application and concentration of auxins between concentration of 0.1 to 0.25 mgL^{-1} were essential for successful rooting.

Hormone free treatments have produced adventitious roots from microcuttings of shoots in species such as *Eucalyptus camaldulensis* in MS (Kumar and Ayyappan, 1987; Kumar and Raman,1989). Hormone free MS media produced a healthy root system in the case of *Hibiscus cannabinus* (Cristofari *et al.,* 1988). Root initiation was reported with *Eucalyptus tereticornis* in hormone free culture media, there was however a variation in the culture medium. The medium used for initiation was half strength MS basal media (Rao, 1988). But another report by Warrag *et al.* (1990), has reported that the level of basal minerals (1/4, ½ or full strength) have little effect on rooting percentages in *Eucalyptus* species.

Cheng *et al.* (1992) also recorded the lowest (40%) rooting percentage in *Eucalyptus sideroxylon* in hormone free half strength MS basal media. Similar findings have been reported in rice, rye and wheat by Whitney (1996) where the explants failed to produce any root developing callus and many of the explants died or failed to grow at all when placed on hormone free media. Similarly in the present study, explants of *Paraserianthes falcataria* failed to adapt very well to hormone free conditions.

IBA on the whole performed better than IAA and NAA in all three media. IBA was better suited *Paraserianthes falcataria*. However, the concentrations at which the optimum for IBA has been recorded vary from species to species. Reports have been published with some plant species reaching an optimum at lower concentrations and otherwise (*Eucalyptus tereticornis* (1.0mgL^{-1} IBA)) (Das and Mitra, 1990). In this study, it was found that as the concentrations of IBA were increased the explants started to show visible signs of necrosis and excessive callus formation was observed. After the second and third subculture some of the explants did not survive, among the few that survived, unhealthy and abnormal looking stunted shoots with sparse axillary buds and leaf formation were the common features that were noticed. Blomstedt *et al.* (1991) published similar findings in *Eucalyptus regnans* with increasing concentrations of IBA. In few other species higher concentrations of IBA had a beneficial effect and increased the rooting percentage. Such findings have been reported by Barakat and Lakany (1992) in *Acacia saligna* with 2.0 mgL^{-1}IBA; Rao (1988) in

Eucalyptus tereticornis with 4.4 mgL^{-1} and 4.9 mgL^{-1}IBA; Zhang *et al.* (1982) in *Eucalyptus botyroides* with >2.0 mgL^{-1} IBA; Cheng *et al.* (1992) in *Eucalyptus sideroxylon* with 2.5-10 mgL^{-1} IBA; Bennett and McComb (1982) in *Eucalyptus marginata* with 5 mgL^{-1} IBA; Warrag *et al.* (1990) in *Eucalyptus grandis* with 2 mgL^{-1}, IBA; Le Roux and Van Staden (1991) in *Eucalyptus* species with 2 mgL^{-1} IBA; Darus (1991)*Acacia mangium* with 0.5 mgL^{-1} IBA; Sandum *et al.* (1986), Tang and Srivastava (1985) and Hamsari and Srivastava (1985) in *Glemina arborea* with 2.5-5 mgL^{-1} IBA. Continual subculture on multiplication medium has been reported to increase the rooting percentage of *Eucalyptus grandis* by upto 18% (Warrag *et al.*, 1990), however it was a common occurrence that repeated subculture the process of rooting was not augmented with *Paraserianthes falcataria* in the present study. The survival rate of the explants after the third and fourth subculture was very low with only four or five explants out of 25 replicates subcultured produced roots in hormone free media and ten to fifteen of the replicates rooted with auxin supplemented media.

According to experiments done on tobacco by Skoog and Miller (1957), manipulation of auxin and cytokinin concentrations in the medium will result in better root and shoot formation than by addition of a single hormone. This has been true for many woody species. In many cases, a mixture of two cytokinins or two auxins has proven to be superior to a single cytokinin or auxin (Thorpe and Patel, 1984). The imbalance of hormones can inhibit the root or shoot formation in the microcuttings. Shoot cultures of *Eucalyptus regnans* have been

established in the past, but zero or unacceptably low rooting frequencies have been reported (Barker et al., 1977; De Fossard *et al.*, 1977; Hartney,1981; Hartney and Barker, 1980). Therefore, the presence of auxins or cytokinins alone do not influence rooting percentages, but is a combination of various conditions such as irradiance, carbohydrate source, cytokinin and auxin combination and the age of the explant. In the case of *Paraserianthes falcataria*, Photoperiod of 16h day at 17 Wm^{-2} and 25^0C, with 3% sucrose, 0.5 mgL^{-1} Zeatin and 0.1-0.75 mgL^{-1} IBA supplemented WPM media were seen to produce the optimum for root initiation and maintenance. Cytokinins have been proved to be inhibitory to root development. However there are some circumstances when at low concentrations they can stimulate lateral roots, probably by suppressing taproot growth. The light spectrum for root initiation is red light but the light spectrum of red and blue light can be manipulated to facilitate shoot initiation but not root initiation.(Murashige 1974).

To conclude seed germination, shoot and root initation trials have proven that micropropagation trials were successful for *Paraserianthes falcataria* explants. Warm water treatment at 70^0C was the best treatment for germination of *Paraserianthes falcataria* seeds. From the present study, the exogenous plant hormones between concentration of 0.1 to 0.5 mgL^{-1} were most suited for initiation studies in *Paraserianthes falcataria.* The adaptation of the explants to the four media (MS, LP, GD and WPM) investigated were different for shoot and root initiation, as well as the reaction of the four cytokinins (zeatin, BAP, 2iP and

kinetin) and three auxins (IAA, IBA and NAA). Shoot initiation was most successful with zeatin supplemented in MS media while the other three media and cytokinins produced lesser number of shoots in comparison, while WPM supplemented with IBA was most suited for root initiation in comparison to the other three media and two auxins. *In vitro* propagation is possible provided suitable environmental conditions and exogenous hormones are supplied in right measures. Given that the effect of exogenous hormones can be dependent on time of application it is pertinent to mention the concept of changes in hormone sensitivity, rather than concentration (Skoog and Miller, 1957) as a regulatory mechanism of organogenesis (Trewavas, 1981, 1982). Also, the presence or absence of endogenous hormones at the required stage of organogenesis plays an important role in the successful establishment of the *in vitro* cultures. This aspect lay beyond the parameters of the present study but is an important facet in the growth and morphogenesis of a plant. Therefore, it is important to state the fact that it is not practical to prove that every possible manipulation of exogenous and endogenous hormone levels will not promote organogenesis ties in closely with the problems of competence or totipotency, *in vitro* (Street, 1979). This is because both these phenomena are measured by a response to exogenous hormone application and it is not possible therefore to prove a lack of totipotency (Rice *et al.,* 1979). Another important factor to be considered is the juvenile genotypic nature of the explants used in culture. The heterozygotic nature of the seed material may have contributed to the different responses to the growth hormones. This aspect needs more research but was not carried out

in the present study because the parameters of the research were beyond the boundaries of this thesis.

It can be inferred that the objectives set out by this study have been achieved by the successful acclimation of *Paraserianthes falcataria* to *in vitro* conditions consisting of seed germination, shoot initiation, root initiation and plant acclimation. The introduction of this species for revegetation and agroforestry practices can be initiated in the near future.

REFERENCES

Note (1): Page numbers of books where the entire book was used for reference have not been listed.

Note (2): French articles carry names in french and not the translated versions in english due to proficiency in the language (the articles were read in french).

- Abdullah, A.A., Yeoman, M.M. and Grace, J. (1989). Calabrian Pine (*Pinus brutia* Tenore). *In* Biotechnology in Agriculture and Forestry, Volume 5, Trees 2, Springer – Verlag, Germany.

- Ahmad, D.H. (1991). Micropropagation of *Acacia mangium* from aseptically germinated seedlings. *Journal of Tropical Forest Science* **3**, 204-208.

- Ahuja, A.A. (1993). Micropropagation a la carte. *In* Cell and Tissue Culture in Forestry, Volume 3, Martinus Nijhoff Publishers, Dordrecht.

- Aitken – Christie, J. and Thorpe, T.A. (1984). Clonal Propagation: Gymnosperms. *In* Cell Culture and Somatic Cell Genetics of Plants, Volume

1, Academic Press.

- Akyeampong, E., Hitimanal. A., Franzel, S. and Munyemana, P.C. (1995). The agronomic and economic performance of banana, bean and tree intercropping in the highlands of Burundi - an interim assessment. *Agroforestry Systems* **31**, 199-210.

- Ammirato, P.V. and Steward, F.C. (1987). Clonal propagation. *Botanical Gazette* **132,** 149-158.

- Anderson, W.C. (1980). Mass propagation by tissue culture, principles and techniques. *In* Proceedings of the Conference on Nursery Production of Fruit Plants through tissue culture - Applications and Feasibility 1-10.

- Andtbacka, C. (1995). Optimising the application of biotechnology to forest plantations. *Eucalypt plantations : Improving fibre yield and quality (*eds. Potts, B.M., Borralho, N.M.G., Reid, J.B., Gromer, R.N., Tibbits, W.N. and Raymond.C.A.). *Proceedings of CRC – IUFRO Conference, Hobart, 19-24 Feb (CRC for temperate Hardwood Forestry, Hobart)* 472-429.

- Anonymous. (1976). International seed testing association. *Seed Science and*

Technology **4**, 3-49.

- Anonymous. (1969). 'Berelex' (Gibberellic acid) for promoting natural plant growth. ICI Plant Protection Ltd, London.

- Anonymous. (1979). *Albizia* species, tropical legumes. *In* Resources for the future. National Academy of Sciences, National Academy Press, Washington DC 171-185.

- Anonymous. (1983). *Albizia falcataria*, firewood crops. *In* Resources for the future. National Academy of Sciences, National Academy Press, Washington DC 4-6.

- Anonymous. (1991). Australian paper and pulp products. *In* Resource Assessment Commission. Forest Timber Inquiry Draft Report, Volume 1, Australian Government Publishing Service, Canberra **43,** 76-81.

- Anonymous. (1992). National Association of Forest Industries, Forest facts. Forest Industries, Canberra.

- Anonymous. (1996). AMCOR Paper group expands to fight imports. *Accent on*

AMCOR **16,** 5-6.

- Awonaike, K.O., Danso, S.K.A. and Zapota, F. (1996). Nitrogen fixation in *Leucaena leucocephala* as affected by rooting volume and competition with *Eucalyptus camaldulensis*. *Agroforestry Systems* **33**, 195-203.

- Bajaj, Y.P.S. (1986). Biotechnology of tree improvement for rapid propagation and biomass energy production. *In* Biotechnology in Agroforestry and Forestry, Volume 5, Trees 2, Springer – Verlag, Germany 1-24.

- Barakat, M.N. and El Lakany, M.H. (1992). Clonal propagation of *Acacia saligna* by shoot tip culture. *Euphytica* **59**, 103-107.

- Barker, P.K., De Fossard, R.A. and Bourne, R.A. (1977). Progress towards clonal propagation of Eucalyptus species by tissue culture techniques. *International Plant Propagators Society Combined Proceedings* **27**, 546-556.

- Barlass, M. (1983). Micropropagation of woody species. *Australian Parks and Recreation* 57-600.

- Barlass, M. (1991). Commercial production in Australia and New Zealand. *In*

Micropropagation Technology and its application, Kluwer Academic Publishers, Netherlands.

- Bell, D.T., Rokich, D.P., Mcchesney, C.J. and Plummer, J.A. (1995). Effects of temperature, light and gibberellic acid on the germination of seeds of 43 species native to Western Australia. *Journal of Vegetation Science* **6**,797-806.

- Bennett, I.J. and McComb, J.A. (1982). Propagation of Jarrah (*Eucalyptus marginata*) by organ and tissue culture. *Australian Forest Research* **12**, 121-127.

- Binkley, D., Dunkin, K., Debell, D. and Ryan, M. (1992). Production and nutrient cycling in mixed plantations of *Eucalyptus* and *Albizia* in Hawaii. *Forest Science* **38**, 393-408.

- Binkley, D. (1997). Bioassays of the influence of *Eucalyptus saligna* and *Albizia falcataria* on soil nutrient supply and limitation. *Forest Ecology and Management* **91**, 229-234.

- Biondi, S. and Thorpe, T.A. (1982). Clonal propagation of forest tree species. Proceedings of COSTED Symposium on Tissue Culture of Economically

Important Plants, 197-204,

- Blomstedt, C., Cameron, J., Whiteman, P., and Chandler, S.J. (1991). Micropropagation of juvenile *Eucalyptus regnans* (Mountain Ash). *Australian Journal of Botany* **39**, 179-186.

- Boisand, J. and Malcoste, R. (1970). Analyse spectrophotometrique du phytochrome dans l'embryon de Courge *(Cucurbita pepo)* et de Potiron *(Cucurbita maxima). Planta* **91,** 54-67.

- Boland D.J., Brooker, M.H. and Turnball, J.W. (1980). *Eucalyptus* seed, Division of Forest Research, CSIRO, Canberra.

- Boland, D.J., Brooker, M.I.H., Chippendale, G.M., Hall, N., Hyland, B.P.M., Johnson, K.D., Kleinig, D.A. and Turner, J.D. (1994). Forest Trees of Australia, CSIRO, Australia.

- Bonga, J.M. (1991). *In vitro* propagation of conifers: fidelity of the clonal offspring. *In* Woody Plant Biotechnology, Plenum Press, New York.

- Bonga, J.M. and Durzan, D.J. (1982). *Tissue Culture in Forestry*, Springer

Verlag, Berlin 1-412.

- Boulay, M. (1987). Preliminary research on somatic embryogenesis of *Eucalyptus gunnii*. *Annales de Recherches Sylvicoles*, AFOCEL, France **23**, 23-37.

- Bradbeer, J.W. (1988). Seed dormancy and germination. *In* Tertiary Level Biology, Blackie Academic and Professional, Melbourne.

- Buck, K. (1989). Brave new botany. *New Scientist* **1667,** 32-34.

- Burger, D.W. (1987). *In vitro* propagation of *Eucalyptus sideroxylon*. *Hortscience* **22**, 496-497.

- Burza,W., Murkowski, A. and Malepszy, S. (1994). Difference in the luminescence of regenerated cucumber plants caused by plant hormones in the medium. *Gartenbauwissenschaft* **59**, 105-108.

- Carswell, G.K. and Locy, R.D. (1984). Root and shoot initiation by leaf, stem and storage root explants of sweet potato. *Plant Cell, Tissue and Organ Culture* **3,** 229-236.

- Caulovitz, P.G.V. (1995). Multipurpose Trees and Shrubs, Sources of Seeds and Innoculants, ICRAF 12.

- Chalupa, V. (1990). Biotechnology in forest tree improvement: Trees of the future. *In* Plant Aging: Basic and Applied Approaches, Plenum Press, New York.

- Chandler, S.F. and Thorpe, T.A. (1982). Hormonal regulation of Organogenesis *in vitro. Journal of Plant Physiology* 1-17.

- Chandler, S.F., Bateman, C., Blomstedt, C., Williams, D. and Young, R. (1989). Forest biotechnology of Calgene Pacific. *Australian Journal of Biotechnology* **3,** 281-284.

- Charomaini, Z.M. (1989). Pre sowing treatment of *Glemina arborea* Roxb seed in assisting germination. *Buletin Penelitiian Hutan* **515,** 29-39.

- Chen, C. (1978). Effects of sucrose concentration on plant production in anther culture of rice. *Crop Science* **18**, 905-906.

- Chen, C.M. (1982). Metabolism and Molecular Activities of Cytokinins.

Springer-Verlag, Berlin.

- Chen, J.C. (1987). Wood properties of *Albizia falcata* grown in Guangdong province. *Hua nang nung yeh da hsueh hsueh pao, Kuangchou, South China Agricultural College* **3,** 59-69.

- Cheng, B., Peterson, C.M. and Mitchell, R.J. (1992). The role of sucrose, auxin and explant source on *in vitro* rooting of seedling explants of *Eucalyptus sideroxylon. Plant Science* **87**, 207-214.

- Cheng, T.Y. and Voqui, T. (1977). Regeneration of Douglas-fir plantlets through tissue culture. *Science* **198**, 306-307.

- Chinearivera, J.D. (1995). Production, dispersal and dormancy of seeds of *Albizia procera* (Roxb) Benth a woody weed of pastures in Puerto Rico. *Journal of Agriculture of the University of Puerto Rico* **79**, 163-171.

- Chylah, A. and Tran Thanh Van, M. (1975). Differential reactivity in epidermal cells of *Begonia rex* excised and grown *in vitro. Physiology of Plants* **35,** 16-20.

- Coleman, G.D. and Ernst, S.G. (1989). *In vitro* shoot regeneration of *Populus deltoides:* effect of cytokinin and genotype. *Plant Cell Reports* **8**, 459-462.

- Coleman, W.K. and Greyson, R. (1977). Promotion of root initiation by gibberellic acid in leaf discs of tomato (*Lycopersicum esculentum*) cultured *in vitro. New Phytology* **78,** 47-54.

- Costacurta, A. and Vanderleyden, J. (1995). Synthesis of phytohormones by plant associated bacteria (Review). *Critical Reviews in Microbiology* **21**, 1-18.

- Cristofari, E., Desantis, F., Milleti, G. and Mencacci, N. (1988). *In vitro* clonal propagation of *Hibiscus cannabinus* L. *Annali della Facolta di Agraria, Universita degli studi di Perugia* 689-693.

- Cross, A.F, Creemers-molenaar, T, Van Den Ende, G, Kemp, A. and Barendse, G.W.M. (1985). Tissue age as an endogenous factor controlling *in vitro* bud formation on explants from the inflorescence of *Nicotiana tabacum* L. *Journal of Experimental Botany* **36,** 1771-1779.

- Curir, P., Damiano, C., Volpi, L. and Ruffoni, B. (1985). Observation on the development of new shoots from axillary buds in the *in vitro* propagation of

Eucalyptus gunnii Hook. *Annali dell' Instituto Sperimentale per la Floricultura – Italy* **16**, 59-68.

- Curir, P., Damiano, C., Esposito, P. and Ruffoni, B. (1986). The *in vitro* propagation of *Eucalyptus gunnii* Hook and *Eucalyptus stuartiana* F.VM. *Annali dell' Instituto Sperimentale per la Floricultura –Italy* **17**, 73-84.

- Curir, P., Vansumere, C.F., Termini, A., Barthe, P., Marchesini, A. and Dolci, M. (1990). Flavonoid accumulation is correlated with adventitious root formation in *Eucalyptus gunnii* Hook micropropagated through axillary bud stimulation. *Plant Physiology* **92**, 1148-1153.

- Damiano, C., Curir, P. and Cosmi,T. (1985). Short note on the effect of sugar on the growth in vitro of *Eucalyptus gunnii. Annali dell'Instituto Sperimentale per la Floricultura* –Italy **16,** 75-80.

- Damiano, C., Curir, P., Esposito, P. and Ruffoni, B. (1989). Present micropropagation research programs at ISF in San Remo, Proceedings of a symposium on growth regulators in ornamental horticulture held at Skierniewice, Poland on 5-10 Sep, 1988. *Acta Horticulturae* **251**, 129-133.

- Dargavel, J. and Semple, N. (1991). Prospects for Australian Forestry

Plantations, Panther Publishing and Printing, Canberra.

- Darus, H.A. (1991). Multiplication of *Acacia mangium* by stem cuttings and tissue culture techniques. Advances in tropical *Acacia* research. *In* Proceedings of an International workshop held in Bangkok, Thailand, 11-15, Feb 1991, ACIAR Proceedings series 1991 **35**, 32-35.

- Darus, H.A. (1992). Micropropagation techniques for *Acacia mangium* and *Acacia auriculiformis*, Proceedings of an international workshop held in Tawau, Sabah, Malaysia, 1-4 July, 1991, ACIAR Proceedings series 1992 **37**,119-121,

- Das, T. and Mitra, G.C. (1990). Micropropagation of *Eucalyptus tereticornis* Smith. *Plant Cell, Tissue and Organ Culture* **22,** 95-103.

- Dean, R.D. (1995). Optimising the application of biotechnology to forest plantations. *Eucalypt plantations : Improving fibre yield and quality (*eds. Potts, B.M., Borralho, N.M.G., Reid, J.B., Gromer, R.N., Tibbits, W.N. and Raymond.C.A.). *Proceedings of CRC – IUFRO Conference, Hobart, 19-24 Feb (CRC for temperate Hardwood Forestry, Hobart)* 472-429.

- De Fossard, R.A. (1974). Tissue culture of *Eucalyptus. Australian Forester* **37**,

43-54.

- De Fossard, R.A., Nitsch, C., Cresswell, R.J. and Lee, E.C. (1974). Tissue and organ culture of *Eucalyptus. New Zealand Journal of Forestry Science* **4,** 267-278.

- De Fossard, R.A., Barker, P.K. and Bourne, R.A.(1977). The organ culture of nodes of four species of *Eucalyptus. Acta Horticulturae* **78**. 157-163.

- De Fossard, R.A. and De Fossard, H. (1988). Micropropagation of some members of Myrtaceae. *Acta Horticulturae* **227,** 346-351.

- De, P.S. and Basu, P.S. (1996). Content of different phytohormones and Indole-3-acetic acid metabolism in root nodules of *Derris scandens* Benth, *Journal of Basic Microbiology* **36,** 299-304.

- Deandrade, A.C.S., Loureiro, M.B., Souza, A.D.D. and Ramos, F.N. (1997). Dormancy break in *Bowdichia virgiloides* seeds. *Pesquisa Agropecuaria Brasileira* **32**, 465-469.

- Debell, D.S., Whitesell, C.D. and Schubert, T.H. (1985). Mixed plantations of

Eucalyptus and leguminous trees enhance biomass production. *USDA Forest Service Research Paper, Berkeley* **175,** 6.

- Debell, D.S., Whitesell, C.D. and Crabb, T.B. (1987). Benefits of *Eucalyptus – Albizia* mixtures vary by site on Hawaii Island. *USDA Forest Service Research Paper, Berkeley* **187,** 5

- Debell, D.S., Whitesell, C.D. and Schubert, T.H. (1989). Using Nitrogen fixing *Albizia* to increase growth of *Eucalyptus* plantations in Hawaii. *Forest Science* **35**, 64-75.

- Debell, D.S., Cole, T.G. and Whitesell, C.D. (1997). Growth and development and yield in pure and mixed stands of *Eucalyptus* and *Albizia. Forest Science* **43**, 286- 298.

- Delarosaibarra, M., Maiti, R.K. and Deleon, B. (1994). Effect of gibberellic acid and 2,4,diphenoxy acetic acid Garlic crop yields (*Allium sativum* L). *International Journal of Experimental Botany* **56,** 91-94.

- Dhawan, V. and Bhojwani, S.S. (1985). *In vitro* propagation of *Leucaena leucocephala* (Lam) de Wit. *Plant Cell Reports* **4**, 315-318.

- Dhawan, V. and Bhojwani, S.S. (1987). *In vitro* nodulation of micropropagated plants of *Leucaena leucocephala* Rhizobium. *Plant and Soil* **103**, 274-276.

- Dillner. (1995). Optimising the application of biotechnology to forest plantations. *Eucalypt plantations : Improving fibre yield and quality (*eds. Potts, B.M., Borralho, N.M.G., Reid, J.B., Gromer, R.N., Tibbits, W.N. and Raymond.C.A.). *Proceedings of CRC – IUFRO Conference, Hobart, 19-24 Feb (CRC for temperate Hardwood Forestry, Hobart)* 472-429.

- Domecq, C.M. (1988). *In vitro* culture of shoot tips of *Melia azedarach* var. *gigantea. Hortscience* **48,** 33-42.

- Dracup, M. (1991). Increasing salt tolerance of plants through cell culture requires greater understanding of tolerance mechanism. *Australian Journal of Plant Physiology* **18,** 1-15.

- Draper, J. and Scott,R. (1991). Gene transfer to Plants. *Plant Genetic Engineering* **1,** 38-81.

- Duguma, B. and Tonye, J. (1994). Screening of multipurpose tree and shrub species for agroforestry in the humid lowlands of Cameroon. *Forest Ecology*

and Management **64,** 135-143.

- Duguma, B., Tonye, J., Kanmegne, J., Manga, T. and Enochi, T. (1994). Growth of ten multipurpose tree species on acid soils in Sangmelima, Cameroon. *Agroforestry Systems* **27**, 107-119.

- Duhoux, E. and Davies, D. (1985). Caulogenès a parti bourgeons cotyledonaires d'*Acacia albida* et influence en saccharose sur la rhizogenèse. *Journal of Plant Physiology* **121,** 176-180.

- Dunlap, J.R., Slovin, J.P. and Cohen, J.D. (1996). Indole-3-acetic acid, ethylene and abscisic Acid metabolism in developing muskmelon (*Cucumis melol)* fruit. *Plant Growth Regulation* **19,** 45-54.

- Durzan, D.J. (1988). Application of cell and tissue culture in tree improvement . CIBA Foundation Sympossium, John Wiley and Sons 36-49.

- Dwyer, P.J., Bannister, P. and Jameson, P.E. (1995). Effects of three plant growth regulators on growth, morphology, water relations and frost resistance in Lemonwood (*Pittosporium eugenioides*). *New Zealand Journal of Botany* **33,** 415-424.

- Ecenomou, A.S. (1982). Chemical and physical factors influencing *in vitro* propagation of hardy deciduous azaleas (*Rhododendron spp*). Discussion, University of Minnesota, St. Paul 1-12.

- Ecenomou, A.S. and Read, P.E. (1980a). Effect of benzyladenine pre-treatments on shoot proliferation from petunia leaf segments cultured *in vitro*. *Proceedings of Plant Growth Regulators Working Group* **7,** 96-103.

- Edwards, K.L. and Scott, T.K. (1974). Regulation of growth. *Planta* **119,** 27-37.

- Ellis, D.D. and Webb, D.T. (1993). Light regimes used in conifer tissue culture. *In* Micropropagation of Woody Plants, Kluwer Academic Press, Netherlands.

- Escalante, B.Z. and Langville, A.R. (1995). Role of growth regulators in *in vitro* rhizome growth of potato *(Solanum tuberosum). Hortscience* **30,** 1248-1250.

- Evans, M.L. (1976). Chemical regulation. *Plant Physiology* **58,** 599-601.

- Evans, D.A., Sharp,W.R. and Flick,C.E. (1981). Growth and behaviour of cell cultures. *Plant Tissue Culture: Methods and Application in Agriculture* 45-113.

- Evans, M.L. and Mulkey, T.J. (1982). Comparitive effects of auxin and abscisic acid on growth, hydrogen ion efflux and gravitropism in primary roots of maize. *Plant growth Substances, Proceedings of the 11th International Conference on Plant Growth Substances, held in Aberstwyth from 12th to 16th July, 1982* 33-42.

- Evans, M.L. (1984). Functions of hormones at the cellular level of organisation. *In* Encyclopaedia of Plant Physiology. New Series Volume 10, Springer-Verlag, Heidelberg and New York.

- Franclet, A. and Boulay, M. (1982). Micropropagation of frost resistant Eucalypt clones. *Australian Forest Research* **13**, 83-89.

- Furze, M.J. and Cresswell, C.F. (1985). Micropropagation of *Eucalyptus grandis and Eucalyptus nitens* using tissue culture techniques. *South African Forestry Journal* **135**, 20-23.

- Galiana, A., Tibok, A. and Duhoux, E. (1991). *In vitro* propagation of the

nitrogen fixing tree legume *Acacia mangium* Willd. *Plant and Soil* **135,** 151-159.

- Gamborg, O.L., Miller, R.A. and Ojima,K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50,** 151-158.

- Gamborg, O.L., Murashige, T., Thorpe, T.A. and Vasil, I.K. (1976). Plant tissue culture media. *In Vitro* **12,** 473-478.

- Gamborg, O.L. and Shyluk,J.P. (1981). Nutrition, media and characteristics of plant cell and tissue cultures. *In* Plant Tissue and Culture Methods and Applications in Agriculture, Academic Press, New York.

- Gan, E. and Liang, S.B. (1992). Nursery identification of hybrid seedlings in open pollinated seedlots. Breeding technologies for tropical acacias. Proceedings of an International Workshop held in Tawau. Sabah. Malaysia. 1-4 July. (1991). ACIAR Proceedings Series 1992 **37,** 76-87.

- Gaspar, T., Kevers, C., Penel, C., Greppin, H., Reid, D.M. and Thorpe,T.A. (1996). Plant hormones and plant growth regulators in plant tissue culture. *In*

Vitro Cellular and Developmental Biology - Plant **32,** 272-289.

- Gaspar, T.H. and Coumans, M. (1987). Root formation. Cell and tissue culture in forestry. Specific Principles and Methods. *Growth and Development* **2,** 202-217.

- George, E.F. and Sherrington, P.D. (1984). Plant propagation by tissue culture. *In* Handbook and directory of commercial laboratories, Exegetics Ltd. Great Britain.

- Gerhards, C.C. (1966). Physical and mechanical properties of Molucca albizia grown in Hawaii. *U.S. Forest Service Research Paper* FLP **55,** 8.

- Goncalves, A., Machado, M., Caldas, L.S., Sharp, W.R. and Do Amaral Mello,H. (1984). Tissue culture of *Eucalyptus*, Chapter 27. *Plant Cell and Tissue Culture* **125,** 20-25.

- Gorst, S.R., Slaytor, M. and de Fossard, R.A. (1983). The effect of indole-3-butyric acid and riboflavin on the morphogenesis of adventitious roots of *Eucalyptus ficifolia*.F.Muell grown *in vitro*. *Journal of Experimental Botany* **34,** 1503-1515.

- Grattapaglia, D., Bertolucci, F.L., Penchel, R. and Sederoff, R.R. (1995). Advances in genetic mapping of *Eucalyptus grandis: Eucalyptus* plantations , improving fibre yield and quality (eds. Potts, B.M., Borralho, N.M.G., Reid, J.B., Gromer, R.N., Tibbits, W.N. and Raymond.C.A.). *Proceedings of CRC – IUFRO Conference, Hobart, 19-24 Feb (CRC for Temperate Hardwood Forestry, Hobart)* 392-397.

- Gresshoff, P.M. and Doy, C.M. (1972). Development and differentiation of haploid *Lycopersicum esculentum. Planta* **107**, 161-170.

- Haddon, L. and Northcote, D.H. (1976). The influence of gibberellic acid and abscisic acid on cell differentiation of bean callus. *Journal of Cell Science* **20,** 47-55.

- Hall, T.C. and Derose, R.T. (1988). Transformation of plant cells in applications of plant cell and tissue culture. CIBA Foundation Symposium. John Wiley and Sons. 123-134.

- Hamsari, S. and Srivastava, P.B.L. (1985). Trials on rooting of cuttings *on Gmelina arborea* Roxb. I. Effect of Hormone treatment. *Malaysian Forester* **48,**

223-239.

- Hartney, V.J. (1980). Vegetative propagation of the Eucalypts. *Australian Forestry Research Paper* **10**, 191-211.

- Hartney, V.J. and Barker, P.K. (1980). The vegetative propagation of *Eucalyptus* by tissue culture. *In* IUFRO symposium and workshop on genetic improvement and productivity of fast growing tree species. *Sao Paulo. Brazil.* 1-5.

- Hartney, V.J (1981). Vegetative propagation of *Eucalyptus in vitro* .*In* colloque International sur la culture in vitro des essences forestieres, (AFOCEL, Nangis) Fontainbleau. France 175-179.

- Hartney, V.J. and Svensson, J.G.P. (1992). The role of micropropagation for Australian tree species. *In* Rapid Propagation of Fast Growing Woody Species, CAB International, England.

- Heller, R. (1953). Recherches sur la nutrition minerale des tissues vegetaux cultives in vitro. *Annales de Science Naturale Botanie.Vegetation* **14**,1-223.

- Horgan, K. (1987). *Pinus radiata. In* Cell and Tissue Culture in Forestry, Volume 3, Martinus Nijhoff Publishers, Drodrecht.

- Hughes, K.W. (1981). 'Propagation of Higher Plants through Tissue Culture: Emerging Technologies and Strategies, Pergamon, Oxford.

- Hussey.G. (1980). *In vitro* propagation . *In* Tissue Culture Methods for Plant Pathologists, Oxford.

- Hutchinson, J.F., Kaul, V., Maheswaran, G. and Moran, J.R. (1992). Genetic improvement of floricultural crops using biotechnology. *Australian Journal of Botany* **40**, 765-87.

- Ikemori, Y.K. (1987). Epicormic shoots from the branches of *Eucalyptus grandis* as an explant source for *in vitro* culture. *Commonwealth Forest Review* **66,** 351-356.

- Ilahi, I. and Jamal, S. (1987). Mass propagation of *Eucalyptus tereticornis* Smith. *Pakistan Journal of Botany* **19**, 67-74.

- Jacobs, W.P. (1979). Plant hormones and plant development. Cambridge University Press. London. 47-100.

- Janick, J. (1986). Horticultural Science Fourth Edition (WH Freeman. New York)

- Jones, O.P., Hopgood, M.E. and O'Farrell, D. (1977). Propagation *in vitro* of M.26 apple rootstocks. *Journal of Horticultural Science* **52,** 235-238.

- Kadiata, B.D., Mulongoy, K. and Isirimah, N.O. (1996a). Time course of biological nitrogen fixation, nitrogen absorption and biomass accumulation in three woody legumes. *Biological Agriculture and Horticulture* **13**, 253-266.

- Kadiata, B.D., Mulongoy, K., Isirimah, N.O. and Amakiri, M.A. (1996b). Screening woody and shrub legumes for growth nodulation and nitrogen fixation potential in low contrasting soils. *Agroforestry Systems* **33,** 137-152.

- Kadiata, B.D., Mulongoy, K. and Isirimah, N.O. (1997). Influence of pruning frequency of *Albizia lebbeck, Gliricidia sepium* and *Leucaena leucocephala* on nodulation and potential nitrogen fixation. *Biology and Fertility of Soils* **24,** 225-260.

- Kai, S., Ohya, T., Moriya, K. and Fujimoto, T. (1995). Growth control and biophoton radiation by plant hormones in red bean (*Phaseolus angularis).* *Japanese Source of Applied Physics, part 1, Regular Papers, Short Notes and Review Papers* **34,** 6530-6538.

- Kaufman, P.B. Ghosheh, N.S. and Ikuma, H. (1968). Growth hormones. Plant Physiology **17,** 23-34.

- Kefeli, V.I. (1978). Natural plant growth inhibitors and phytohormones, BV Publishers.

- Kleinschmit, J. and Meier-Dinkel, A. (1990). Biotechnology in forest tree improvement: Trees of the future. *In* Plant Aging: Basic and Applied Approaches, Plenum Press, New York.

- Klimazewska, K. and Keller, W.A. (1985). High frequency plant regeneration from thin cell layer explants of *Brassica napus. Plant Cell, Tissue and Organ Culture* **4,** 183-197.

- Klopfenstein, N.B. and Kerl, J.G. (1995). The potential of biotechnology in

temperate agroforestry practices. *Agroforestry Systems* **32**, 29-44.

- Krishnamoorthy, H.N. (1977). Gibberellins and Plant Growth, Halsted Press, New York.

- Kumar, R.R. and Ayyappan, P. (1987). Rapid clonal multiplication of *Eucalyptus camaldulensis* Dehn through *in vitro* culture. *Planter's Chronicle* **81**, 225-227.

- Kumar, R.R. and Raman, K. (1989). Callus induction and recovery of plantlets from seedling explants of *Eucalyptus camaldulensis* Dehn. *Journal of Plantation Crops* **16,** 21-26.

- Larbi, A., Smith, J.W., Adekunle, I.O. and Kurdi, I.O. (1996). Studies on multipurpose fodder trees and shrubs in West Africa - variation on determinants of forage quality in *Albizia* and *Paraserianthes* species. *Agroforestry Systems* **33**, 29-39.

- Le Poivre. (1977). Medium for plant propagation. *Plant Cell Physiology* **10,** 10-15.

- Le Roux, J.J. and Staden, J.V. (1991a). Micropropagation of *Eucalyptus* species. *Horticultural Science* **26**, 199-200.

- Le Roux, J.J. and Staden, J.V. (1991b). Micropropagation and tissue culture of *Eucalyptus* - a review. *Tree Physiology* **9,** 435-477.

- Libby, W.J. (1974). The use of vegetative propagation in forest genetics and tree improvement. *New Zealand Journal for Forest Science* **4,** 440-447.

- Lindey, K. and Jones, M.G.K. (1989). Plant Biotechnology in Agriculture, Open University Press, Milton, Keynes.

- Lloyd, G. and Mccown, B. (1980). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia,* by use of shoot tip culture. *International Plant Propagators' Society Combined Proceedings* **30,** 421-7.

- Logan, A.F., Phillips, F.H., Williams, M.D. and Eddowes, P.J. (1984). Pulpwood Potential of some fast growing tropical hardwoods [*Acacia auriculiformis, Albizia falcataria, Eucalyptus deglupta, Eucalyptus tereticornis, Gmelina arborea* and *Terminalia brassil* for use in the production of sulphate, NSSC and high yield (refiner mechanical, groundwood from impregnated billets, cold soda semichemical and chemithermochemical) pulps]. *APPITA,*

Parkville **35,** 391-399.

- Longkamp, P. (1987). Germination of Australian Native Plant Seed, Inkata Press, Melbourne.

- Lorilla, E.B. (1992). Direct sowing technology for *Acacia mangium* Willd, *Paraserianthes falcataria* (L.) Nielsen and *Pterocarpus vidalianus* Rolfe. *College Laguna (Philippines)* May 1992.

- Lorz, H. and Brown. P.T.H. (1986). Variability in tissue culture derived plants – possible origins, advantages and drawbacks. *In* Genetic manipulation in Plant Breeding, Walter de Gruyler, Berlin.

- Maghembe, J.A. and Prins, H. (1994). Performance of multipurpose trees for agroforestry two years after planting at Makoka, Malawi. *Forest Ecology and Management* **64**, 171-182.

- Mantell, S.H., Matthews, J.A. and McKee, R.A. (1985). Principles of plant biotechnology. *In* An Introduction to Genetic Engineering in Plant, Blackwell Scientific Publications, Oxford.

- Martin, C. (1985). Plant breeding *in vitro*. *Endeavour New Series.* **9,** 81-86.

- Mascarenhas, A.F., Hazara, S., Potdar, U., Kulkarni, D.K. and Gupta, P.K. (1982). Rapid clonal multiplication of mature forest trees through tissue culture. Proceedings of the Fifth International Congress. *Plant Tissue and Cell Culture* **119,** 719-720.

- McComb, J.A. and Bennett, I.J. (1986). *Eucalyptus (Eucalyptus spp). In* Biotechnology in Agriculture and Forestry, Volume I: Trees, Springer-Verlag, Berlin.

- McCown, B.H. and Lloyd, G.B. (1983). A survey of the response of *Rhododendron* to *in vitro* culture. *Plant Cell, Tissue and Organ Culture* **2,** 77-85.

- McLean, K.S., Lawrence, G.W. and Reichert, N.A. (1992). Callus induction and adventitious organogenesis of Kenaf (*Hibiscus cannabinus* L). *Plant Cell Reports* **11**, 532-534.

- Mehra-Palta, A. (1982). Clonal propagation of *Eucalyptus (nova-anglica* sp.) by tissue culture. *Plant Science Letters* **26,** 1-11.

- Menzies, M.I. (1992). *In* Increasing value through technology. Improving the productivity of radiata pine plantations. Adding value to the New Zealand timber and forest products, 2-3 Dec .

- Meyer, H.J. and Van Staden, J. (1987). Regeneration of *Acacia melanoxylon* plantlets *in vitro*. *South African Journal of Botany* **53**, 206-209.

- Mizuguchi, S. and Ohkawa, M. (1994). Effects of napthalene acetic acid and benzyladenine on growth of bulblets regenerated from white callus of mother scale of *Lilium japonicum* Thunb. *Journal of Japanese Society for Horticultural Science* **63**, 429-437.

- Mohammed, G.H. and Vidaver, W.E. (1988). Root production and plantlet development in tissue-cultured conifers. *Plant Cell, Tissue and Organ culture* **14**,137-160.

- Mok, M.C., Mok, D.W.S., Turner.J.E. and Mujer.C.V. (1987). Biological and biochemical effects of cytokinin-active phenylurea derivatives in tissue culture systems. *Hortscience* **22,** 1194-1197.

- Moloney, R.A., Aitken, R.L. and Gutteridge, R.C. (1990). The effect of phosphorus and nitrogen applications on the early growth of *Adenanthera pavonina, Albizia falcataria* and *Schleinitzia insularum. APPITA, Parkville* **43,** 193-195.

- Muralidharan, E.M., Gupta, P.K.and Mascarenhas, A.F. (1989). Plantlet production through high frequency somatic embryogenesis in long-term cultures of *Eucalyptus citriodora. Plant Cell Reports* **8**, 41-43.

- Muralitharan, M.S., Van Steveninck, R.F.M. and Chandler, S.F. (1993). Physiological adaptation to high ion concentrations or water deficit by callus cultures of highbush blueberry *Vaccinium Corymbosum. Australian Journal of Plant Physiology* **20,** 159-172.

- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays in tobacco tissue culture. *Physiology of Plants* **15**,473-497.

- Murashige, T. (1974). Plant propagation through tissue culture. *Annual Review of Plant Physiology* **25,** 135-166.

- Murashige, T. (1977). Clonal crops through tissue culture. *Plant Tissue*

Culture and its Bio-Technological Applications 392-403.

- Murashige, T. (1978). The impact of tissue culture on agriculture. *In* Plant Tissue Culture and Biotechnological Applications, Springer-Verlag, Berlin and New York.

- Napoli, C. and Klee, H. (1993). Phenotype modification in horticultural crops through hormonal control. *Scientia Horticulturae* **55,** 161-175.

- Nash, D.T. and Davies, M.E. (1972). Some aspects of growth and metabolism of Paul's scarlet rose cell suspensions. *Journal of Experimental Botany* **23,** 75-91.

- Negrutiu, I., Jacobs, M. and Cachita, D. (1978). Some factors controlling *in vitro* morphogenesis of *Arabidopsis thaliana. Zeitung für Pflanzenphysiologie* **86,** 113-124.

- Newbigin, E., Smyth, D.R. and Clarke, A.E. (1995). Understanding and controlling plant development. *Trends in Biotechnology* **13,** 338-343.

- Ohira, K., Ikeda, M. and Ojima, K. (1976). Plant Culture. *Plant Cell Physiology*

17, 583.

- Otsamo, A., Adjers, G., Hadi, T.S., Kuusipalo, J., Tuomela, K. and Vuokko, R.(1995). Effect of site preparation and initial fertilisation on the establishment and growth of four plantation tree species used in reforestation of *Imperata cylindrica* (L) dominated grasslands. *Forest Ecology and Management* **7**,271-277.

- Palit, S. (1980). Trials of *Albizia falcataria*. Fosberg and *Leucaena Leucocephala* de wit in North Bengal. *Indian Forestry* 456-465.

- Panjaitan, M., Stur, W.W. and Jessop, R. (1993). Growth of forage tree legumes at four agroclimatic sites in Indonesia. *Journal of Agricultural Science* **120**, 311-317.

- Pence, V.C. and Caruso, J.L. (1984). Effects of IAA and four IAA conjugates on morphogenesis and callus growth from Tomato leaf discs. *Plant Cell, Tissue and Organ Culture* **3,** 101-110.

- Phillips.R.L., Plumkett.D.J. and Kaeppler.S.M. (1992). Novel approaches to the induction of genetic variation and plant breding implications. *Plant*

Breeding in the 1990's (eds. Stalker,H.T. and Murphy, J.P.) 389-408.

- Poissonnier, M., Dumant, M.J. Franclet, A. (1984). Acclimation of *Eucalyptus* clones propagated *in vitro. Annales de Recherches Sylvicoles, AFOCEL* 54-83.

- Profumo, P., Gastaldo, P., Caffaro, L., Dameri, R.M., Michelozzi, G.R. and Bennici, A. (1985). Callus induction and plantlet regeneration in *Cichorium intybus* L. *Protoplasma* **126,** 215-220.

- Pryor, L.D. (1976). The biology of Eucalypts. *In* Institute of Biology's Studies in Biology No.61. Edward Arnold, London.

- Rao, A.N. and Lee, S.K. (1982). Importance of tissue culture in tree propagation. *In* Plant Tissue Culture, Proceedings of the 5th International Congress on Plant Tissue and Cell Culture.

- Rao, K.S. (1988). *In vitro* meristem cloning of *Eucalyptus tereticornis* Sm. *Plant Cell Reports* **7**, 546-549.

- Raven, P.H., Evert, R.F., Ray, F. and Eichhorn, S.E. (1992). *Biology of Plants.* Worth Publishers, New York.

- Rayle, D.L. and Cleland, R.E. (1977). Tissue culture. *Current Topics in Developmental Biology* **11,** 187-214.

- Redig, P., Shaul, O., Inze, D., Vanmontagu, M. and Vanonckelen, H. (1996). Levels of endogenous cytokinin. Indole-3-acetic acid and abscisic acid during the cell cycle of synchronised Tobacco by –2 cells. *FEBS Letters* **391**, 175-180.

- Reynolds, J.F. (1987). Chemical regulation in tissue culture: An overview. *Hortscience* **22,** 1192-1193.

- Rice, T.B, Reid, R.K. and Gordon, P.N. (1979). Morphogenesis in field crops. *In* Propagation of Higher Plants Through Tissue Culture: a Bridge Between Research and Application, US Technology and Information Service, Springfield, Vancouver.

- Richards, D. and Beardsell, D. (1987). Seed dormancy. *In* Germination of Australian Native Plant Seed, Inkata Press.
- Ridgeway, J. and Kingsley, A. (1989). The mechanics of life. Southside Publishers. Oxford. 211-239.

- Roberts, J.A. and Hooley, K. (1988). Plant Growth Regulators, Bell and Bain Limited, Glasgow.

- Roesel, H.A. and Haber, A.H. (1964). Grain crops. *Plant Cell Physiology* **14,** 523-532.

- Rucker, W. (1982). Combined influence of indoleacetic acid, gibberellic acid and benzylaminopurine on callus and organ differentiation in *Digitallis purpurea* leaf explants. *Zeitung für Pflanzenphysiologie* **107,** 103-140.

- Rumary, C. and Thorpe, T.A. (1984). Plantlet formation in black and white spruce. I. *In vitro* techniques. *Canadian Journal of Forest Research* **14**,10-16.

- Salisbury, F.B. and Ross, C.W. (1992). *Plant Physiology.* Wadsworth Publishing Company, Belmont, California 94002.

- Sandum, A.K.I., Srivastava, P.B.L. and Doraisingam, M. (1986). Trials on rootings of cuttings of *Gmelina arborea.* III. Effects of source, hormone treatment, media and frequency of misting. *Malaysian Forester* **49**, 332-351.

- Sangwan, R.S., Norreel. and Harada, H. (1976). Effect of kinetin and gibberellin A_3 on callus growth and organ formation in *Limnophila chinensis* tissue culture. *Biology of Plants* **18,** 126-131.

- Sankara. R. and Venkateswara, R. (1985). Tissue culture of forest trees. Clonal multiplication of *Eucalyptus grandis. Plant Science* **40,** 51-55.

- SAS User Guide: Statistics 1985. Version 5 Edition. By SAS Institute Inc., Cary, NC.

- Schenk, R.U. and Hildebrandt, A.C. (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* **50**, 199-204.

- Schubert, T.H., DeBell, D.S. and Whitesell, C.D. (1988). *Eucalyptus*/legume mixtures for biomass productionin Hawaii. *Nitrogen fixing trees, Thailand Institute of Scientific and Technological Research* **6,** 26-27.

- Semana, J.A., Lasmarias, V.B. and Ballon, C.H. (1982). Hardboard from Moluccan Sau (*Albizia falcataria). Forest Products Research and Industries Development Commission* **11,** 20-26.

- Singh, B.P., Dhyani, S.K., Chauhan, D.S. and Prasad, R.N. (1995). Effect of multipurpose tree species on chemical properties of an acid alfisol in Meghalaya. *Indian Journal of Agricultural Sciences* **65**, 345-349.

- Sita, G.L., Sobha-Rani. and Rao, K.S. (1986). Propagation of *Eucalyptus grandis* by tissue culture . *Eucalyptus* in India. Past, present and future. Proceedings of the national seminar held at Kerela. Forest Research Institute, Peechi, Kerela, India. Jan 30-31. 1984 (Ed. Sharma.J.K.Nair.C.T.S. Kedharnath.S. and Kalidas.S). 318-321.

- Skolmen, R.G. (1986). *Acacia* (*Acacia koa* Gray). Biotechnology in Agriculture and Forestry. Vol 1. (Ed. Y.R.S. Bajaj) Springer - Verlag Berlin. 375-384.

- Skoog, F. and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. *Symposium of Social Experimental Biology* **11**, 118-131.

- Smith, D.R., Horgan, K. and Aitken, J. (1980). Micropropagation – a new aid in tree improvement. *In* What's New in Forest Research, No 87.

- Smith, D.R., Walter, C., Hargreaves, C.L. and Grace.L.J. (1994). Somatic embryogenesis joins the plantation forestry revolution in New Zealand. *Biological Science Symposium 3-6 Oct* 19-29.

- Sommer, H.E. and Caldas, L.S. (1981). *In vitro* methods applied to forest trees. Plant Tissue Culture - Methods and Applications in Agriculture (ED. T.A. Thorpe). Academic Press Inc. New York. 349-358.

- Soni, S.L. and Kaufman, P.B. (1972). Grains. *Canadian Journal of Botany* **50,** 1185-1190.

- Steward, F.C. and Krikoran, A.D. (1971). Plants, Chemicals and Growth. Academic Press, New York.

- Street, H.E. (1977). Plant Tissue and Cell Culture. Blackwell Scientific Publications, Oxford.

- Subbiah, M.M. and Minocha, S.C. (1990). Shoot regeneration from stem and leaf callus of *Eucalyptus tereticornis. Plant Cell Reports* **9,** 370-373.

- Takahashi,Y., Ishida, S. and Nagata, T. (1993). Functions and modulations of

auxin regulated genes. *Journal of Plant Research* **106,** 357-367.

- Tang, K.S. and Srivastava, P.B.L. (1985). Trials on rooting of cuttings of *Gmelina Arborea* Roxb. II. Effect of sucrose, hormone treatment and position. *Malaysian Forester* **48,** 298-313.

- Teasdale, R.D. (1995). Optimising the application of biotechnology to forest plantations. *In* Eucalypt plantations: improving Fibre Yield and Quality, Proceedings of CRC - IUFRO Conference, Hobart (19-24 Feb).

- Teklehaimanot, Z. and Animkwapong, G. (1996). Tolerance of *Albizia zygia* (DC) Macbride - a shade tree for cocoa to soil acidity and water stresses. *Journal of the Science of Food and Agriculture* **70**, 389-395.

- Teulieres, C., Alibert, G., Marien, J.N. and Boudet, A.M. (1988). Isolation and frost resistance screening of protoplasts from different clone of *Eucalypts* 1-4.

- Teulieres, C.D, Ferrand, D. and Boudet, A.M. (1989). Obtention de suspensions cellulaires d'*Eucalyptus gunnii*: survie des cellules et de leurs protoplastes à basse temperature. *Annales Recherches, Association Forét Cellulose* 117-131.

- Texier, F. and Faucher, M. (1986). *In vitro* culture of shoot tips of a mature Eucalypt, *Eucalyptus parvifolia* Camb. *Annales de Recherches Sylvicoles,* AFOCEL. France. 1986. 7-23.

- Thorpe, T.A. (1982). Carbohydrate utilisation and metabolism. *Tissue Culture in Forestry*. 325-368.

- Thorpe, T.A. (1983). Biotechnological applications of tissue culture to forest tree improvement. *Biotechnological Advances* **1**, 263-278.

- Thorpe, T.A. and Patel, K.R. (1984). Clonal propagation: adventitious buds. *Cell Culture and Somatic Cell Genetics* **1**, 49-59.

- Thorpe, T.A., Harry, I.S. and Kumar, P.P. (1991). Application of micropropagation to forestry. *In* Micropropagation technology and Applications, Kluwer Academic Publishers, Netherlands.

- Tonye, J., Duguma, B. and Tikimanga, T. (1994). Stepwise approach to alley cropping technology development and transfer in the forest zone of Cameroon. *Agroforestry Systems* **28**, 269-278.

- Tran Thanh Van, K. and Trinh, H. (1978). Morphogenesis in thin cell layers. Concept. Methodology and results. *Frontiers of Plant Tissue Culture* 37-48

- Trewavas, A. (1981). How do plant growth substances work? *Plant Cell Environment* **4,** 203-208.

- Trewavas, A. (1982). Growth substance sensitivity: the limiting factor in plant development. *Physiology of Plants* **55**, 60-72.

- Trindade, H., Ferreira, J.G., Pais, M.S. and Aloni, R.. (1990). The role of cytokinins and auxins in rapid multiplication of shoots of *Eucalyptus globulus* grown *in vitro. Australian Forester* **53,** 221-223.

- Van den Ende, G., Croes, A.F., Kemp, A. and Barendse, G.W.M. (1984). Development of flower buds in thin – layer cultures of floral stalk tissue from tobacco: role of hormones in different stages. *Physiology* **61,** 114-118.

- Vincent, A.J., Mitchell, B.A. and Sandrasegan, K. (1964). Permanent sample plot information on the stocking growth and yield for pulpwood for Batai (*Albizia falcata* Back.) grown in Malaya. *Malayan Forester* **27,** 327-353.

- Voesenek, L.A.C.J., Bonga, M., Rijnders, J.G.H.M., Visser, E.J.W. and Blom, C.W.P.M. (1996). Hormone sensitivity and plant adaptations to flooding. *Folia Geobotanica and Phytotaxonomica* **31**, 47-56.

- Walters, G.A. (1971). A species that grew too fast - *Albizia falcataria. Journal of Forestry* **6**, 168.

- Warrag, E.I., Lesney, M.S. and Rockwood, D.L. (1990). Micropropagation of field tested superior *Eucalyptus grandis* hybrids. *New Forests* **4**, 67-79.

- Warrag, E.I., Lesney, M.S. and Rockwood, D.L. (1991). Nodule culture and regeneration of *Eucalyptus grandis* hybrids. *Plant Cell Reports* **9**, 586-589.

- White, P.R. (1943). Nutrient deficiency studies and an improved inorganic nutrient for cultivation of excised tomato roots. *Growth* **7**, 53-65.

- White, T.L. (1987). A conceptual framework for tree improvement program. *New Forests* **4**, 325-342.

- Whitney, P.J. (1996). Hormone independent root organ cultures of rye

(*Secale cereale*). *Plant Cell, Tissue and Organ Culture* **46,** 109-115.

- Wilcox, M.D. (1994). Towards clonal forestry with *Pinus radiata* in New Zealand. Asia – Pacific Symposium on Forest Genetic Improvement, 19-22, October 1-8.

- Williams, C.G. and Neale, D.B. (1992). Conifer wood quality and marker aided selection: a case study. *Canadian Journal of Forest Resources* **22,**1009-1017.

- Wong, C.Y. and Haines, R.J. (1992). Multiplication of families of *Acacia mangium* and *Acacia auriculiformis* by cuttings from young seedlings. Breeding technologies for tropical *Acacias*. Proceedings of an International Workshop held in Tawau. Sabah. Malaysia. 1-4 July. 1991. ACIAR Proceedings series 1992 **37,** 112-114.

- Yamaguchi, T., Fukuzomi, T.and Yoshomoto, T. (1986a). Phenolics of tissue cultures from *Eucalyptus* spp. II. Effects of phytohormones on phenolic action of *Eucalyptus robusta*. *Mokuzai Gakkaishi - Journal of the Japan Wood Research Society* **32**, 209-212.

- Yamaguchi, T., Fukuzomi, T. and Yoshomoto, T. (1986b). Phenolics of tissue

cultures from *Eucalyptus* spp. III. Influence of inorganic elements and sucrose on the growth. phenolic accumulation and composition of *Eucalyptus polybractea* suspension cultures. *Mokuzai Gakkaishi - Journal of the Japan Wood Research Society* **32,** 366-372.

- Yuan, L. and Knauf, V.C . (1997). Modifications of plant component: A review. *Current Opinion in Biotechnology* **8(2),**227-233.

- Zar, JV. (1984). Biostatistical analysis. Prentice Hall. Englewood Cliffs. NJ.

- Zeevart, J.A.D. and Boyer, G.L. (1982). Metabolism of Abcisic acid in *Xanthium strumarium* and *Ricinus communis. Plant growth Substances.* Proceedings of the 11th International Conference on Plant Growth Substances, held in Aberstwyth from 12th to 16th July, 335-342.

- Zhang, N.G., Yong, J.W.H., Hew, C.S. and Zhou, X. (1995). The production of cytokinins ABA and auxin by CAM orchid aerial roots. *Journal of Plant Physiology* **147**, 371-377.

- Zhang, P.F., Ni, D.X., Wang, K.J. and Bao, Z.H. (1982). Effects of plant hormones on callus growth and organogenesis in *Eucalyptus botyroides in*

vitro. *Fudan Journal Fudan Xuebao* **21**, 445-452.