In vitro Propagation of Bijasal (*Pterocarpus marsupium* Rxob.) Through Tissue Culture

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THESIS

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FACULTY OF AGRICULTURE
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Dedicated to my parents...

It's their hopes that
I strive to achieve today
and in their dreams lie
my tomorrows

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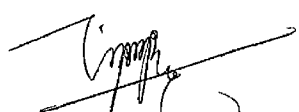
CERTIFICATE

Certified that this thesis entitled "*In vitro* propagation of bijasal (*Pterocarpus marsupium* Roxb) through tissue culture " is a record of research work done independently by Sri Santhosh Kumar, A V under my guidence and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him



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We, the undersigned members of the advisory committee of Sri Santhosh Kumar, A V , a candidate for the degree of Master of science in Forestry, agree that the thesis entitled '*In vitro* propagation of bijasal (*Pterocarpus marsupium* Roxb) through tissue culture ' may be submitted by Sri Santhosh Kumar, A V in partial fulfilment of the requirements for the degree

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Santhosh Kumar A V

CONTENTS

LIST OF TABLES

LIST OF PLATES

ABBREVIATIONS

BA	Benzyladenine
cv	Cultivar
Cycocel/CCC	2 Choloroethyl trimethyl ammonium chloride
2,4-D	2,4 Dichlorophenoxy acetic acid
FeEDTA	Ferric salt of ethylenediaminetetra acetic acid
GA	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoogs's (1962) medium
ppm	Parts per million ; mg/l
v/v	Volume in volume
w/v	Weight in volume
WPM	Lloyd and McCowns's (1980) woody plant medium

a

INTRODUCTION

INTRODUCTION

Bijasal (Pterocarpus marsupium Roxb.) is a large, nearly evergreen tree of the moist deciduous forests They grow between 88° and 72° Western longitudes and hardly occurs to the North-west of a line joining Baroda and Sahjehanpur (Rodger, 1913) This tree is a characteristic of Southern moist mixed deciduous, Moist peninsular high level Sal and Moist peninsular low level Sal forests The timber is extensively used for construction and furniture purposes After teak and rose wood it is the most valued timber for these uses P. marsupium is an important multipurpose tree of Kerala's forests and forms an ideal fuel wood with a calorific value of 4900 calories for sap wood and 5141 calories for its heart wood (Krishna and Ramaswami, 1932) The bark, leaves and flowers have various medicinal uses and leaf forms an ideal manure especially for arecanut plantations (FRI, 1983) The fodder of this species is classed as good An MFP called 'gum kino' obtained from the bark exudation has medicinal properties and is officinal in Indian Pharmacopia It also finds application in dyeing, tanning, printing and is of potential use for paper industry

P. marsupium due to its multipurpose utilities, nitrogen fixing nature and wide adaptability to different soil conditions is an ideal species for afforestation and agroforestry programmes This tree, however, yields only a moderate seed crop and does not yield annually equal crop (Rodger, 1913)

Germination of seeds too is rather poor (Singh, 1982) Artificial propagation is done mainly through planting of stumps as it does not respond favorably to other conventional modes of vegetative propagation

Until very recently, forests were considered typically as a renewable resource However, several factors have lead many forest specialists to classify forest resource as non-renewable, or at best semi-renewable This fact coupled with the large scale deforestation and the 'regeneration delay ' has made people to search for forest products out side the natural forest If historical patterns are any indication, an important part of forest products in the future might come from plantations However, forest plantation programmes face many problems, in particular inadequate supply as well as poor quality of seedlings

There has been tremendous interest during the last two decades on the propagation of crop plants through tissue culture. However, in most of the tree species, the application of this technique is not fully achieved Micro propagation through *in vitro* techniques is one of the possibilities of overcoming the problems of regenerating bijasal in large scale Tissue culture opens exciting opportunities for propagation of plants vegetatively on a mass scale through rapid production of plantlets This technique offers the possibility of producing large number of seedlings of known superior genotypes with

uniform quality Further , Callus production, cell cultures and protoplast fusion offers exciting possibilities for augmenting tree improvement programmes

The study was taken up with a view to develop a protocol for mass propagation of bijasal through in vitro technique so that enough quantity of genotypically superior seedlings of P. marsupium are available for extensive planting programmes in future

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Origin of plant tissue culture could be traced back to the early experiments of Haberlandt (1902) in which, he tried to grow cells under aseptic conditions in liquid media supplemented with sugar Identification and purification of indole-3-acetic acid (IAA), the first known growth regulator, by Kogl (1934) and then by Thimman (1935), made it possible to control growth of plants, tissues and cells The early development of plant tissue culture owes to the relentless effort by the pioneering investigators including White (1934), Gautheret (1934), Nobecourt (1939), Miller *et al* (1956), Reinert (1958), Steward *et al* (1958), Bergmann (1960), Vacin and Went (1949) and many others.

The best commercial application of tissue culture has been in the production of true-to type plants at a very rapid rate compared to the conventional methods (Levy, 1981) Micropropagated plants grew faster and matured earlier than their seed propagated progenies (Vasil and Vasil, 1980).

According to Murashige (1974), there are three possible routes for *in vitro* propagule multiplication

i) Enhanced release of axillary buds

ii) Production of adventitious buds through organogenesis

iii) Somatic embryogenesis

In the first route, primary meristems like shoot tips and axillary buds are cultured, which assures genetic uniformity of the progeny to a great extent (Chand and Roy, 1980; Rao and Lee, 1986) The second route, callus mediated somatic organogenesis, is generally not recommended for clonal multiplication, but may be ideal for recovering useful mutant lines The last path, somatic embryogenesis, is the least understood of the three and is promising conceptually as it results in the most rapid and assured way of propagation Few plants have shown the ability to form somatic embryos in culture

Advantage of plant tissue culture as a propagation and breeding tool has not been fully realised in forestry till date However, there are several reports on successful tissue culture in many species of conifers like Pinus pinaster (David et al , 1982), Pinus nigra (Kolveska-pletikapis et al , 1983), Euphedra foliata (Bhatnagar and Singh, 1984) and Pinus radiata (Horgan, 1987) and a few broad leaved species

A comprehensive review of the work done in tissue culture propagation of broad leaved trees is presented below

2 1. Clonal propagation

Mathuri and Chandra (1983) noticed induction of shoot buds in Acacia nilotica under in vitro conditions, in MS media supplemented with IAA (0 1 - 10 0 ppm) Mittal et al (1989)

reported multiple shoot formation from excised axillary bud explants of the same species on MS containing 50 per cent (v/v) coconut water and 1 μM of BA The capacity for shoot morphogenesis in internodal segments of Acacia holosericea and A saligna were demonstrated by Jones et al. (1990) through indirect organogenesis In the same study A divenosa, A holosericea, A salicina, A sclerosperma and A saligna showed enhanced release of axillary buds in MS medium Acacia mangium nodal segments of seedling origin could give rise to plantlets when cultured in MS containing 0 5 ppm BA (Ahamed, 1990)

Multiple shoot formation was induced from excised seedling segments of Anogeissus pendula through indirect organogenesis (Joshi et al , 1991) Cotyledon segments were noticed to have a higher organogenetic potential than epicotyl segments, when cultured in MS supplemented with 1.0 ppm BA and 0 1 ppm IAA. About 800 plants could be regenerated in 200 days through this method Hardened plantlets were planted out

In vitro propagation protocols were established for two leguminous trees Bauhinia variegata and Parkinsonia aculeata from explants taken from mature trees (Mathur and Mukunthakumar, 1992) Kumar (1992) reported successful micropropagation of 15-18 year old Bauhinia purpurea trees through callus culture Stem segments cultured in MS with 10μM 2,4_D developed callus, which regenerated into plantlets with 2-3 leaves in the same basal medium supplemented with 5μM kinetin

Grellier et al (1984) has stated that multiplication of Betula pendulosa and Betula pubescens was achieved on MS medium containing low levels of cytokinins Pseudoterminal buds of B uber when placed in medium containing 0 6 and 0 05 ppm of BA and IAA respectively opened in four to five days and produced upto three leaves in a week (Vijaykumar et al., 1990) The plantlets regenerated have been field planted.

Aboel-nil (1987) reported that in vitro propagation method has been found successful in Casuarina species, namely, C cunninghamiana, C glauca and C equisetifolia. Callus was induced from juvenile and mature stem segment explants on MS medium supplemented with 0 5 μM each of 2ip and NAA Buds were regenerated from callus tissue and stem segment explants on MS medium containing BA at 2 2-11 0 μM combined with IAA at 0 5 μM.

Callus mediated shoot bud formation and rooting of shoots in Dalbergia latifolia have been reported (Rao, 1986; Sita et al , 1986) Successful induction of multiple shoots on excised hypocotyl segments and shoot tips of in vitro germinated seedlings of Indian rosewood on MS supplemented with cytokinins and auxins has been achieved by Rai and Chandra (1989) Elite Indian rose wood trees of 60-80 years age could be micropropagated through axillary bud culture in MS supplemented with 1 0 ppm BA and 0 05 ppm NAA or 0 5 ppm IAA (Swamy et al , 1992) Protocol for clonal propagation of mature rose wood through axillary bud culture has been standardised (Mahato

1992) Indian rose wood could be regenerated from callus tissue of cambial origin too (Kumar *et al* , 1991)

Multiple shoots from nodal segments of mature trees of *Dendrocalamus strictus*, *Bambusa arundinacea* and *B vulgaris* were obtained by Nadgir *et al* (1984) Explants cultured in MS medium containing BA (0.02 ppm) and coconut water (5 %, v/v) produced shoots *Dendrocalamus strictus* embryos started callusing soon after inoculation in B_5 having 2,4-D at 10-30 μM The callus on subculture gave rise to somatic embryos and germinated in the same medium (Rao and Rao, 1988)

Ashok (1985) reported success in *in vitro* propagation of *Eucalyptus citriadora* He has suggested that activated charcoal increases shoot development in this species Juvenile tissues of *in vitro* germinated seedlings of *E camaldulensis* showed multiple shoot production on MS supplemented with BA, adenine sulphate and sodium dihydrogen phosphate (Kumar and Ayyappan, 1987) According to Gupta and Mascarenhas (1987), the nodal explants of *E tereticornis* and *E golbulus* produced multiple shoots on MS medium containing kinetin (0 2 ppm), BA (0 3 ppm), calcium pentathenate (0 1 ppm) and biotin (0 1 ppm) Maximum multiple shoot induction from axillary buds of *E tereticornis* was noticed on modified MS supplemented with BA at 0 1 ppm and NAA at 0 1 ppm (Das and Mitra, 1990)

Shoot tip explants of Ficus lacor saplings could be induced to form shoots at a rate of 20-30 in MS containing 1 0 ppm BA, 1 0 ppm kinetin and 1000 ppm casein hydrolysate (Amatya and Rajabhandary, 1990)

Mascarenhas et al (1982) carried out micropropagation of Havea brasiliensis and found out that terminal buds from 10-20 year old trees when cultured on MS medium along with 0 5 ppm kinetin, 2 0 ppm BA, 200 ppm casein hydrolysate, 0.1 ppm calcium pentathenate and 0 1 ppm biotin induced three shoots per explant Datta and Datta (1985) reported that shoots were developed from nodal explants on MS medium containing 1 0 ppm BA whereas multiple shoots could be induced with 2.0 ppm BA.

In Leucaena leucocephala, for tissue culture propagation terminal bud explants were cultured in MS media with BA (3.0 ppm) and NAA (0 05 ppm) This method was found to be best suited for shoot multiplication (Goyal et al , 1985) Successful in vitro multiplication technique for clonal propagation of Leucaena leucocephala through axillary bud culture was reported by Datta and Datta (1985)

Rapid clonal multiplication of mulberry plant could be obtained by culturing the axillary buds in MS with 2 0 ppm BA (Tewary and Rao, 1990)

In vitro shoot multiplication of *Populus glandulosa* through indirect organogenesis could be obtained in a variety of combinations involving different media and growth regulators (Jang *et al*, 1988). Sixty day old excised roots of *Populus alba* x *P. grandidenta* cultured in WPM containing 22.0 μM zeatin yielded about 111 shoots per root segment (Son and Richard, 1990). Shoot cultures of *Populus deltoides* too have been successfully regenerated from internodal stem explants (Coleman and Ernst, 1990).

Differentiation could be obtained from hypocotyl segments of *Prosopis cineraria* cultured in MS supplemented with 4.5 ppm kinetin and 0.25 ppm IAA/NAA (Goyal and Arya, 1989). Krackar *et al* (1990) could obtain enhanced release of axillary buds from mature trees of the same species in MS supplemented with 3.0 ppm of naphthoxyacetic acid (NOA) and NAA. Callus cultures of *P. tamarugo* were established from hypocotyl and cotyledon on MS medium containing 2.0 ppm NAA and 0.2 ppm BA (Nandwani and Ramawat, 1992). Regeneration from callus was observed when it was subcultured to MS containing 5.0 ppm BA.

Callus induction followed by differentiation was reported by Sita *et al* (1980) in *Santalum album* using endosperm tissues from seeds of green fruits. The explants were cultured on MS supplemented with 2,4-D (1.0-2.0 ppm), kinetin (0.1-0.2 ppm), BA (0.5-2.0 ppm) and NAA (1.0 ppm).

Callus of Sesbania bipinosa could be obtained from both cotyledon and mature leaf explants (Sinha and Malick, 1991), in MS containing BA at 0 5 ppm and 2,4-D at 2 0 ppm Callus could be later regenerated into shoot in MS medium supplemented with 2 0 ppm BA and 15 per cent (v/v) coconut milk Sesbania grandiflora could be micropropagated by using hypocotyl and cotyledon segments (Shankar and Ram, 1990)

In vitro propagation of tamarind has been achieved from nearly all parts of seedlings (stem, leaf, root and cotyledon) when cultured on MS containing kinetin (0 2 ppm), BA (0.5 ppm), NAA (0 5 ppm) and biotin (0 1 ppm) (Mascarenhas et al 1987). Four to five shoots developed from hypocotyl, one from nodal and three from shoot tip explants Kopp and Nataraja (1990) reported shoot tip cultures in tamarind on MS with 0 5-5 0 ppm BA as effective for shoot induction Optimal culture conditions for high frequency plant regeneration from excised cotyledons of tamarind were established by Jaiwal and Gulati (1991). Cotyledon segments cultured in MS supplemented with 5 M of BA formed 34 to 95 shoots in a period of 4 months

Yadav et al (1990) reported that multiple shoots could be induced in shoot tip explants of Syzygium cumini seedlings The authors used MS medium supplemented with 0 23-8 9 μM of BA singly or in combination with auxins (NAA, IBA or IAA) at the range of 0 12-1 0 μ M.

Gupta et al (1980) reported the successful induction of multiple shoot formation from excised terminal buds of 100 year old teak trees They have suggested that over 500 plants could be produced from a single bud of a selected elite tree on MS containing kinetin (0 15 ppm) and BA (0 15 ppm)

Mazanera and Pardos (1990) reported successful propagation of juvenile and adult oak (Quercus suber) It was observed that the explants from old and young sources differed greatly in their optimum culture conditions

Axillary buds of Ziziphus cv Geumsung showed the best shoot and root growth after 8 weeks when 500 ppm activated charcoal was added to half strength MS with 0 5 ppm BA However, cv Bokjo responded best to 1000 ppm activated charcoal in the above medium (Kim and Lee, 1988)

Among the genus Pterocarpus, P indicus was the first one to be attempted for micropropagation Lee and Rao (1980) however, could only obtain callus in their experiments Callinawan and Halos (1981) could obtain organogenesis from shoot callus in MS medium after irradiating the calli at 10-60 Kr Patri et al (1988) experimenting on P santalinus observed that shoots could be obtained through callus culture in 1/4 MS supplemented with 3 0 ppm BA and 40 ppm adenine. Sita et al. (1992), however, could enhance the rate of multiplication and produce rooted plantlets of red sanders Till date, no work on P marsupium has appeared in literature

2.2 Factors affecting success of *in vitro* propagation.

2.2 1. Explant

It is widely recognised that success in plantlet formation is dependent to a large extent on the careful selection of the explant (Murashige, 1974 , Sommer and Caldas, 1981) Considerable difference in organogenic capability often exists among different parts of the plant (Bonga, 1982a) Such difference occur between neighboring tissues or cells (Tra Thanh Van, 1980, Bilkey and Cocking, 1981; Binns, 1981) and even within such a small organ as the shoot apical meristem (Ball, 1950 , Barlass and Skene, 1980) Further more, morphogenic potential of a tissue is often either stimulated or inhibited by neighboring tissues (Tra Thanh Van, 1980) Aittken *et al*. (1981) noted the importance of explant selection on adventitious bud formation in radiata pine. By using excised cotyledons from embryos they could double the shoot forming capacity of whole embryos and by using cotyledons excised from newly germinated seeds, shoot forming capacity was increased 12 5 fold over that of cotyledons from embryos It has been argued that in mature trees, possibly only those somatic cell lines that eventually enter meosis have the capacity for organogenesis retained (Bonga, 1982a) Several workers have tried to use a variety of explants like immature inflorescence (Dale *et al* , 1981), stalks of immature female cones (Bonga, 1982b), anthers (Rajasekaran and Mullins, 1979), nucellus, ovule and ovary (Brink, 1982 ;

Spiegel-Roy and Kochba, 1980) For clonal propagation, however, axillary and terminal buds have been routinely employed (Bonga, 1981)

Size of explant used for culturing has a profound influence on the success in plant tissue culture works As a rule, larger the size of the explant, the more rapid the growth and greater are the rates of survival (Hussey, 1983) When tissues are cut, the cut surface turns brown due to the oxidation of phenols in the damaged cells (Monaco et al ,1977). If the explant size is small, the cut surface : volume ratio is high and adversely affects explant survival However, larger the explant size more will be the chance of it harbouring contaminant microorganisms The smallest explants possible, meristem tips with a few leaf primodia, are used for culturing to get virus free plants (Hussey, 1978)

As the tree matures, the morphogenic ability of its cells become progressively more repressed (Bonga, 1981). Most of these changes involve cytoplasmic and nuclear DNA (Bonga, 1981) In general, the more juvenile the tissue and thus in a state of more active growth, the better will it respond to in vitro treatments leading to de novo primodium initiation and subsequent organogenesis Sita et al (1980) and Rao (1986) found that callus could be induced from young tissues of Dalbergia latifolia, but not from the mature tissues Pierik and Steegman (1975) observed that the ability of stem segments of Rhododendron

sp to produce roots decreased with the age of the stem In the experiments of Takayama and Misawa (1982) most young leaf explants of Begonia sp produced buds and roots, where as those from mature leaves dried Nevertheless, examples of tissues taken from mature trees being capable of adventitious primodium formation are aplenty in literature Stem segments with buds from 20-100 year old Sequoia sempervirens (Boulay, 1979), 5-50 year old Norway spruce (van Arnold and Eriksson, 1979) have all been used as explants, and have produced viable plantlets

2 2.2 Surface sterilisation.

Surface sterilisation of explants is always necessary prior to culture inoculation (George and Sherrington, 1984) It is done with an objective of removing all microorganisms present on the explant with minimum damage to the explant tissues Explants for surface sterilisation are usually cut to a size larger than that of of the final explant and trimmed to the appropriate size after sterilisation and before inoculation (Hussey, 1979)

To check bacterial and fungal contamination, fungicides and antibiotics are often used either as surface sterilants or incorporated into the medium Ethyl alchohol, mercuric chloride, chlorine water, bromine water, silver nitrate, commercial bleach etc are some of the other common surface sterilants used in tissue culture Maroti and Levi (1977) advanced a view wherein explants are surface sterilised in a series of steps involving a rinse in ethyl alchohol (45 % v/v) for three minutes followed by

a 10 minute bleach treatment (0 5 - 10 0 % w/v) and finally three rinses in distilled water In case of Dalbergia latifolia use of mercuric chloride (0 1 % w/v) for 10- 12 min has been demonstrated to be effective (Sita et al , 1980; Mahato, 1992) Mercuric chloride gave better sterilisation of explants than sodium hypochlorite and absolute alchohol in cardamom (Reghunath, 1989)

An aqueous solution of sodium hypochlorite (5-20 % w/v) or calcium hypochlorite (5-10 % w/v) solution are the most commonly employed surface sterilants In order to make a good contact between sterilant and the explant surface a surfactant (teepol, tween etc) is also used along with the sterilant The sterilants used are often toxic to the explant and hence, are washed off from the treated tissues twice or thrice with sterile distilled water (Hu and Wang, 1983) Concentrations ranging from 1 0 per cent (Minocha, 1980) to 10 0 per cent (Kuo and Tsay, 1977) of sodium hypochlorite have been reported to be efficient for various species Several workers have reported the use of various fungicides in the culture medium for reducing fungal contamination (Brown and Sommers, 1982; Shields et al , 1984) However, most of the systemic fungicides and some of the antibiotics inhibit growth of explant in culture Dodds and Roberts (1985) suggested to avoid the use of antibiotics for sterilisation since they or their degradation products may be metabolised by plant tissues with unpredictable results Consequent to addition of systemic fungicides in the medium,

explants showed chlorophyll degradation and vitrification in leaves (Reghunath, 1989) Davey et al (1980) suggested that application of antibiotics like streptomycin, ampicillin or nystatin may be done when the material is infected with known bacterial or fungal contaminants. Antibiotics have also been noticed to increase the growth rate of cultured tissues sometimes in a quite spectacular way (Carew and Patterson, 1970) Such unpredictable effects have resulted in quite cautious use of these chemicals

2.2.3 Culture medium.

Success in plant tissue culture is mainly influenced by the nature of media used A proper medium should contain not only adequate quantity of nitrogen, potassium, calcium, phosphorus, magnesium and sulfur as well as minor nutrients like salts of iron, manganese, zinc, boron, copper, molybdenum and cobalt, but also a carbohydrate, usually sucrose, trace amounts of organic compounds like vitamins, aminoacids and plant growth regulators Some cultures perform better with the addition of some undefined organic compounds like coconut water, fruit juices, yeast extract, casein hydrolysate etc Generally for plant tissue culture, semi-solid media are employed in which case a gelling agent like agar or gelrite too is added to the media

2.2.3 1 Basal media

A plethora of formulations of media for the culture of woody plants including forest trees have appeared in the literature. The response of the species to media may often be highly species specific and it ranged from little difference in growth between several media for Rosa sp to a life-or-death situation for Kalmia latifolia (McCown and Salmar, 1987) Recently complexity of in vitro nutrition has been considered in a more unifying fashion The earlier media were characterised by a low overall concentration of inorganic ions, especially those of potassium, nitrate and by providing nitrogen solely as nitrate Later on, media having ammoniacal form of nitrogen appeared (Knudson, 1946; Vacin and Went, 1949), which were used in orchid propagation Further development in plant nutrition under in vitro conditions lead to development of White's medium (White, 1943) and Heller's medium (Heller, 1953) Since 1980, however, most researchers have been using MS (Murashige and Skoog, 1962), B_5 (Gamborg et al , 1968) or SH (Schenk and Hilderbrandt, 1972) media But after 1980, the most popular media have been DCR (Gupta and Durzan, 1985) and WPM (Llyod and McCown,1980) media, especially for woody plants

2.2.3.2. Growth regulators.

Growth regulators are organic compounds (other than a nutrient) which in small amounts promote, inhibit or qualitatively modify growth and development (More, 1979). The

commonly recognised classes of plant hormones are auxins, cytokinins, gibberelins abscisic acid and the growth inhibitor, ethylene as well as the hypothetical compounds like florigen and anthesins The most important factor in successful tissue culture is the addition of plant growth regulators (Krikorian, 1982) The growth and morphogenesis in vitro are regulated by the interaction and balance between the growth regulators supplied to the medium and the growth substances produced endogenously by the cultured cells

Plant tissue culture works although started quite early in this century, did not begin in earnest until the 1930's when serious attempts were made by several workers to obtain continuously growing cultures from a variety of plants including tree species such as Acer pseudoplatanus, Sambucus nigra and Salix carparea (Gautheret et al , 1932, 1933) Cultures could not maintain growth activity beyond six months, indicating deficiency in nutrient medium Following the discovery of auxin by Went (1926) and its chemical characterisation by Kogl et al (1934), several workers found out its use in cell cultures for purposes like callus induction, rooting etc Another milestone in establishing the importance of growth regulators in plant tissue culture was the discovery of cytokinins as the regulators for cell division (Miller et al , 1955) It was soon followed by a wide use of these chemicals in plant tissue culture Skoog and Miller (1957) found that the shoot formation could be induced predictably from callus using low levels of auxin and a high

level of cytokinin in the medium Since this discovery, many aspects of cellular differentiation and organogenesis in tissue and organ cultures have been found to be controlled by an interaction between cytokinins and auxin concentration For axillary shoot proliferation, cytokinins have been utilized to overcome the apical dominance of shoot and to enhance the branching of lateral buds from leaf axils (Murashige, 1974).

Other growth regulators like GA and ABA have some times shown dramatic influence on overcoming bud dormancy and achieving organogenesis (Bokowska and Habdas, 1982; Yostuya et al , 1984)

2.2.3.3. Carbon energy source

Normally, for the culture of cells, tissues or organ, it is necessary to incorporate a carbon energy source in most tissue culture media Sucrose in the range of 2 to 30 g l^{-1} is the main carbon source for most of the plant tissue culture media (Oka and Ohyama, 1982) Many other carbon sources are found used, instead of sucrose Gautheret (1939) studying on this line observed that sucrose was superior to glucose, maltose, raffinose, fructose, galactose, mannose and lactose

2.2 3.4. Vitamins.

Vitamins are the accessory food factors required by plant cells in very small quantities to perform certain very essential role in metabolism The most common vitamins used in plant

tissue culture are pyridoxine, nicotinic acid, biotin, riboflavin, folic acid and thiamine Among these, thiamine is very essential and most frequently added in plant tissue culture media at levels of 0 1 ppm to 1 0 ppm Nicotinic acid and pyridoxine too are often added Addition of biotin at 1 0 ppm level was found to have a complementary effect on growth and development of leaves (Reghunath, 1989). Rucker (1982) recorded favourable effect of arginine on in vitro rhizogenesis Linsmair and Skoog (1965) demonstrated that, most vitamins are not essential for callus growth in tobacco Pyridoxine, biotin and nicotinic acid could be deleted from the medium without serious impact on growth Letham (1974) found that myo-inositol, a plant vitamin, interacted with cytokinins to promote cell division in carrot phloem explants

2.2.3.5. Other organic compounds.

Many undefined supplements were employed in early tissue culture media Their use has slowly declined over the years as the balance between inorganic salts have been improved and the effect of growth regulators has been more understood. Nevertheless, several supplements of uncertain and variable composition are still in common use Conger (1981) observed their role in successful growth and differentiation in tissues and organs Some of these are casein hydrolysate coconut water, yeast and malt extract and fruit juices Coconut water is reported to be having promoting effect on growth and

differentiation in a wide variety of excised tissues (Vanoverbeek et al , 1943) Hawker et al (1973) reported that replacement of casein with coconut milk could double growth rate of callus Many researchers have warned against the indiscriminate use of these compounds, as it will lead to little control over the experiment (Gamborg and Shyluk 1981) In cases where nutritional requirements have not been established, mixtures of amino acids like casein hydrolysate may be added in concentrations ranging from 0.1 to 0 5 per cent (w/v) (Huang and Murashige, 1977) The discovery by Pollard et al (1965) that coconut water contained myo-inositol lead to the inclusion of this chemical in many culture media

Addition of activated charcoal in tissue culture media may have beneficial or harmful effects Growth, rooting, organogenesis and embryogenesis are reported to be stimulated in a wide variety of species and tissues (Wang and Huang, 1976) and inhibited in certain others on the other hand (Fridborg and Eriksson, 1975) In addition it may also prevent browning of tissues (Tisserat, 1979) Harmful effects of activated charcoal include binding of plant growth regulators and other metabolites (Weatherhead et al , 1978). Activated charcoal added to liquid MS reduced IAA and IBA concentration by over 97 per cent (Scott et al., 1990)

Certain other organic compounds like adenine and its more soluble form adenine sulphate are often added to the tissue culture media They can enhance growth and organ formation due

to their cytokinin like activity (Skoog and Tsui, 1948) Amino acids are routinely added to the media as they provide an immediately available source of nitrogen and their uptake can be much more rapid than that of inorganic nitrogen in the same medium (Simpkins et al , 1970) Polyphenolic compounds like phloroglucinol in the medium too has been found to have a beneficial role in organogenesis and growth (Hunter , 1979; Mallika et al , 1992)

2.2.4. Culture conditions.

The culture conditions play a vital role in the success of plant tissue culture Physical conditions as well as pH of the medium, light, temperature and relative humidity of the culture room, season of culturing etc all have been found to play a profound influence on the rate of growth and extent of differentiation exhibited by the cultured cells

Changes in the physical condition of the medium is brought about by changing the concentration of the gelling agent in the medium. Importance of physical condition on culture is evident in case of Dalbergia latifolia cultures (Mahato, 1992) In the experiments on rosewood, liquid cultures with shaking gave a performance far superior to semisolid media Plant cells in culture require an acidic pH of 5 5 - 5 8 (Gamborg and Shyluk, 1981). Bonga (1982a) remarked that pH of the medium is usually set at about 5 0 for liquid cultures and at 5 8 for semi-solid

The most visible and direct effect of pH on the major constituents of commonly used media is the precipitation of certain cations at higher pH values (Minocha, 1982)

Light requirement involves a combination of several components like intensity, quality and duration An optimum combination of these are required for certain phytomorphogenic events According to Murashige (1977), the optimum day light period is sixteen hours for a wide range of plants Hawker *et al*. (1973) reported the favourable effect of increasing the illumination period on bud proliferation.

Mean culture temperature is usually higher than those which would be experienced by plants of the same species *in vivo* (George and Sherrington, 1984) Yeoman (1986) reported that the usual environmental temperature of the plant should be taken into account. However, most tissue culture experiments are done at a temperature of around 25 $\pm$ 2 ^{o}C, with successful results Temperature affects culture primarily through its effect on chemical reactions (Went, 1943) Effect of temperature assumes greater importance in tissue culture of recalcitrant species

Relative humidity is rarely a problem except in arid climates, where rapid drying occurs Hu and Wang (1983) reported that air humidity is infrequently controlled and when it is controlled, 70 per cent has been found to be the most frequent setting

It has been found that tissues taken from field grown plants are not equally amenable to tissue culture conditions throughout the year Borrod (1971) determined that explants form one clone of chestnut formed callus at best in March and the worst in December, whereas, another clone showed just the reverse response

2.2.5. Genotype.

The growth of cultured tissues or organs, and *in vitro* morphogenesis are probably more influenced by genotype, than any other factor The media and the culture environment often need to be varied from one genus or species of plant to another, and even closely related varieties of plants can differ in cultural requirements (George and Sherrington, 1984). There were large differences in the capacity of the explants from different selections of mature *Eucalyptus marginata* trees to survive in culture (McComb and Bennet, 1982)

2.2.6. Root induction.

Rhizogenesis of *in vitro* shoots are usually achieved through subculturing to a medium containing auxin and lacking cytokinin or treating the shoots with rooting hormone as a conventional cutting (Yeoman, 1986) Generally, auxin favors root formation Among the auxins, NAA has been the most effective one for induction of rooting (Ancora *et al* , 1981) The concentration of

auxin required is often critical to provide sufficient stimulus to initiate roots while preventing the excessive formation of callus (Yeoman, 1986) Sometimes a combination of auxins may give a better response (Gupta et al , 1980) The root elongation phase also has been found to be very sensitive to auxin concentration High concentration of auxin inhibited root elongation (Thimman, 1977)

Several researchers have observed that in vitro rooting can successfully be achieved by reducing salt concentration in the media, particularly in MS, B_5 and LS which contain high salt concentrations Abundant rooting was observed when salt concentration was reduced to one-half or one third in the medium (Lane, 1979) Lowering nitrogen content too has been observed to promote rooting(Rucker, 1982)

Rooting of the shoots in vitro and in vivo has been reported by the following simple, highly efficient and more economical methodology by Yeoman (1986), Schwarz et al (1988) and Vijaykumar et al (1990) This is achieved by using vermiculite, sand and peat singly or in combination in tubes or pots as the rooting medium and maintaining it in high humid conditions

2.2.7. Acclimatisation and planting out.

Acclimatisation is one of the last phases of plant tissue culture, wherein the tissue culture plantlet is made conditioned for normal growth in natural environment It is one of the most

important steps because *in vitro* plants are not used to the *ex vitro* conditions (Brainerd and Fuchigami, 1981) The physiological abnormalities like vitrification may adversely affect plant survival outside

Physical, chemical and biological properties of the planting medium are important for plant establishment (Kyte and Briggs, 1979) Thorough washing of the plantlet to remove traces of nutrient sticking to it is essential for eliminating the problems of fungal infection (Anderson, 1977) Recent work by Shackel *et al* (1990) indicated that stomata of apple shoots do not close after being removed from the culture Such non functional leaves will have to be hardened during this phase for achieving success in planting out

The literature available clearly indicate that the work done on tropical tree species is far from satisfactory and much remains to be desired Trees ,especially the leguminous one ,had been resisting the attempts for micropropagation till a very recent time With the advancement in knowledge that scientists have drawn from various sources including agriculture, the work on micropropagation in commercially important trees will have to be strengthened if the full potential of tissue culture is to be realised in forestry

• *MATERIALS AND METHODS*

MATERIALS AND METHODS

The present investigations on *in vitro* propagation of bijasal (*Pterocarpus marsupium*) through tissue culture were carried out at the College of Forestry Vellanikkara during the period 1991-93 The details regarding the experimental materials and the methods adopted for the study are described in this

3.1 Materials.

3 1 1. Explant

Nodal segments of about one cm size collected from mature trees of age 10 years grown in the Botanical Garden, Kerala Agricultural University Vellanikkara were used as the explants

3.1.2. Media.

The basic media used for the study include MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980) and Hellers (Heller, 1953) The technical composition of the media are given in Table 1 The basic media were supplemented with plant growth regulators and other additives in the different experiments

The chemicals used for preparing the media were of analytical grade from British Drug House (BDH), Sisco Research Laboratories (SRL), Merck or Sigma

Table 1 Chemical composition of various culture media used for *in vitro* propagation in *Pterocarpus marsupium*

Compound	Amount (mg/l)		
	Murashige and Skoog (MS)	Woody plant Medium(WPM)	Hellers Medium
INORGANIC			
ammonium nitrate	1650 0	400 0	----
aluminum chloride	----	----	0 03
boric acid	6.2	6 2	1 0
calcium chloride-2 hydrate	440 0	96 0	75 0
calcium nitrate-4 hydrate	----	556 0	----
cobalt chloride-6 hydrate	0 025	----	----
copper sulphate-5 hydrate	0 025	0 025	0 03
ferric chloride-6 hydrate	----	----	1 0
ferrous sulphate-7 hydrate	27 8	27 8	----
manganese sulphate-1 hydrate	22 3	22 3	0 1
magnesium sulphate-7 hydrate	370 0	370 0	250 0
Na_2EDTA-2 hydrate	37 3	37 3	----
nickel chloride-6 hydrate	----	----	0 03
potassium chloride	0 83	----	750 0
potassium iodide	----	----	0 01
potassium nitrate	1900 0	----	----
potassium sulphate	----	990 0	----
sodium dihydrogen phosphate	170 0	170 0	125 0
sodium molybdate-2 hydrate	0 25	0 25	----
sodium nitrate	----	----	600 0
zinc sulphate- 7 hydrate	8 6	8 6	1 0
ORGANIC			
inositol	100 0	100 0	100 0
nicotinic acid	0 5	0 5	----
thiamine HCl	0 1	0 1	1 0
pyridoxine HCl	0 5	0 5	----
glycine	2 0	2 0	----
OTHERS			
sucrose (in per cent W/V)	3 0	2 0	2 0
agar (in per cent (W/V)	0 8	0 8	0 8
pH	5 6	5 6	5 6

1/2 MS/WPM and 1/4 MS denotes 1/2 and 1/4 the amounts of the inorgan constituents per liter respectively

MS(a) is MS with 2527 5 mg^1 pottassium nitrate and 134 mg^1 ammonium sulphat and lacking ammonium nitrate

3.2. Methods.

3.2.1. Collection and preparation of explant

Since the mother trees selected for collecting the explants were growing in the open, chances of harboring microbial populations were quite high To control the rate of contamination in cultures, the branches of the mother trees were sprayed with a mixture of a systemic fungicide 'Bavistin' 50 per cent WP (Carbendazim) and the contact fungicide 'Indofil M-45' (Mancozeb) 0 3 per cent once in 10 to 15 days

Stem segments of approximately 25 to 30 cm with 10 to 12 nodes were excised from the mother tree using a sharp razor blade and brought to the lab as quickly as possible The leaves were then removed leaving 0.5 cm of the rachis Stem segments were washed in tap water using a detergent to remove traces of fungicide and dust adhering to it After drying the segments in blotting paper, they were swabbed with a cotton dipped in 70 per cent (v/v) alchohol Further sterilisation procedures were carried out under perfect aseptic conditions in a 'Klenzaids' laminar air flow cabinet The stem segments were then cut into nodal segments of size 1 5 cm (approx) and subjected to surface sterilisation using different chemicals described in Table 2 For all the treatments the explants were immersed in the given chemical for the stipulated period with occasional stirring The explants after surface sterilisation were removed from the chemical and rinced thrice in sterilised distilled water to remove traces of sterilant sticking to the surface The cut ends

Table 2 Chemicals used, their concentration and duration of treatment for surface sterilisation of explants *Pterocarpus marsupium*

Sterilant	Concentration	Duration (minutes)
Mercuric chlorid	0 1 %	5-15
Chlorine water	3 5 mg l^{1}	5-15
Hydrogen peroxide	15 0 %	5-15

of the explants were then further trimmed to give a final size of approx one cm to the explant

An investigation for identifying indigenous bacteria in the initial explant was made by growing pure cultures of the bacteria isolated from contaminated culture tubes, in nutrient agar media After identification these bacteria were tested for sensitivity various antibiotics viz , dexicycline, chloramphynicol norfloxacin, oxytetracycline, pencillin, ciphaleurin and ampicillin

Another experiment was conducted to estimate the optimum size of the explant for enhanced release of axillary buds Three sizes of explants viz <0 5 cm 0 5-1 5 cm >1 5 cm were used for the study

3.2.2. Preparation of culture media.

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the media Stock solutions of major and minor nutrients were prepared by dissolving the required quantity

of the chemicals in distilled water and were stored in amber coloured bottles under refrigirated conditions The stock solution of nutrients were prepared fresh every four weeks and that of vitamins, amino acids and growth regulators every week

Specific quantities of the stock solutions of the chemicals and growth regulators were pipetted out into a 1000 ml beaker Sucrose and inositol were added fresh and dissolved The volume was then made upto about 1000 ml by adding distilled water The pH of the solution was checked with a pH meter and adjusted to the range 5 5 to 5 8 using 1 N NaOH or 1 N HCl Agar was then added to the medium and the final volume made upto 1000 ml.

The solution was then boiled for melting the agar by keeping over a burner Twenty ml each of the media was poured hot to the oven dried culture tubes (150 x 25 mm), which were previously washed and rinsed in distilled water The containers with the medium were then tightly plugged with absorbent cotton wool plugs. The media was then autoclaved for 15-20 min at 15 psi pressure and 121^{o}C temperature (Dodds and Robert 1982) After sterilisation, the culture vessels were immediately transferred to the culture room

3.2.3 Inoculation and culturing of the explants.

All the inoculation operations were carried out under perfect aseptic conditions in a laminar air flow cabinet

To inoculate the explants to the culture medium, the cotton wool plug of the culture vessel was removed and the vessel neck

was first flamed over a gas burner kept in the chamber The sterile explants were quickly transferred into the medium using sterile forceps The neck of the culture vessels was once again flamed and the cotton wool plug replaced

The culture vessel was then transferred to the culture room where they were incubated at a temperature of 27± 2°C Artificial illumination was provided using cool white fluorescent lamps The light intensity was maintained at 2000 lux Photo period was fixed as 16 h per day

3.2.4. Shoot induction.

3.2.4.1. Standardisation of medium supplements.

Studies were conducted to evaluate the effect of various plant growth regulators on bud break, shoot induction multiple shoot production and growth from axillary buds For this study separate experiments were conducted using WPM and MS as basal medium Details regarding the various plant growth regulators tried and concentrations used are furnished in Table 3 Apart from growth regulators, various organic and inorganic additives were evaluated for their capacity to produce shoots from axillary bud explants of Pterocarpus marsupium Details regarding the organic supplements other than growth regulators tried are presented in Table 4

Table 3.Treatments of plant growth regulators tried for shoot induction from axillary buds of Pterocarpus marsupium

Basal medium	Treatment
WPM	kinetin (1 0 2 0, 3 0 ppm) BA (1 0, 2 0, 3 0 ppm) and 2-ip (1 0, 2 0, 3 0 ppm)
WPM	3x3 combinations of kinetin (1.0, 2 0, 3 0 ppm), IAA (0 1, 0.5, 1 0 ppm)
WPM	3x3 combinations of kinetin (1 0, 2.0, 3.0 ppm), IBA (0 1, 0.5, 1 0 ppm)
WPM	3x3 combinations of kinetin (1 0, 2 0, 3.0 ppm), NAA (0 1, 0 5, 1 0 ppm)
WPM	3x3 combinations of BA (1 0, 2 0, 3 0 ppm), IAA (0.1, 0 5, 1 0 ppm)
WPM	3x3 combinations of BA (1 0, 2 0, 3 0 ppm), IBA (0 1, 0 5, 1 0 ppm)
WPM	3x3 combinations of BA (1 0 2 0, 3 0 ppm), NAA (0 1, 0 5, 1 0 ppm)
WPM	3x3 combinations of 2-ip (1 0, 2 0, 3 0 ppm), IAA (0 1, 0 5, 1 0 ppm)
WPM	Gibberellic acid (1 0, 2 0, 3 0 ppm) in combination with 2 ppm kinetin and 0.1 ppm IAA
WPM	ABA (0 1, 0 25, 0 5 0 75, 1 0 ppm) in combination with 2 ppm kinetin and 0 1 ppm IAA
MS	kinetin (1 0 2 0, 3 0 ppm) and BA (1 0 2 0, 3 0 ppm)
MS	3x3 combinations of kinetin (1 0, 2 0 3.0 ppm) IAA (0 1 0 5, 1 0 ppm)
MS	3x3 combinations of BA (1 0, 2 0, 3 0 ppm), IAA (0.1, 0 5, 1 0 ppm).
MS	3x3 combinations of BA (1 0, 2 0, 3 0 ppm), IBA (0 1, 0 5, 1 0 ppm)
MS	3x3 combinations of BA (1 0 2 0 3 0 ppm), NAA (0 1, 0 5 1 0 ppm)
MS	3x3 combinations of BA (1 0 2 0, 3 0 ppm), 2 4-D (0 1 0 5, 1 0 ppm).

Table 4 Treatments of media additives tried for shoot induction from axillary buds of Pterocarpus marsupium

Media	Treatment
WPM	Casein hydrolysate (200, 300, 500 ppm) in combination with 2 0 ppm kinetin and 0 1 ppm IAA
	Cycocel (0 1, 0 5, 0 75, 1 0, 2.0, 3 0 ppm) in combination with 2 ppm kinetin and 0 1 ppm IAA
	Adenine sulphate (20, 40, 60 ppm) in combination with 2 0 ppm kinetin and 0 1 ppm IAA
	Activated charcoal (1, 2, 3, 4 per cent W/V) in combination with 2 0 ppm kinetin and 0 1 ppm IAA
	Silver nitrate (5, 10, 20 ppm) in combination with 2 0 ppm kinetin and 0 1 ppm IAA
	Copper sulphate (5, 10, 20 ppm) in combination with 2 0 ppm kinetin and 0 1 ppm IAA
MS	Adenine sulphate (10, 20, 30, 40 ppm) in combination with 2 0 ppm BA and 0 1 ppm NAA
	Coconut water (10, 20, 30 per cent V/V) in combination with 2.0 ppm BA
	Silver nitrate (5, 10, 15, 20 ppm) in combination with 2 0 ppm BA and 0.1 ppm IAA
	Copper sulphate (5, 10, 15 ppm) in combination with 2 0 ppm BA and 0 1 ppm NAA
	Cobalt chloride (1, 2, 3 ppm) in combination with 2 pm BA and 0 1 ppm NAA

culture inoculation All the data were calculated based on cultures that remain with out contamination after the required peroid of incubation

The following observations were recorded from various experiments

2.4.2. Standardisation of physical condition

As physical condition of the culture play a profound influence on the shoot induction and growth from axillary explants, a study on the influence of these conditions were carried out An experiment was conducted where varying levels of agar (0%, 0 6%, 0 7%, 0 8%) were incorporated and their influence studied Study on the influence of pH of the medium on culture establishment was done by adjusting pH of the medium to 5 0, 5 5 and 6 0 Another trial was conducted to study the influence of light on shoot induction and growth of Pterocarpus marsupium axillary bud explants Treatments tried included culture incubation in the dark, in white fluorescent light at 500 lux, 1000 lux, 2000 lux, as well as red light and sun light

3.2 4.3. Establishment of continuous cultures.

Shoots produced from the primary explants were cut and removed to be used as the source of explants for establishing continuous cultures These shoots were used as such or cut into nodal segments and inoculated into fresh medium, details of which are given in Table 5

3.3.5 Observations.

All the trials were conducted with a minimum of three replications, each replication having seven tubes The observation were recorded between third and fourth weeks after

Table 5 Various media combinatons tried for production of continuous culture from axillary buds of *Pterocarpus marsupium*

Media	Additives
WPM	Combinations of 2 0 and 3.0 ppm kinetin + 0.5 ppm IBA
	Combinations of 2 0 ppm kinetin + 0 1 and 0 5 ppm IAA
	Combinations of 2 0 ppm kinetin + 0 1 ppm IAA + 500 and 1000 ppm glutamin
	Combinations of 2 0 ppm kinetin + 0 5 ppm IAA + 10 and 100 ppm adenine sulphate
	Combinations of 2 0 ppm kinetin + 0 1 ppm IAA + 10, 40 ppm $AgNO_3$
	2 0 ppm kinetin + 0 5 ppm IAA + 40 ppm $AgNO_3$
	2 0 ppm kinetin + 0 1 ppm IAA + 1000 ppm casein hydrolysate
	2 0 ppm kinetin + 0 1 ppm IAA + 500 ppm casein hydrolysate + 10 $AgNO_3$
	2 0 ppm kinetin + 0.5 ppm IAA + 80 ppm glutamic acid
	1 0 ppm kinetin + 10 ppm $AgNO_3$ + 20 ppm glutamic acid
	Combinations of 1 0 ppm kinetin + 40, 60, 100 and 200 ppm glutamic acid
	2 0 ppm kinetin + 3 % sucrose
	Combinations of 2 0 ppm kinetin + 50, 200, 250 and 300 ppm phloroglucinol
	1 0, 2 0, 3 0 ppm 2-ip
	Combinations of 1 0 and 2 0 ppm BA + 0.1 ppm IAA.
	1 0, 2 0 ppm BA
	Combinations of 1 0 and 2 0 ppm BA + 1 ppm NAA
MS	Combinations of 2 0 ppm BA + 0 1 and 0 5 ppm IAA
	Combinations of 1 0 and 3 0 ppm BA + 0.5 IAA
	Combinations of 2 0 ppm BA + 0 5 IAA with 10, 15, 20 ppm $AgNO_3$
	Combinations of 2 0 ppm kinetin with 0 5 and 0 1 ppm IAA
1/2 MS	Combinations of 2 % maltose with 1 0, 2 0 and 3 0 ppm BA
	2 % maltose + 1 0 ppm BA + 0 1 ppm NAA
	Combinations of 0 5 ppm BA + 5 10, 15 and 20 ppm adenine sulphate
	1 0 ppm BA + 0 5 ppm NAA
	1 0, 2 0, 3 0 and 4 0 ppm BA
	1 0, 1 5 and 2 0 ppm kinetin
	1 0 ppm kinetin + 10 ppm adenine sulphate
	1 0 ppm 2-ip
1/4 MS	Combinations of 1 0, 3 0 and 4 0 ppm BA + 500 ppm glutamine
	Combinations of 3 0 ppm BA + 5, 10 and 20 ppm adenine
	3 0 ppm BA
Hellers	2 ppm kinetin
	2 ppm kinetin + 0 5 ppm IAA
	2 ppm BA + 0 5 ppm IAA
	2 ppm 2-ip + 0 5 ppm IAA
	2 ppm 2-ip

a Number of explant showing shoot proliferation. Number of cultures showing bud proliferation were expressed as a percentage of total number of surving cultures

b Number of explants with multiple shoot: Number of explants with multiple shoot were expressed as a percentage of total number of surviving cultures

c Number of cultures showing leaf expansion: The number of cultures that had leaf growth were expressed as a percentage of total surviving cultures

d Average number of shoots per expalnt. This character was expressed based on number of cultures showing shoot proliferation among the different replications

e Average shoot length: This was expressed as average of all the shoots produced in the different replications

- *RESULTS*

RESULTS

Results of the various experiments conducted to standardise the procedure for clonal propagation of Pterocarpus marsupium through axillary bud culture are presented below

4.1. Explant choice and sterilisation.

4.1.1. Explant choice.

The observations on the effect of explant size on culture establishment is presented in Table 6 Nodal explants of 0 5 to 1 5 cm were found to be better for culture establishment in which case 83 per cent of the cultures showed release of axillary buds. Bigger explants of size more than 1 5 cm had lower number of cultures showing bud sprout More chance of contamination was also noticed in these cultures Only 37 per cent of the cultures from smaller explants of less than 0.5 cm showed bud burst and many of these had slight callusing around the bud

4.1.2. Sterilisation procedure.

Data on the trial conducted to find out the optimum surface sterilisation procedure for the explant is given in Table 7 Among the three sterilants tried, mercuric chloride (0 1 %) was found to be most effective in controlling surface contamination of explants Though at short duration treatments this chemical was not very effective, as the duration increased a gradual increase in the number of uncontaminated cultures was noticed. Treatment with mercuric chloride (0 1 %) for 10 min gave the optimum result with only 52 per cent of the cultures showing

Table 6. Effect of size of explants in axillary bud cultures of *Pterocarpus marsupium* on initiating shoot bud release

Explant size	% cultures showing response
< 0 5 cm	37
0 5 cm - 1 5 cm.	83
> 1.5 cm	76

Culture medium WPM + 2.0 ppm K + 0.1 ppm IAA

Table 7 Effect of various surface sterilants on culture establishment in *Pterocarpus marsupium*

Sterilisation chemical	Time (minutes)	% of cultures contaminated	% of cultures not infected Living	Dead
$HgCl_2$ (0 1 %)	5	100	0	0
	7	100	0	0
	9	85	15	0
	10	52	48	0
	12	50	41	9
	15	45	20	35
Chlorine water (3.150mg l^{-1}	5	100	0	0
	7	100	0	0
	9	100	0	0
	10	90	10	0
	12	85	15	0
	15	82	18	0
H_2O_2(15 %)	5	100	0	0
	10	100	0	0
	15	100	0	0

contamination along with a culture survival of 48 per cent Though with increase in treatment time, the contamination rate could be brought down to 45 per cent, the survival of cultures was found to be drastically reduced (20 %) While chlorine water (3 15 g l^{-1}) controlled contamination to a certain extent, hydrogen peroxide (15 %) was found to be totally ineffective even after 15 minutes of treatment

During subculturing and production of continuous cultures latent infection by systemic bacteria were noticed, inspite of the sterilisation treatments adopted The bacterial cultures made from explant ooze was put for study and based on the characters mentioned in Table 8, it was identified to be of two species of genus Bacillus The cultures were further put for antibiotics sensitivity test and the results obtained are presented in Table 9

4.1.3 Seasonal influence on explant contamination and bud break.

A seasonal influence on the extent of explant contamination is evident from the study (Table 10) Total loss of all the cultures due to contamination occurred during the rainy months (June, July, August, October and November), whereas, only 78 per cent and 75 per cent of the cultures were contaminated during the dry months of March and April, respectively A prophylactic spraying of fungicides given to the mother plants, however, considerably reduced the rate of explant contamination The per centage of contaminated cultures was reduced to as low as 10 in

Table 8 Identification of contaminant bacteria obtained from axillary bud cultures of Pterocarpus marsupium

Reaction/Observation	Character of isolate
Morphology of the colony	Medium sized, translucent
Gram reaction	Gram +
Shape	Bacillus
Motility	–
Growth aerobically	+
Growth anaerobically	–
Catalyse reaction	+
Oxidase reaction	+
Glucose utilisation	Fermentative

Table 9 Results of the antibiotic sensitivity test carried out on the bacterial culture obtained from axillary bud cultures of Pterocarpus marsupium

Antibiotic	Response
Doxycycline (D)	+++
Chloramphenicol (C)	+++
Norflexacin (Nx)	++
Oxytetracycline (O)	++
Penicillin (P)	+
Ciphalosporin (Cp	+
Ampicillin (A)	+

+++ strong inhibition
++ moderate inhibition
+ light inhibition

Table 10 Seasonal influence on contamination and culture establishment in axillary bud cultures of Pterocarpus marsupium

Month	Contamination		Establishment
	Sprayed (%)	Unsprayed (%)	(%)
Jan	20	83	65
Feb	23	80	66
Mar	18	78	70
Apr	10	75	72
May	12	85	73
Jun	28	100	68
Jul	32	100	63
Aug	25	100	62
Sep	26	98	58
Oct	25	100	60
Nov	20	100	55
Dec	20	95	56

the month of April as against 75 per cent in the unsprayed material during the same period In general, in both sprayed and unsprayed materials, the rate of contamination was high during the rainy months

Extent of cultures showing bud break too was influenced by season In general, explants collected during the months of September, October, November and December showed a higher tendency to form callus whereas bud break and culture establishment was maximum during the months of March, April and May (70, 72 and 73 per cent respectively)

4.2 Standardisation of basal medium.

The data on the effect of different basal media on nodal cultures of P. marsupium are presented in Table 11 Among the different media tried namely, MS, MS(a), 1/2 MS, 1/4 MS, WPM, 1/2

WPM and Hellers + 1 micromol of ammonium sulphate, all in combination with 2 0 ppm BA, 1/2 MS had the highest number of explants showing shoot proliferation (86 %) and 1/2 WPM the lowest (45 %) The two media MS and MS(a) were also noticed to give similar results, the per cent of explants showing shoot proliferation being 85 per cent and 84 per cent, respectively These in addition were the only media capable of inducing multiple shoot proliferation in combination with 2 0 ppm BA. Average shoot length was noticed to be maximum in MS (a) medium (0 7 cm) Leaf proliferation, however, was absent in all the media tried except in MS (a) where 25 per cent of the cultures exhibited leaf expansion and proliferation

4.3. Standardization of media supplements.

4.3.1. Plant growth regulators.

In order to find out the effect of plant growth regulators on bud break and shoot proliferation from axillary buds of P. marsupium, WPM as well as MS medium were supplemented with different levels of cytokinins like kinetin, BA and 2ip independently and in combination with auxins, namely, IAA, IBA, NAA and 2,4-D Effect of different levels of ABA as well as GA were also studied The results obtained are presented below

4.3.1.1. Effect of kinetin and auxins.

Data on the effect of different concentrations of kinetin and auxins supplemented to WPM are given in Table 12

Table 11 Effect of basal media (in combination with 2 ppm BA) on culture of nodal segments of *Pterocarpus marsupium*

Media	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
MS	85	14	1 1	0	0 50
1/4 MS	62	0	1 0	0	0 25
1/2 MS	86	0	1 0	0	0 50
MS(a)	84	8	1 2	25	0 70
Hellers + 1 micromole of $(NH_4)_2SO_4$	75	0	1 0	0	0 25
WPM	65	0	1 0	0	0 25
1/2 WPM	45	0	1 0	0	0.25

Axillary bud explants cultured in WPM containing 1 0, 2 0, and 3 0 ppm kinetin in the absence of any auxin failed to respond Neither bud break nor shoot growth could be obtained in any of the levels of this cytokinin when tried independently

Kinetin in combination with IAA, however, could result in enhanced release of axillary buds from the initial explants Production of single shoots having an average length of 1 6 cm was observed in 93 per cent of the cultures when kinetin and IAA were added to the basal medium at concentrations of 1.0 ppm and 0 5 ppm, respectively. Lower concentration of the auxin (0 1 ppm) with the same level of kinetin resulted in a marginal decrease in number of cultures producing shoots, however, this combination gave the longest shoots (1 8 cm) among the combinations of IAA tried with 1 0 ppm kinetin Higher concentration of IAA (1 0 ppm) at this level of kinetin (2 0 ppm) could not induce bud break and shoot production Kinetin at 2 0 ppm with IAA concentration of 0 1 ppm was the best among the different combinations of kinetin and IAA tried in WPM media This combination induced multiple shoot production in all the cultures producing shoots of average length of 2.5 cm Higher concentration of IAA (0 5 ppm) with the above level of kinetin resulted in 94 per cent of cultures producing single shoots of average length 1 8 cm Further increase in IAA concentration to 1 0 ppm concentration had negative influence on culture establishment with none of the cultures showing shoot proliferation Auxin IAA at a concentration of 0 5 ppm with 3 0 ppm of kinetin in WPM resulted in 66 per cent of the cultures

Table 12 Effect of kinetin with auxin combinations on the axillary bud cultures of Pterocarpus marsupium in WPM media

Concentration of kinetitin (ppm)	Auxin	Concentration(ppm)	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
1 0			0	0	0	0	0
2 0			0	0	0	0	0
3 0			0	0	0	0	0
1 0	IAA	0 1	90	0	1 0	0	1 80
		0 5	93	0	1 0	0	1 60
		1 0	0	0	0	0	0
2 0		0 1	96	96	3	0	3 80
		0 5	94	0	1 0	0	1 80
		1 0	0	0	0	0	0
3 0		0 1	0	0	0	0	0
		0 5	66	11	1 3	0	1 70
		1 0	0	0	0	0	0
1 0	IBA	0 1	0	0	0	0	0
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
2 0		0 1	0	0	0	0	0
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
3 0		0 1	12	0	1 0	0	0 30
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
1 0	NAA	0 1	20	0	1 0	0	0 50
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
2 0		0 1	32	0	1 0	0	0 25
		0 5	25	0	1 0	0	0 25
		1 0	0	0	0	0	0
3 0		0 1	0	0	0	0	0
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0

producing shoots with an average of 1 3 shoots per culture and having an average shoot length of 1 7 cm Other combinations of IAA with 3 0 ppm kinetin failed to induce shoot production

Among the nine combinations of kinetin and IBA tried in WPM shoot proliferation was observed only in the combination involving kinetin at 3 0 ppm with 1 0 ppm of IBA This combination resulted in 12 per cent of the cultures producing single shoots of average length 0 3 cm

Along with 0 1 ppm of NAA in the WPM media, kinetin at 1 0 ppm produced single shoots with average length of 0.5 cm in 20 per cent of the cultures But shoot proliferation was absent when the NAA concentration was increased to either 0 5 ppm or 1 0 ppm and maintaining the kinetin level at 1 0 ppm Two ppm of kinetin in combination with 0 1 ppm or 0 5 ppm NAA resulted in the production of single shoots with an average length of 0 25 cm (32 % and 25 % of cultures, respectively) whereas no response was obtained along with 1 0 ppm NAA Still higher concentration of kinetin failed to induce shoot production in combination with any of the three levels of NAA tried

Data obtained from the study of the influence of kinetin alone or in combination with auxin in MS media are presented in Table 13 All levels of kinetin (1 0, 2 0, 3 0 ppm) tried in this media failed to induce shoot proliferation Kinetin at 1 0 ppm in combination with any of the three levels of IAA tried failed to induce shoot production However, some of the higher doses of kinetin in combination with lower doses of IAA showed

Table 13 Effect of kinetin with auxin combinations on the axillary bud cultures of *Pterocarpus marsupium* in MS media

Concentration of kinetin (ppm)	Auxin	Concentration(ppm)	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av. no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
1 0			0	0	0	0	0
2 0			0	0	0	0	0
3 0			0	0	0	0	0
1 0	IAA	0 1	0	0	0	0	0
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
2 0		0 1	16	0	1.0	0	0 5
		0.5	0	0	0	0	0
		1 0	0	0	0	0	0
3 0		0 1	50	0	1 0	0	0 25
		0 5	28	0	1 0	0	0 25
		1 0	0	0	0	0	0

favorable response Maximum percentage (50 %) response was noticed in a combination involving 3 0 ppm kinetin and 0 1 ppm IAA where the shoot length was 0 25 cm Although the shoot length remained the same shoot proliferation was observed only in 28 per cent of the cultures when the concentration of IAA was increased to 0 5 ppm, keeping the kinetin content at 3 0 ppm The only combination that induced shoot production at 2 0 ppm level of kinetin with IAA was a concentration of 0 1 ppm wherein 16 per cent of the explants produced 0 5 cm long shoots None of the combination of kinetin with IAA in MS induced multiple shoot production

4 3.1.2. Effect of BA and auxins.

BA alone was effective in producing single shoots when incorporated into WPM (Table 14) Number of responding cultures was highest (60 %) when the concentration of BA in the medium was the lowest (1 0 ppm) But this was found to decrease with increasing levels of BA

All levels of BA and IAA tried except 1 0 ppm each of the growth regulators were found to exhibit positive response to shoot induction Along with 1 0 ppm BA, 0 1 ppm of IAA in WPM produced single shoots The auxin IAA at a concentration of 0 1 ppm with 1 0 ppm BA resulted in 50 per cent of the cultures producing single shoots of average length 0 25 cm Increase in IAA concentration to 0 5 ppm at the same level of BA resulted in an increase in average shoot length to 0 5 cm compared to the former with no change in the number of responding cultures

Table 14 Effect of BA with auxin combinations on the axillary bud cultures of *Pterocarpus marsupium* in WPM media

Concentration of BA (ppm)	Auxin	Concentration(ppm)	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
1 0			60	0	1 0	0	0 50
2 0			35	0	1 0	0	0 25
3 0			32	0	1 0	0	0 25
1 0	IAA	0 1	50	0	1 0	0	0 25
		0 5	50	0	1 0	0	0 50
		1 0	0	0	0	0	0
2 0		0 1	73	6	1 1	0	1 45
		0 5	80	16	1 2	0	1 70
		1 0	20	2	1 1	0	0 25
3 0		0 1	65	7	1 2	0	1 80
		0 5	79	23	1 2	0	1 00
		1 0	25	3	1 1	0	0 75
1 0	IBA	0 1	88	23	1 4	0	1 70
		0 5	53	26	1 1	0	0 25
		1 0	2	0	1 0	0	0 25
2 0		0 1	26	0	1 0	0	0 50
		0 5	76	8	1 4	0	1 60
		1 0	0	0	0	0	0
3 0		0 1	46	13	1 1	0	0 50
		0 5	52	38	1 4	0	0 50
		1 0	81	7	1 2	0	0 25
1 0	NAA	0 1	0	0	0	0	0
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
2 0		0 1	30	0	1 0	0	0 50
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
3 0		0 1	26	0	1 0	0	0 50
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0

Further increase in IAA concentration, along with 1 0 ppm BA, failed to induce shoot production All combinations involving 2 0 ppm BA along with IAA resulted in multiple shoot production. The cytokinin BA at this strength along with 0 1 ppm IAA resulted in proliferation of an average of 1 1 shoots of 1 45 cm per explant in 73 per cent of the cultures Increase in IAA concentration resulted in an increase in number of cultures showing bud break (80 %), number of shoots per explant (1 2) and average shoot length (1 7 cm) Still higher concentration of the auxin (1 0 ppm) resulted in the lowest number cultures showing shoot proliferation (20 %) and average shoot length (0 25 cm) among the combination involving 2 0 ppm BA with IAA in WPM All combinations of 3 0 ppm BA along with IAA in WPM also resulted in multiple shoots The lowest concentration of IAA tried (0 1 ppm) along with 3 0 ppm BA produced an average of 1 2 shoots per culture, with an average shoot length of 1 8 cm in 65 per cent of the cultures Increase in IAA concentration (0.5 ppm) resulted in an increased number of responding cultures (85 %) but a lower shoot length (1 0 cm) compared to the former Further increase in the auxin concentration to 1 0 ppm had a negative influence on number of cultures showing shoot proliferate (25 %), number of shoots per culture (1 1) and average shoot length (0 75 cm).

A general enhancement of release of axillary buds was noticed when WPM was supplemented with BA along with different levels of IBA With the concentration of BA at 1 0 ppm, average number of responding culture, average number of shoots per culture and average shoot length decreased with increase in the

level of IBA concentration While at 0 1 ppm IBA 88 per cent of the cultures produced shoots with average of 1 4 shoots of length 1 7 cm per culture Higher concentration of IBA (0 5 ppm) resulted in 53 per cent of the cultures producing 1 1 shoots per culture with an average length of 0 25 cm Further increase in IBA concentration to 1 0 ppm suppressed shoot production in majority of the cultures with only 2 per cent of the cultures showing bud release The auxin IBA at a concentration of 0 5 ppm along with 2 0 ppm of BA in induced multiple shoot production at a rate of 1 4 shoots per explant in about 76 per cent of the cultures The average shoot length at this concentration was 1 6 cm Lower levels of IBA (0 1 ppm) resulted in a reduction in the number of responding cultures (26 %), shoots per explant (1 0) and shoot length (0 5 cm) where as a higher level (1 0 ppm) completely suppressed shoot production The auxin at the level of 0 1 ppm with 3 0 ppm of BA resulted in 46 per cent of the cultures producing 1 1 shoots of average length 0 5 cm per culture Combination of IBA at 0 5 ppm along with 3 0 ppm of BA resulted in an increase in number of shoots per culture (1 4) , while the average shoot length remained the same At the highest concentration of IBA (1.0 ppm) tried along with 3 0 ppm of BA 81 per cent of cultures showed shoot proliferation However, the number of cultures producing multiple shoots decreased and the average number of shoots per culture was decreased to 1 2

In general NAA was not found very effective for culture establishment along with BA in WPM While all combinations of NAA with 1 0 ppm BA failed to induce shoot production NAA at

0 1 ppm along with 2 0 ppm BA produced single shoots of 0 5 cm in 30 per cent of the cultures Higher concentrations of NAA with 2 0 ppm of BA failed to induce shoots The lowest concentration of NAA tried (0 1 ppm) with 3 0 ppm BA produced single shoots in 26 per cent of the cultures with an average shoot length of 0 5 cm Higher concentrations of the auxin along with 3 0 ppm BA also did not produce shoots from the cultures

Data on the effect of BA in combination with different levels of auxin in medium MS on shoot proliferation are presented in Table 15 Different levels of BA alone in MS media was seen to favor release of axillary buds of bijasal At a concentration of 1 0 ppm 75 per cent of the cultures produced single shoots of average length 0 25 cm The cytokinin BA at 2 0 ppm resulted in a marginal decrease in number of responding cultures to 60 per cent, but the number of shoots per culture and the shoot length remained the same At a still higher concentration of BA (3 0 ppm) multiple shoots were formed Out of 42 per cent of the cultures which produced shoots 20 per cent had multiple shoots of average length 0 30 cm

The auxin IAA at a concentration of 0 1 ppm along with BA at 1 0 ppm in MS media resulted in single shoot production in 90 per cent of the cultures, with an average shoot length of 0 25 cm Higher concentration of IAA (0 5 ppm) along with the above concentration of BA resulted in lower number of responding cultures (50 %) but a higher average shoot length (0 5 cm) Further increase in IAA concentration to 1.0 ppm resulted in total suppression of shoot production from the initial explant

Table 15 Effect of BA with auxin combinations on the axillary bud cultures of *Pterocarpus marsupium* in MS media

Concentration of BA (ppm)	Auxin	Concentration(ppm)	% of cultures showing shoot proliferation	% of cultures with multiple shoots	av no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
1 0			75	0	1 0	0	0 25
2 0			60	0	1 0	0	0 25
3 0			42	20	1 4	0	0 30
1 0	IAA	0 1	90	0	1 0	0	0 25
		0 5	50	0	1 0	0	0 50
		1 0	0	0	0	0	0
2 0		0 1	71	7	1 2	0	1 45
		0 5	85	21	1 2	0	1 70
		1 0	25	0	1 0	0	0 50
3 0		0 1	70	8	1 3	0	1 80
		0 5	85	30	1 3	0	1 00
		1 0	50	0	1 0	0	0 50
1 0	IBA	0 1	98	25	1 5	0	1 80
		0 5	50	20	1 3	0	0 50
		1 0	0	0	0	0	0
2 0		0 1	78	0	1 0	0	0 25
		0 5	80	10	1 5	0	1 80
		1 0	0	0	0	0	0
3 0		0 1	50	20	1 2	0	0 50
		0 5	55	43	1 6	0	0 50
		1 0	90	10	1 3	0	0 50
1 0	NAA	0 1	96	55	2 9	1	0 75
		0 5	93	28	2 3	0	0 75
		1 0	0	0	0	0	0
2 0		0 1	85	8	1 5	0	1 70
		0 5	90	0	1 0	0	0 25
		1 0	20	0	1 0	0	0 25
3 0		0 1	92	9	1 4	0	1 20
		0 5	90	8	1 2	0	0 25
		1 0	35	0	1 0	0	0 25
1 0	2 4-D	0 1	25	0	1 0	0	0 25
		0 5	50	0	1 0	0	0 25
		1 0	33	0	1 0	0	0 50
2 0		0 1	30	0	1 0	0	0 25
		0 5	50	0	1 0	0	0 30
		1 0	25	0	1 0	0	0 25
3 0		0 1	32	0	1 0	0	0 30
		0 5	55	0	1 0	0	0 25
		1 0	40	0	1 0	0	0 25

Addition of BA at a concentration of 2 0 ppm along with 0.1 ppm of IAA resulted in 71 per cent of the cultures producing shoots of average length 1 45 cm at the rate of 1 2 shoots per culture Increased amount of IAA (0 5 ppm) along with 2 0 ppm of BA further increased the number of cultures producing shoots to 85 per cent This treatment resulted in 21 per cent of cultures producing multiple shoots with an average shoot length of 1 7 cm Highest concentration of IAA (1 0 ppm) tried along with same concentration of BA resulted in lesser number of cultures producing shoots (25 %) Higher concentration of BA (3 0 ppm) along with 0 1 ppm IAA produced shoots at a rate of 1 3 shoots of average length 1 8 cm per culture Increase in IAA (0 5 ppm) with the same concentration of BA did not change the average number of shoot per culture, although the number of cultures producing shoots increased to 85 per cent This treatment resulted in a decrease in shoot length (1 0 cm) compared to the former Still higher concentration of IAA (1 0 ppm) resulted in 50 per cent of the cultures producing single shoots of 0 5 cm

A combination of BA (1 0 ppm) along with IBA at 0 1 ppm in MS resulted in 98 per cent of the cultures producing shoots This treatment resulted in cultures producing shoots at a rate of 1 5 shoots per culture with average length of 1 8 cm The auxin IBA at 0 5 ppm along with the above concentration of BA had deleterious influence on the culture, with reduced number of shoots per culture (1 3), lesser average shoot length (0 5 cm) as well as culture response (50 %) Further increase in IBA (1 0 ppm) concentration failed to produce any shoots The cytokinin

BA at 2 0 ppm with 0 1 ppm of IBA resulted in 78 per cent cultures producing single shoots of average length of 0 25 cm. Higher concentration of IBA (0 5 ppm) resulted in multiple shoot production in 80 per cent of the cultures The average number of shoots per explant was 1 5 and length 1 8 cm Highest concentration of the auxin (1 0 ppm) with the above level of BA failed to produce shoots from the primary explants At the lowest level of IAA (0 1 ppm) tried with the highest level of BA (3 0 ppm) an average of 1 2 shoots each were formed in 50 per cent of the cultures Addition of 0 5 ppm of IBA with 3 0 ppm BA resulted in a higher number of shoots per culture and a marginal increase in number of cultures producing shoots A maximum of 90 per cent culture response was noticed when medium MS was supplemented with 3 0 ppm BA and 1.0 ppm IBA The rate of shoot production per explant, however, was reduced to 1 3 in the culture

Combination of BA along with different lower concentrations of NAA in MS was beneficial for culture establishment from primary explants of bijasal With 0 1 ppm NAA and 1 0 ppm BA, 96 per cent of the cultures produced shoots at a rate of 2 9 per culture, with an average shoot length of 0 75 cm This treatment resulted in 1 per cent of cultures producing leaves This was the only treatment which induced leaf morphogenesis at least in some of the cultures Higher concentration NAA with the same level of BA resulted in a marginal decrease in number of cultures producing shoots (93 %) and number of shoots per culture (2 3 cm), while the average shoot size remained the same Still

higher strength of NAA (1 0 ppm) in the medium along with 1 0 ppm BA suppressed shoot production from the explants Increasing the BA concentration to 2 0 ppm along with 0.1 ppm of NAA resulted in 85 per cent of the cultures producing shoots at the rate of 1 8 shoots per culture The shoots produced had an average length of 1 8 cm which was the highest recorded among the treatments of BA with NAA in MS NAA at a strength of 0 5 ppm with the same level of BA induced single shoots in 90 per cent of the cultures This treatment further decreased the average shoot size too (0 25 cm) compared to the earlier treatment Further increase in NAA to 1 0 ppm markedly decreased the number of culture showing shoot proliferation (20 %) with no change in shoot length Combination of BA at a strength of 3 0 ppm with NAA at 0 1 ppm produced multiple shoots at the rate of 1 4 shoots of average length 1 2 cm per explant Increase in NAA concentration (0 5 ppm) brought about a drastic decrease in average shoot length to 0 25 cm, while other observations recorded only a marginal decrease Further increase in NAA concentration in MS suppressed multiple shoot initiation and resulted in 35 per cent of the cultures producing single shoots of average size 0 25 cm

All combinations of BA with 2,4-D in MS produced single shoots which did not vary much with regard to the shoot size The combinations differed only with respect to the number of cultures showing shoot proliferation Combination of BA at 1 0 ppm with NAA at 0 1 ppm resulted in 25 per cent of the cultures showing bud release Increase in the auxin content to 0.5 ppm resulted in an increase in number of cultures producing shoots

(50 %) Further increase in 2,4-D (1 0 ppm) reduced the number of cultures producing shoots to 33 per cent, compared to the former Among the treatments involving 2,4-D with 2 0 ppm BA, 0 5 ppm of 2,4-D yielded highest number of responding cultures (50 %) Higher (1 0 ppm) and lower (0 1 ppm) concentrations of the auxin with 2 0 ppm of BA resulted in decreased number of responding cultures In combinations of 2,4-D with 3 0 ppm BA also the combination of 0 5 ppm 2,4-D exhibited the maximum explant response (55 %) In the lower (0 1 ppm) and higher levels (1 0 ppm) levels of the auxin giving comparatively lesser response (32 and 40 per cent respectively)

4.3.1.3. Effect of 2ip and auxins.

Addition of the cytokinin 2ip in WPM at a concentration of 3 0 ppm resulted in 80 per cent of the cultures producing single shoots of average length 0 5 cm (Table 16) However, lower levels of 2ip (1 0 and 2 0 ppm) did not induce any favorable response to the axillary bud explants of P. marsupium

Lower levels of IAA, in general, is found to exert synergistic effect on 2ip to inducing shoot proliferation from the explants Addition of IAA at 0 1 ppm along with 1 0 ppm 2ip resulted in production of single shoots of average length 0 5 cm in 60 per cent of the cultures Higher levels of IAA did not produce any shoots With an increase in 2ip concentration to 2 0 ppm, IAA at the lowest level tried (0 1 ppm) could result in multiple shoot production Average number of shoots per culture in this combination was 1 2 and the average shoot length 0 75 cm

Table 16 Effect of 2ip with IAA combinations on the axillary bud cultures of *Pterocarpus marsupium* in WPM media

Concentration of 2ip (ppm)	Auxin	Concentration(ppm)	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av. no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
1 0			0	0	0	0	0
2 0			0	0	0	0	0
3 0			80	0	1 0	0	0 50
1 0	IAA	0 1	60	0	1.0	0	0 50
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
2 0		0 1	73	7	1 2	0	0 75
		0 5	0	0	0	0	0
		1 0	0	0	0	0	0
3 0		0 1	75	0	1 0	0	0 50
		0 5	20	0	1.0	0	0 25
		1 0	0	0	0	0	0

Higher concentrations of IAA at this concentration of 2ip also failed to induce shoot production The cytokinin 2ip at 3 0 ppm with 0 1 ppm of IAA resulted in 75 per cent of the cultures producing single shoots of average length, 0 5 cm Further increase in IAA (0 5 ppm) concentration resulted in a decrease in both the number of responding cultures (20 %)and average shoot length (0 25 cm) Still higher concentration of the auxin prevented shoot emergence totally

4.3.1 4 Effect of abscissic acid.

The plant growth regulator ABA added at concentrations of 0 1 , 0 25, 0 5, 0 75 and 1 0 ppm in WPM media supplemented with 2.0 ppm kinetin and 0 1 ppm IAA suppressed shoot production completely from the explants

4.3.1.5. Effect of gibberelic acid

The influence of GA_3 for enhanced release of axillary buds in bijasal were carried out in WPM media supplemented with 2 0 ppm kinetin and 0 1 ppm IAA When 1 0 ppm of GA_3 was incorporated in the basal formulation 59 per cent of the cultures produced single shoots of size 0 25 cm Increased presence of gibberelic acid to 2 0 ppm and 3 00 ppm completely suppressed shoot proliferation from axillary bud explants

4.3.2. Other supplements

Data on the trials conducted to evaluate supplements other than plant growth regulators in WPM and MS are given in Table 17 and Table 18, respectively

4.3 2.1. Silver nitrate

Influence of silver nitrate as a media supplement was studied in WPM containing 2 0 ppm kinetin and 0 1 ppm IAA Data recorded observations presented in Table 17 Silver nitrate at 5 ppm added to this media resulted in 53 per cent of the cultures producing single shoots of average size 0 75 cm At 10 ppm concentration of silver nitrate, number of responding cultures increased (89 %) while the average shoot size decreased (0 65 cm) At the highest concentration of this supplement (20 ppm) tried in WPM both number of cultures showing shoot initiation and the average shoot length recorded a reduction

When $AgNO_3$ incorporated at a level of 5 ppm in media MS, 90 per cent of the cultures were seen to produce single shoots of average size 0 25 cm (Table 18) Increased amount of silver nitrate lead to an increase in average shoot size to 0 5 cm, while only a marginal increase was observed with respect to the number of cultures producing shoot initial (93 %) Silver nitrates when supplemented at 15 ppm in MS lead to a marked decrease in number of cultures with shoot initials along with no change in the average shoot length Still higher concentration of the chemical lead to a further decrease in the number of cultures

Table 17 Effect of media suppliments on the axillary bud cultures of *Pterocarpus marsupium* in WPM media containing 2 0 ppm kinetin and 0 1 ppm IAA

Treatments	Concentration	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no shoots/ explant	% of cultures with leaf expansion	av.shoot length (cm)
Silver nitrate	5 ppm	53	0	1 0	0	0 75
	10 ppm	89	0	1.0	0	0 65
	20 ppm	20	0	1 0	0	0.25
Copper sulphate	5 ppm	85	14	1 3	0	0 25
	10 ppm	82	25	1 4	0	0 25
	20 ppm	32	0	1 0	0	0 25
Adenine sulphate	20 ppm	52	0	1.0	0	0 50
	40 ppm	61	20	1.2	0	0 25
	60 ppm	56	18	1 2	0	0 25
Casein hydrolysate	200 ppm	62	0	1 0	0	0 50
	300 ppm	57	0	1 0	0	0 50
	500 ppm	58	0	1.0	0	0.50
Cycocel	0 10 ppm	50	0	1 0	0	0 25
	0 50 ppm	92	0	1 0	0	0 25
	0 75 ppm	88	0	1 0	80	0 25
	1 00 ppm	90	0	1 0	78	0 25
	2 00 ppm	88	0	1 0	78	0 25
	3 00 ppm	95	0	1 0	80	0 25
Activated charcoal	1 % (w/v)	91	0	1 0	0	0 25
	2 % (w/v)	45	0	1 0	0	0 25
	3 % (w/v)	0	0	0	0	0
	4 % (w/v)	0	0	0	0	0

Table 18 Effect of media suppliments on the culture establishment from the primary nodal segments of Pterocarpus marsupium in MS media

Treatments	Concentration.	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
Silver nitrate[a]	5 ppm	90	0	1 0	0	0 25
	10 ppm	93	0	1 0	0	0 50
	15 ppm	54	0	1 0	0	0 50
	20 ppm	48	0	1 0	0	0 25
Copper sulphate[b]	5 ppm	83	0	1 0	0	0 25
	10 ppm	85	0	1 0	0	0 25
	15 ppm	43	0	1 0	0	0 25
Cobalt chloride[b]	1 ppm	16	8	1 2	0	0 50
	2 ppm	0	0	0	0	0
	3 ppm	0	0	0	0	0
Adenine sulphate[b]	10 ppm	56	0	1 0	0	0 25
	20 ppm	54	0	1 0	0	0 25
	30 ppm	48	0	1 0	0	0 25
	40 ppm	50	0	1 0	0	0 25
Coconut water[c]	10 % (v/v)	78	0	1 0	0	0 25
	20 % (v/v)	72	0	1 0	0	0 25
	30 % (v/v)	74	0	1 0	0	0 25

a - MS + 2 0 ppm BA + 0 1 ppm IAA
b - MS + 2 0 ppm BA + 0 1 ppm NAA
c - MS + 2 0 ppm BA

showing shoot proliferation (43 %) and the average shoot length (0 25 cm)

4.3.2.2 Copper sulphate

Effect of copper sulphate in culture establishment was studied in both MS and WPM. The data generated in experiments involving copper sulphate added to WPM supplemented with 2 0 ppm kinetin and 0.1 ppm IAA are presented in Table 17 With 5 ppm of copper sulphate added to the basal formulation, 85 per cent of the cultures produced shoots at a rate of 1 3 shoots of size 0 25 cm per culture Enhanced amount of copper sulphate (10 ppm) in the medium resulted in 82 per cent of the cultures producing shoots at a rate of 1 4 shoots per culture Average shoot length remained the same in this treatment compared to the former. Twenty ppm of the chemical in the medium brought about a reduction in number of cultures producing shoots to 32 per cent. In addition all the cultures initiated single shoots which did not vary in size compared to the former treatments.

In MS supplemented with 2 0 ppm BA and 0.1 ppm NAA, 5 ppm of copper sulphate resulted in 83 per cent of the cultures producing single shoots of average size 0 25 cm (Table 18) Increased amount of copper sulphate to 10 ppm did not change either the number of shoots per culture or the average shoot length The change in number of cultures producing shoots too was only marginal (85 %) With further increase in copper sulphate content in the media to 15 ppm reduction in number of cultures

showing shoot initiation was observed (43 %), while all the other observations were same compared to the former treatments

4.3.2.3. Cobalt chloride.

Influence of cobalt chloride in *in vitro* culture of bijasal was studied in MS supplemented with 2 0 ppm BA and 0 1 ppm NAA (Table 18) When 1 0 ppm of cobalt chloride was incorporated in the basal media 16 per cent of the cultures produced shoots at a rate of 1 2 shoots of average size 0 5 cm per culture Increased amount of the chemicals (2 0 and 3 0 ppm) in the media suppressed shoot initial formation completely

4.3.2.4 Adenine sulphate.

Adenine sulphate at 20 ppm added to WPM supplemented with 2 0 ppm kinetin and 0 1 ppm IAA resulted in 52 per cent of the cultures producing single shoots of length 0 5 cm (Table 17) Increased presence of adenine sulphate (40 ppm) in the same basal combination lead to an increase in both number of cultures producing shoots (61 %) and the average number of shoots per culture (1 2) Increase in the level of adenine sulphate, however, resulted in a decreased shoot length to 0 25 cm Further increase in adenine sulphate level to 60 ppm did not change either the number of shoots per culture or their average length.

All levels of adenine sulphate (10 ppm, 20 ppm, 30 ppm and 40 ppm) added in MS supplemented with 2 0 ppm BA and 0 1 ppm NAA exhibited shoot morphogenesis from the explants (Table 18)

There was no marked difference in the per cent response of the cultures. It ranged from 48 per cent to 56 per cent for 30 ppm and 10 ppm of adenine sulphate, respectively In all combinations, the number of shoots per culture as well as the average shoot length remained the same

4.3.2.5 Coconut water.

Effect of coconut water on culture establishment was studied in MS supplemented with 2 0 ppm BA The data generated are presented in Table 18 When 10 per cent (v/v) of coconut water was incorporated in the medium 78 per cent of the cultures produced single shoots of average length 0 25 cm Increasing the amount of coconut water to 20 and 30 per cent did not bring about any substantial change from the first treatment

4.3.2.6. Casein hydrolysate.

Effect of adding casein hydrolysate in culture establishment was studied in WPM supplemented with 2 0 ppm kinetin and 0 1 ppm IAA As shown in Table 17, with 200 ppm casein hydrolysate 62 per cent of the cultures produced single shoots of average size 0 5 cm Further increase in the dose of the chemical to 300 ppm and 500 ppm showed only slight difference in the characters studied compared to the former

4.3.2.8 Cycocel.

Cycocel was tried at six different levels in WPM supplemented with 2 0 ppm kinetin and 0 1 ppm IAA Data generated is presented in Table 17 At the lowest level of the

chemical tried (0 1 ppm), half of the cultures produced single shoots with an average shoot length of 0 25 cm Increased presence of CCC in the same basal formulation increased the number of cultures producing shoots to 92 per cent, but the average shoot length remained the same Further increase in CCC (0 75 ppm) resulted in 88 per cent of the cultures producing single shoots, out of which 80 per cent had leaves Average shoot length did not vary from the above treatments here too Further increase in cycocel content in the medium to 1, 2 and 3 0 ppm did not bring about any appreciable difference in any of the parameters studied The most important observation made in this experiment is that cycocel at concentration from 0 75 ppm to 3 0 ppm could induce leaf morphogenesis in about 80 per cent of the shoots produced in culture

4.3.2.8. Activated charcoal.

Effect of activated charcoal supplemented to WPM was studied and data presented in Table 17 Activated charcoal at 1 per cent (W/V) incorporated to WPM containing 2.0 ppm kinetin and 0 1 ppm IAA resulted in 91 per cent of the cultures producing single shoots of average size 0 25 cm Increased presence of charcoal (2 %) in the medium resulted in decreased number of responding cultures Further increase in charcoal to 3 per cent and 4 per cent completely suppressed shoot production

4 3 3 Carbon source.

Data on the trials conducted to evaluate the effect of various carbon sources are given in Table 19 Sucrose at a strength of one per cent (W/V) when added to WPM supplemented with 2 0 ppm BA and 1 0 ppm IAA, induced shoot proliferation in 20 per cent of the cultures The average length of shoot was only 0 25 cm Doubling the sucrose level to two per cent increased the number of cultures showing shoot initials (44 %), and rate of shoot production per culture (1 2), the mean shoot length (0 25 cm) remaining the same One per cent of maltose added to two per cent sucrose increased the number of cultures producing shoots to 60 per cent and mean shoot length to 0 40 cm while decreasing the rate of shoot production (1 1) Three per cent sucrose resulted in 40 per cent of the cultures producing shoots maintaining the same number of shoots per culture but a lower shoot length (0 25 cm). When 1 per cent of maltose was added to the same combination the number of shoots per culture and number of cultures producing shoots improved slightly (1 2 and 45 % respectively)

4.4 Standardisation of physical conditions

4.4 1. Physical state of the medium.

Data on the trials on the influence of physical state of the medium conducted in MS supplemented with 2 0 ppm BA and 0 1 ppm IBA is given Table 20 Liquid cultures recorded single shoot initial formation in 60 per cent of the cultures The mean shoot length was 1 0 cm When agar was incorporated in the medium at

Table 19 Effect of carbon source on axillary bud culture of *Pterocarpus marsupium* in WPM containing 2 0 ppm BA and 1 0 ppm IAA

Treatments	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no. shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
1 % (W/V) Sucrose	20	0	1 0	0	0 25
2 % (W/V) Sucrose	44	9	1 2	0	0 25
2 % (W/V) Sucrose+ 1 % (W/V) Maltose	60	6	1 1	0	0 40
3 % (W/V) Sucrose	40	5	1.1	0	0 25
3 % (W/V) Sucrose+ 1 % (W/V) Maltose	45	8	1 2	0	0 25

Table 20 Effect of Physical condition of media on axillary bud cultures of *Pterocarpus marsupium* in WPM containing 2.0 ppm BA and 1.0 ppm IAA

Treatments	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
Liquid medium	60	0	1.0	0	1.00
Semi solid medium with					
Agar 0.6 %	86	68	2.5	0	1.25
Agar 0.7 %	89	72	2.5	0	1.25
Agar 0.8 %	98	80	2.6	0	1.50

a strength of 0 6 per cent, there was an increase in number of cultures producing shoots (86%) average shoots per culture (2 5) and average shoot length (1 25 cm) Agar at 0 7 per cent did not produce any marked change in number of cultures producing shoots average shoot length or the average number of shoots per culture. Highest level of agar tried, however, gave the best results with 98 per cent of the cultures producing shoots at a rate of 2 6 shoots per explant having a mean length of 1 5 cm

4.4.2. pH of the medium.

Observations recorded on the influence of pH of the medium MS supplemented with 0 1 ppm IAA and 1 0 ppm BA on shoot proliferation from the axillary buds of bijasal are presented in Table 21. When the pH was adjusted to 5 0, 40 per cent of the cultures gave single shoots of average size 0 5 cm At 5 5 pH, number of cultures inducing shoots marginally improved (46 %) while the average shoot size remained the same . Higher pH (6 0) lead to a decreased mean shoot length (0 2 cm) while the number of cultures producing shoots was negatively affected to a marginal extent (44 %)

4.4.3. Light.

Investigations on influence of light were carried out in MS supplemented with 2 0 ppm BA and 0 5 ppm NAA The data is presented in Table 22 When the cultures were kept in total dark conditions, only 12 per cent of them showed shoot initiation with single shoots of mean length 0 25 cm In the presence of

Table 21 Effect of pH on axillary bud cultures of Pterocarpus marsupium

pH	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
5 0	40	0	1 0	0	0 50
5 5	46	0	1 0	0	0 50
6 0	44	0	1 0	0	0 20

Basal medium MS + 1 0 ppm BA + 0 1 ppm IBA

Table 22 Effect of light intensity on axillary bud cultures of Pterocarpus marsupium

Treatments	% of cultures showing shoot proliferation	% of cultures with multiple shoot	av no shoots/ explant	% of cultures with leaf expansion	av shoot length (cm)
Dark	12	0	1	0	0 25
500 lux	56	0	1	0	0 25
1000 lux	76	20	1 2	0	1 25
2000 lux	98	80	2 6	0	1 50
Red light	56	0	1	0	0 50
Sun light	30	0	1	0	0 50

Basal medium MS+ 2 0 ppm BA + 0 5 ppm NAA

500 lux light there was marked improvement in the number of cultures producing shoots to 58 per cent, while the number of shoots per culture and the average shoot size recorded no change 1000 lux of light intensity induced shoot production in 76 per cent of the cultures, at a rate of 1 2 shoots of mean length 2 6 cm per culture Still higher intensity of light (2000 lux) induced shoot production in 98 per cent of the cultures Cultures showing multiple shoot also increased drastically to 80 per cent Red light lead to the production of single shoots in 56 per cent of the cultures, and the mean length per shoot was 0 5 cm Sun light also was found to be not very favorable for shoot induction and growth When the cultures were incubated in bright sun light, only 30 per cent of the cultures produced shoots with mean length 0 5 cm None of the cultures produced multiple shoots

4.5 Standardisation of basal media and supplements for continuous culture.

The effect of various media and supplements on the production of continuous cultures are presented in Table 23 In general it was noticed that 21 per cent of cultures on an average showed latent bacterial contamination whenever continuous cultures were made Among the various treatments tried in WPM media, the extent of cultures dead varied from 100 per cent (2 0 ppm kinetin + 0 5 ppm IBA 1 0 ppm BA + 0 1 ppm IAA 1 0 ppm BA + 1 0 ppm NAA 2 0 ppm BA+ 1 0 ppm NAA 2 ppm kinetin + 200 ppm phloroglucinol 3 0 ppm kinetin + 300 ppm phloroglucinol) to 21 per cent (2 0 ppm kinetin + 0 1 ppm IAA + 500 ppm glutamin

Table 23 Effect of various media and additives on continous culture of *Pterocarpus marsupium*

Media	Additives	--% of cultures-- Dead	living
WPM	2 0 ppm kinetin + 0 5 ppm IBA	100	0
	3 0 ppm kinetin + 0 5 ppm IBA	87	13
	2 0 ppm kinetin + 0 1 ppm IAA	49	51
	2 0 ppm kinetin + 0.1 ppm IAA + 500 ppm glutamin	21	79
	2 0 ppm kinetin + 0 1 ppm IAA + 1000 ppm glutamin	21	79
	2.0 ppm kinetin + 0 5 ppm IAA + 10 ppm adenine sulphate	55	45
	2.0 ppm kinetin + 0 5 ppm IAA	83	17
	2 0 ppm kinetin + 0 1 ppm IAA	75	25
	2 0 ppm kinetin + 0 1 ppm IAA + 10 ppm $AgNO_3$	40	60
	2 0 ppm kinetin + 0.1 ppm IAA + 40 ppm $AgNO_3$	60	40
	2 0 ppm kinetin + 0 5 ppm IAA + 40 ppm $AgNO_3$	84	16
	2 0 ppm kinetin + 0 5 ppm IAA + 100 ppm adenine sulphate	85	15
	2.0 ppm kinetin + 0 1 ppm IAA + 1000 ppm casein hydrolysate	59	41
	2 0 ppm kinetin + 0 1 ppm IAA + 500 ppm casein hydrolysate + 10 $AgNO_3$	89	11
	2 0 ppm kinetin + 0 5 ppm IAA + 80 ppm glutamic acid	73	27
	1 0 ppm kinetin + 10 ppm $AgNO_3$ + 20 ppm glutamic acid	64	36
	1 0 ppm kinetin + 40 ppm gutamic acid	74	26
	1 0 ppm kinetin + 60 ppm glutamic acid	80	20
	1 0 ppm kinetin + 100 ppm glutamic acid	73	27
	1 0 ppm kinetin + 200 ppm glutamic acid	60	40
	2 0 ppm kinetin + 3 % sucrose	59	41
	2 0 ppm kinetin + 50 ppm phloroglucinol	57	43
	2 0 ppm kinetin + 200 ppm phloroglucinol	100	0
	2 0 ppm kinetin + 250 ppm phloroglucinol	100	0
	2 0 ppm kinetin + 300 ppm phloroglucinol	100	0
	1 0 ppm 2ip	68	32
	2 0 ppm 2ip	77	23
	3 0 ppm 2ip	88	12
	1 0 ppm BA + 0 1 ppm IAA	100	0
	2 0 ppm BA	45	55
	1 0 ppm BA	34	66
	2 0 ppm BA + 0.1 ppm IAA	88	12
	1.0 ppm BA + 1 0 ppm NAA	100	0
	2 0 ppm BA + 1 0 ppm NAA	100	0

----contd---

Table 23 (Contd)

MS	2 0 ppm BA + 0 1 IAA	38	63
	2 0 ppm BA + 0 5 IAA	45	55
	1 0 ppm BA + 0 5 IAA	40	60
	3 0 ppm BA + 0 5 IAA	61	39
	2 0 ppm BA + 0 5 IAA + 10 $AgNO_3$	54	46
	2 0 ppm BA + 0 5 IAA + 20 $AgNO_3$	62	38
	2 0 ppm BA + 0 5 IAA + 15 $AgNO_3$	53	47
	2 0 ppm kinetin + 0 5 IAA	83	17
	2 0 ppm kinetin + 0 1 IAA	69	31
1/2 MS	2 % maltose + 1 0 ppm BA	77	23
	2 % maltose + 2 0 ppm BA	80	20
	2 % maltose + 3 0 ppm BA	85	15
	2 % maltose + 1 0 ppm BA + 0 1 ppm NAA	89	11
	0 5 ppm BA + 5 ppm adenine sulphate	92	8
	0 5 ppm BA + 10 ppm adenine sulphate	89	11
	0 5 ppm BA + 15 ppm adenine sulphate	80	20
	0 5 ppm BA + 20 ppm adenine sulphate	58	42
	1 0 ppm BA + 0 5 ppm NAA	73	28
	1 0 ppm BA	58	42
	2 0 ppm BA	80	20
	3 0 ppm BA	82	18
	4 0 ppm BA	90	10
	1 0 ppm kinetin	64	36
	1 5 ppm kinetin	71	29
	2 0 ppm kinetin	76	24
	1 0 ppm kinetin +10 ppm adenine sulphate	54	46
	1 0 ppm 2-ip	68	32
1/4 MS	1 ppm BA + 500 ppm Glutamine	100	0
	3 ppm BA + 500 ppm Glutamine	31	69
	4 ppm BA + 500 ppm Glutamine	32	68
	3 ppm BA + 5 ppm Adenine	100	0
	3 ppm BA + 10 ppm Adenine	62	38
	3 ppm BA + 20 ppm Adenine	77	23
	3 ppm BA + 20 ppm Adenine	77	23
	3 ppm BA	100	0
Hellers	2 ppm kinetin	100	0
	2 ppm kinetin + 0 5 ppm IAA	53	47
	2 ppm BA + 0.5 ppm IAA	66	34
	2 ppm 2-ip + 0 5 ppm IAA	53	47
	2 ppm 2-ip	100	0

2 ppm kinetin + 0 1 ppm IAA + 100 ppm glutamin) Among the combinations involving additives in MS, the highest number of cultures dead was noticed in the combination involving 2 0 ppm kinetin and 0 5 ppm IAA (83 %) The lowest number of dead cultures after three weeks were in the combination having 2 0 ppm BA and 0 1 ppm IAA (38 %) All the combinations of media supplements in 1/2 MS resulted in more than half the cultures dead in three weeks time The maximum dead culture occurred when 2 0 ppm BA was added to the medium (90 %), where as the lowest was observed in a combination involving 1 0 ppm kinetin and 10 ppm adenine sulphate (54 %) In 1/4 MS per cent of growing cultures varied form 0 % (1 0 ppm BA + 500 ppm glutamin, 3 0 ppm BA + 10 ppm adenine, 3 0 ppm BA) to 69 per cent (3 0 ppm BA + 500 glutamin) three weeks after culturing Among the five combinations involving growth regulators in Hellers medium death occurred in all the cultures when kinetin and 2ip were added alone at 2 0 ppm concentration Lowest extent of death was observed in media having 2 0 ppm kinetin + 0 5 ppm IAA and 2 0 ppm 2ip + 0 5 ppm IAA

All the cultures that were living did not show any appreciable amount of growth except that they were remaining green Subsequently all the cultures were found to degenerate further with time

- *DISCUSSION*

DISCUSSION

Availability of sufficient quantity of planting material of superior genotypes is a pre requisite for any extensive planting programme to be successful Production of clones through vegetative propagation of selected plus' trees is the simplest, most economical and least time consuming method of tree improvement In addition, vegetatively propagated plants exhibit lesser period for maturation (Winton and Huhtinen, 1976). Conventional vegetative propagation methods are often not possible in most of the tree species and where feasible, the number of seedlings that can be produced will be quite insufficient to meet the requirement of hundreds of thousands of planting material

Bijasal (Pterocarpus marsupium) is no exception to the above problems and micro propagation through in vitro techniques is one of the possibilities of overcoming these This technique offers the possibility of producing large number of seedlings of known superior genotypes with uniform quality In the present study, an attempt was made to standardise the protocol for in vitro propagation of bijasal The results obtained are discussed below

5.1. Explant

Among the various characteristics associated with the technique of micropropagation, the frequency of culture survival as well as development into a reculturable structure have been directly related to the initial explant size (Murashige, 1974)

As a general rule, larger the size of the explant, greater are the chances of survival (Hussey, 1983). Smaller explant results in a larger surface : volume ratio resulting in adversely affecting explant survival. Flynn et al (1990) experimenting on cocoa has emphasised this point However, the probability of getting a clean tissue, free from microbial contaminants, is inversely related to explant size (Murashige, 1974) In the present experiment explants of size 0 5 to 1 5 cm were found to be ideal, as smaller or larger explants did not give results as good as this (Table 6) Smaller explants often developed calli around the them while larger explants had higher chances of contamination

As the trees used for collecting the explants were remaining in the open field, chances of them harboring microbial contaminants were quite obvious This necessitated a thorough and effective surface sterilisation of the explants before culturing Doublin (1984) observed that when the explants were collected from the field the percentage of infection was over 90, regardless of the procedure used for explant sterilisation An initial treatment of the mother trees in the pre-culture stage (stage 0) with fungicidal spray was recommended by Legrand and Mississo (1986) to reduce culture contamination In the present study, a prophylactic spray with a combination of fungicides, Bavistin -50 percent WP (Carbendazim) and Indofil M-45 (Mancozeb) 0 3 per cent, given to the mother plants could control contamination to a considerable extent (Table 10) Mahato (1992)

has earlier reported that the same combination of fungicides brought about a remarkable reduction in the contamination rate in Dalbergia latifolia cultures For surface sterilisation of the explants mercuric chloride (0 1 %) for 10 min followed by three rinses in sterile distilled water was found to be ideal (Table 7) Increased duration with this sterilant resulted in increased explant mortality and lesser duration resulted in higher culture contamination Although chlorine water (3.15 $\overset{m}{\wedge}g\ l^{-1}$) treatments at the longest duration tried (15 min) could control contamination only slightly, chances of it being an effective sterilant at higher duration is not ruled out However, tissue damage and cell death can be the probable handicap of such a long duration treatment Hydrogen peroxide (15 %) even at the longest duration tried failed to control culture contamination completely Mercuric chloride as an effective sterilant for surface sterilisation is well documented (George and Sherrington, 1984 ; Mahato, 1992) A seasonal influence was also noticed in culture contamination with the peak contamination occurring during the rainy months (June, July August, October and November) and the least during dry spells (March and April) Contamination rate even in cultures using explants taken from the pretreated mother plants followed a similar pattern Seasonal influence on culture contamination was evident in rose wood cultures also (Mahato, 1992)

The season in which explants are obtained can influence the regenerative characteristics of the tissue (Murashige, 1974) Seasonal influence on the physiological state of the plant and

its effect on culture establishment has been reported by several workers (Borrod, 1971 , Seabrook *et al* , 1976) In *Pterocarpus marsupium* the season between January and August was found to be ideal for culture establishment, and over 62 per cent of the cultures showed bud break during this season as against only 55 per cent in November (Table 10) Almost the same season was ideal for *Dalbergia latifolia* too (Mahato, 1992)

5.2. Bud break and shoot proliferation.

Most plants show a considerable difference in quality of growth when cultured in different basal formulations of inorganic salts An attempt to find out the effect of different concentrations of inorganic salts on shoot proliferation from axillary buds of *P. marsupium* was made Considerable differences were evident between various basal media combinations (Table 11) The media MS(a) which differed from MS principally with regard to a lower amount of ammonium and higher amount of nitrate, potassium and sulpher gave the best performance Moreover, this was the only medium which produced leaves in at least some of the cultures Ammonium ion is toxic to plant cells in higher amounts and cannot be accumulated in cytoplasm (Kirby and Cheng, 1979) Gamborg *et al* (1968) observed that ammonium ions above 2mM affected soybean cultures deliteriously and when $(NH_4)_2SO_4$ was used instead of NH_4No_3 and the concentration of ammonium ions reduced to one third of that of MS, soybean cells grew better (Evans *et al* , 1981) Thus the lower amount of ammonium ions might have played a role in making medium MS(a) more favorable for shoot proliferation of *P. marsupium*

An axillary bud is a predetermined organ with great potential to give rise to a shoot in the absence of apical dominance The basic phenomenon involved in the induction of bud break and subsequent plantlet production *in vitro* is reported to be the action of plant hormones. Though little is known how a hormone evoke a particular response (Thorpe, 1980), the favorable effects on apical bud bursting and shoot production by cytokinins had been demonstrated by Murashige (1974) Kinetin is a common natural cytokinin widely used in plant tissue culture works (Sita *et al* , 1980 and Mascarenhas *et al* , 1982) In the present study kinetin alone added to the media MS and WPM at strengths ranging from 1 0 to 3 0 ppm did not result in shoot formation from the axillary buds of bijasal (Tables 12 and 13)

Auxin added to the medium has already been reported to nullify the suppressive effect of high cytokinin content on axillary shoot growth (Lundergan and Janick, 1980) Combinations of kinetin with different auxins could induce bud break In WPM, IAA was a better auxin along with kinetin than both NAA and IBA in inducing bud break While IBA and NAA had only a very moderate effect in shoot proliferation, IAA at lower concentrations exhibited a remarkable effect on bud break and growth. Kinetin at 2 0 ppm along with 0 1 ppm of IAA was found to be the best combination with about 96 per cent of the cultures showing shoot proliferation. Multiple shoot formation with an average of 3 shoots per culture and a range of 2-5 shoots per culture was observed in this combination (Table 12). In all these combinations, however, leaf expansion was either totally

absent or leaf drop after a few days in culture was prevalent This medium was ideal for *in vitro* culturing of bijasal except for this reason Further experiments were conducted with a view to solve this problem and to increase the number of multiple shoots per culture , if possible Combination of IBA with kinetin failed to produce shoot formation from axillary buds except 3 0 ppm kinetin with 0 1 ppm IBA Among the nine combinations that involved kinetin and NAA, three resulted in shoot formation Supplementing of kinetin with auxin IAA in MS medium could induce bud break only in three combinations among the nine tried Single shoots that resulted from these combinations were all less than 1 0 cm in size Further more these combinations of kinetin and IAA in MS did not result in any additional gain as they neither produced leaves nor induced increased multiple shoot formation from the primary nodal explants of bijasal

Unlike kinetin, BA when supplemented in both MS and WPM could induce bud break in *P. marsupium* (Tables 14 and 15) In WPM best results were obtained by using 1 0 ppm of BA Increased concentration brought down the number of responding cultures and shoot length However, increase in BA concentration lead to an increased number of shoots per culture and average shoot length in MS medium The number of responding cultures decreased here too Among the various cytokinins used in plant tissue culture, BA is the cheapest and one of the most effective (Aboel-nil 1987; Jang *et al* , 1988; Scott *et al* 1988; Rai and Chandra 1989, Vijaykumar *et al* 1990) In the present investigation also cytokinin BA was found to be effective in inducing

organogenesis in bijasal while kinetin in various doses tried were completely ineffective It has already been established that apart from the quantity present the effect of cytokinin may vary according to the compound used (George and Sherrington 1984)

In an effort to find out the effect of different auxins along with BA it was found that all combinations of BA with IBA in WPM except the one involving lower levels of BA (1 0 and 2 0 ppm) with high concentration of IBA (1 0 ppm) yielded multiple shoots Shoot length between treatments varied and the maximum was noticed when 1 0 ppm of BA was combined with 0 1 ppm IBA Combinations of NAA with BA in WPM were not very effective in inducing bud break in bijasal Presence of IAA with BA in MS medium enhanced organogenetic potential of the medium Maximum number of shoots were obtained when 3 0 ppm of BA was combined with 0 1 or 0 5 ppm of IAA, with the former yielding shoots of larger size In MS IBA also acted synergistically to bring about an increased rate of differentiation and growth in axillary buds of P. marsupium Maximum number of shoots were obtained when 3 0 ppm of BA was combined with 0 5 ppm of IBA while largest shoots were obtained when 1 0 ppm of BA was combined with 0 1 ppm IBA and when 2 0 ppm BA was used with 0 5 ppm IBA Auxin NAA at lower concentrations (0.1 and 0 5 ppm) with BA at 1 0 ppm gave the largest number of shoots per explant among all the treatments tried in MS medium However, largest shoots among BA and NAA combinations in MS was obtained when 2 0 ppm of BA was used along with 0 1 ppm of NAA All combinations of BA with 2,4-D produced

single shoots that did not vary much with regard to the average shoot size too In Dalbergia latifolia it was observed that presence of auxins inhibited BA's capacity for inducing morphogenesis (Mahato 1992) However, in the present study it was observed that auxins in general except 2 4-D enhanced the effect of BA in inducing bud break from axillary bud cultures Among the combinations of plant growth regulators tried BA at 1 0 ppm along with NAA at 0 1 ppm was the best in terms of number of shoots per culture in MS medium These combinations involving the cytokinin BA too failed to produce either leaves in the shoots or improve the number of shoots per culture Higher levels of cytokinin has been proved to have deleterious effect on shoot growth

The naturally occurring cytokinin, 2ip has been reported to be more effective than BA or kinetin in a number of species like Rhododendron (Anderson, 1975) blue berry (Cohen 1980) and garlic (Bhojwani 1980a) This cytokinin was not very affective in inducing multiple shoot formation in P. marsupium (Table 16) When used alone it could induce bud break only at a high concentration (3 0 ppm) Efficiency of 2ip in inducing bud break could be enhanced by supplementing the cytokinin with an auxin in the media Best results were obtained when 2ip at 2 0 ppm was combined with 0 1 ppm of IAA There are earlier reports of 2i being not very effective in inducing bud break in hybrid willow (Bhojwani, 1980b) and white clover (Bhojwani 1981) Situation with regard to leaf production too did not improve with these treatments

Many aspects of cellular differentiation and organogenesis from tissues have been found to be controlled by an interaction between cytokinin and auxin concentrations (Skoog and Miller, 1957) A higher cytokinin: auxin ratio promotes shoot formation while the reverse induces rhizogenesis The interaction of cytokinin and auxin, however is more complex and more than one combination of these two substances are likely to promote optimum results as evident from the present study Evidences of different kinds of chemicals of the same class of hormones, evoking different response is well documented Vietez (1980) observed that axillary bud proliferation could be induced by BA in chestnut while kinetin was without any effect Both kinetin and BA were effective in inducing bud break in P. marsupium nodal segments, kinetin performed better in WPM medium while BA was equally effective in both WPM and MS media Although was effective, kinetin gave only lesser amount of growth and differentiation in Dalbergia latifolia cultures (Mahato 1992) From the present study 2ip could be rated only next to both BA and kinetin with regard to its potential for inducing bud break in bijasal None of these combinations involving cytokinins and auxins could improve the problem of lack of leaf expansion in culture

Plant tissue cultures can generally be induced to grow and differenciate without gibberellins When GA_3 is added it often produces the effects which are similar to that of auxins (Stuart and Street, 1973) In the present study an inhibitory effect of GA_3 on morphogenesis is evident as higher concentration of the

compound completely suppressed shoot formation from the axillary buds of bijasal Inhibitory effects of GA in combination with auxins and cytokinins on concentration which would otherwise promote morphogenesis were reported by Thorpe and Meier (1973) It is presumed that the enhancement of endogenous auxin level in the explant in the presence of GA_3 might have suppressed the shoot proliferation potentials of the media This growth regulator too did not have any effect on leaf production in culture

Abscisic acid (ABA) is another naturally occurring growth regulator that is synthesised in the plastids (Milborrow 1974) It is generally recognised as a plant growth inhibitor as its effect is frequently contrary to that of other growth promoting regulants such as auxins cytokinins and gibberelic acid Abscisic acid has been observed to inhibit morphogenesis in a number of plants (Heide, 1974) In the present study it was observed that ABA completely blocked morphogenesis from axillary buds of P. marsupium in media where multiple shoot formation was noticed in its absence

Ethylene gas is usually produced in considerable quantities in culture vessels by the explants This gas classed as a separate group of growth regulator, often modifies the morphogenetic potential of cells in culture Ethylene can lead to lack of leaf expansion and their abscission in culture (Biddington 1992) The possible role of this growth regulator in inhibiting leaf production in culture was investigated by

suppressing its action through the addition of ethylene inhibitors in media

Silver ion is a potent inhibitor of ethylene action (Beyer, 1976) and its compounds are added to the tissue culture media to prevent the accumulation of ethylene in culture vessels Addition of silver nitrate has often resulted in enhanced shoot morphogenesis and growth in cultures in a number of species (Purnmauser et al 1987, Songstad et al , 1980; Vasil et al , 1989) Coleman et al (1980) found that silver nitrate could enhance rooting in tomato Although silver ions inhibit the action of ethylene it does not interfere with the ethylene biosynthesis (Liberman 1979) Several bits of evidences show that when silver compounds are added, ethylene binds to the metal containing receptor sites, thus forming a complex (Beyer, 1976) However it is well known that silver compounds at higher concentrations are toxic to plants In the present investigations it was found that silver nitrate did not influence the number of responding cultures (Table 17 and 18) At concentrations of 5 to 20 ppm tried in WPM silver nitrate suppressed multiple shoot production, which occured in its absence In MS too silver nitrate had a similar effect on culture However, the antiethylene properties of the compound did not improve the leaf morphogenesis and retention in the cultures Cultures generated in both WPM and MS supplemented with different levels of silver nitrate did not produce leaves Similar effect of silver nitrate deliteriously affecting cultures has been reported earlier (Vain et al , 1979, Pius et al 1993)

In the present study cobalt chloride added to the media inhibited shoot regeneration slightly at lower strengths and completely at higher strengths (Table 18) Cobalt chloride is a well known inhibitor of ethylene production Unlike silver nitrate, cobalt chloride acts through inhibiting ethylene biosynthesis Cobalt ions inhibit ethylene production by blocking the conversion of 1- aminocyclopropane-1-carbolic acid to ethylene (Yang and Hoffmann 1984) However from the literature it is evident that silver ions are more preferred to cobalt to this end The problem of lack of leaf expansion too could not be solved using cobalt chloride

Used in chemistry for its strong adsorptive properties both for dissolved solids and gases, activated charcoal has frequently improved plant tissue culture (George and Sherrington, 1984), by adsorption of growth inhibitors, prevention of unwanted callus growth and promotion of morphogenesis Ethylene gas in culture could be controlled by activated charcoal added to the media (Biddington, 1992) Kim and Lee (1988) reported that addition of 500 mg l^{-1} in half strength MS media containing BA at 0 5 ppm showed best shoot and root growth from axillary buds of ziziphus In the present study, activated charcoal (1 to 4 %, w/v) incorporated into WPM medium suppressed morphogenesis from the axillary buds of P. marsupium (Table 17) Moreover presence of activated charcoal could not solve the problem of lack of leaf morphogenesis in culture Activated charcoal exhibiting a negative influence on bud break was earlier reported by Mahato (1992) It is suggested that activated charcoal can exhibit an

inhibitory effect on morphogenesis probably due to the adsorption of growth promoting substances in the media (George and Sherrington 1984) Evidences of removal of plant growth regulators, vitamins, amino acids etc from the media have been recorded earlier too (Wang and Huang, 1976; Weatherhead et al , 1978)

Adenine, a nitrogenous base of DNA, when added to cultures could control leaf drop in P. santalinus (Patri et al , 1988) The possible growth regulatory effect of adenine and its more soluble form adenine sulphate has been exploited in tissue culture earlier also Possible role of adenine in enhancing apical dominance was suggested by Davis et al (1977) Addition of adenine at concentrations ranging from 1 0 to 8 0 ppm was found to be beneficial in Dalbergia latifolia (Mahato 1992) Synergistic effect of adenine on cytokinins has been suggested by Nitsch et al (1967) Adenine sulphate, however, performed better when alone than with cytokinin in Dalbergia latifolia (Mahato,1992) Adenine was helpful in promoting organogenesis in Pterocarpus santalinus along with a cytokinin (Patri et al 1988) Adenine sulphate in both MS and WPM resulted in a decreased number of shoots per culture and shoot length compared to the media lacking them (Tables 17 and 18) Leaf production was totally absent in all the combinations having adenine sulphate In this connection, Jarret et al (1980) observed the inhibitory role of adenine sulphate in shoot formation Nickerson (1978) observed that the enhancement of adenine in the medium prevented shoot desiccation

Coconut water contains a number of cell division factors and free amino acids (Shantz and Steward 1952) The favorable effect of coconut water in the promotion of growth and differentiation of excised tissues and organ have been attributed to the presence of cytokinins and gibberelic acid like substances in it (Straus and Rodney, 1960) Both shoot growth and multiple shoot formation in medium containing coconut water has been reported in a number of tree species (Nadgir et al , 1984; Mittal et al , 1989; Rai and Chandra, 1989) Coconut water did not have any beneficial effect on either shoot formation or on leaf production in P. marsupium as evident from the present study (Table 18) Coconut water in Dalbergia latifolia cultures produced callus at cut ends (Mahato, 1992)

Amino acid supplements in plant tissue culture media has been tried by several workers Casein hydrolysate is a complex mixture of several amino acids Murashige and Skoog (1962) observed that casein hydrolysate allowed vigorous organ development over a broad range of IAA and kinetin in MS media In Hevea brasiliensis casein hydrolysate tended to induce multiple shoot production in culture (Mascarenhas et al 1982) Beneficial effect on multiple shoot formation was evident in Dalbergia latifolia cultures too (Mahato, 1992) However in the present study casein hydrolysate had no beneficial effect as multiple shoot formation and growth of shoot in culture were lower than in the media with the same combination of growth regulators, but lacking casein hydrolysate (Table 17) The problem of lack of leaf expansion too could not be solved using

this amino acid mixture In an earlier report by George and Sherrington (1984) no beneficial influence of casein hydrolysate was noticed on cell growth of either monocots or dicots in culture

Cycocel (Chloromequat chloride/ CCC) is a choline derivative containing substituted chlorine Results of the investigations carried out to study the effect of this growth inhibitor showed that it negatively influenced bud break from axillary buds of bijasal However concentrations above 0 75 ppm of CCC in the medium resulted in leaf production in about 80 per cent of the cultures (Table 17) This compound is a well known inhibitor of GA biosynthesis, but not its action Usually CCC treated plants have shorter inter nodes and thicker, greener leaves (Davis and Curry, 1991). Cycocel has been reported to influence leaf morphogenesis and leaf anatomy including palliside cell length, spongy parenchyma layer thickness and mesophyll air space (Gausman, 1986) These observations together with the generally observed increase in chlorophyll concentration (Davis and Curry 1991) had possible implication in culture In addition to these, CCC may sometimes prolong the leaf retention by delaying leaf senescence (Davis and Curry, 1991) which would again be a factor that played a role in the axillary bud cultures of bijasal

Cells in culture being fully heterotrophic or partially autotrophic need a carbon energy source to grow Specific carbohydrate requirement may be related to the specific carbohydrate metabolism through which water relations and endogenous phytohormones are regulated This is an important

factor that can change the success of plant tissue culture by affecting morphogenesis and growth. The selection of sucrose as the carbon energy source in plant tissue culture was the result of comparisons among several alternatives In cardamom sucrose and glucose gave equal performance (Reghunath 1989) In Indian rose wood sucrose at 2 per cent (w/w) was found to be the best carbon energy source (Mahato, 1992) Varying effects were found in using different sugars as the carbon energy source in culture (Reghunath, 1989; Mahato, 1992) In the present study three per cent sucrose with one per cent maltose recorded highest number of responding cultures and largest shoot length (Table 19) However, none of the treatments could induce leaf production in culture Sucrose apart from supplying carbon energy, is the most important component contributing to the osmoticum of the medium Gamborg et al (1974) has suggested That the metabolic roles of sucrose may include its effect on efficiency of nitrate and ammonium ion in the medium and the effect of cytokinin on cell division

Physical condition of the medium plays a critical role in the success of plant tissue culture In an attempt to standardise the physical condition of the media it was found that agar at a strength of 0 8 per cent was the best with regard to all the parameters studied except leaf production which did not occur in any of the treatments (Table 18) In the study a distinct advantage of agar was noticed with cultures showing higher performance with increasing concentration of agar Bhojwani and Razdan (1983) have reported that almost all

commercially available sources of agar contained impurities especially Ca Mg and trace elements in varying amounts Presence of these impurities, which are growth promotory in agar could be a reason for the better performance in agar media Moreover, agar is reported to have adsorptive properties which have not been fully evaluated White (1943) thought that it might remove harmful cell metabolic products in the media Agar medium will be having a lower water potential than its liquid equivalent (Debergh and Maene, 1981) and this may be partially responsible for the higher morphogenic response noticed preferentially in agar medium

Hydrogen ion concentration of the growth medium often has a significant influence on growth and differentiation of cells in culture Plant cells in culture require an acidic pH and initial pH of the medium is usually set at 5 6-5 8 (Gamborg and Shyluk, 1981) For liquid cultures pH is set about 5 0 (Bonga, 1982a) However, differences in culture establishment, growth and morphogenesis were not significantly different in the range of pH 5 0-6 0 in this present study (Table 21) indicating that bijasal cultures can withstand a wider range of pH conditions than many species This range of pH had no influence on leaf expansion too Minocha (1982) remarked that growth rates need not be influenced by a wide range of initial pH ranging from 3 0-8 0, except for the extremes pH at the range of 5 0 to 5 8 did not influence Dalbergia latifolia cultures (Mahato, 1992)

Possible role of light affecting culture growth was investigated with an aim to identify if it could induce leaf formation in culture Light requirement for differentiation involves a combination of several factors, namely, intensity, quality and duration An optimum combination of these is necessary for certain morphogenic events Optimum day length period is 16 hrs for a number of plants in culture (Murashige, 1977) Shoot tip explants are normally maintained in an illuminance of about 500 to 3000 lux for micropropagation (George and Sherrington, 1984) In the present study, influence of light on axillary bud cultures of P. marsupium was evident with regard to number of shoots per culture and shoot growth An illuminantion with 2000 lux light gave the best performance with regard to shoots per culture and shoot growth (Table 22) Lower levels, red light and sun light gave an inferior performance compared to the former Various light treatments tried did not induce leaf morphogenesis in culture

One of the prerequisites for commercially feasible and economically viable micropropagation is the constant supply of *in vitro* propagules This can be achieved by producing a continuous culture system, where shoots can be regularly harvested and put for further cultural operations In order to achieve this objective, experiments were carried out to standardise a medium for continuous culturing However though a wide range of media compositions involving different basal compositions growth regulators and additives were tried none was proved to be efficient (Table 23) No clear cut interpretation could be made

from the data obtained and all cultures seemed to die off with time An important observation that was made from the study was the presence of systemic bacterial ooze in about 21 per cent of the cultures whenever the ends of the explants were cut during subculturing Cultures in which end of explants was not cut did not show this ooze The bacteria were identified as belonging to the genus Bacillus, which are nonpathogenic (Tables 8 and 9) It is hypothesized that the bacteria which multiply inside the vascular bundles of the tissue block the metabolite movement in the explant leading to the degeneration of the culture Similar effect of bacteria interfering with the culture has been observed elsewhere (Nazeem et al, 1992) An antibiotic sensitivity test was done during the study and it was found that the bacteria could be controlled by a number of antibiotics However no antibiotics was tried during the study to control this problem

One of the major obstacles faced in making a standard protocol for in vitro propagation of bijasal was the absence of leaf production and the abscission of leaf a few days after the expansion took place In this connection, abnormal leaf fall was identified as a major problem in P. santalinus cultures established from explants of seedling origin (Patri et al , 1988) In their study they observed that adenine added to the medium could control this problem However the authors did not attempt to explain the exact principle behind this Adenine added to the mediau over a range of concentrations did not improve the situation in case of P. marsupium cultures Presence of ethylene has been suggested to be a possible reason for lack

of leaf expansion and abscission in culture (Biddington, 1992) Some of the known ethylene inhibitors like cobalt chloride , activated charcoal and silver nitrate added to the medium too failed to bring about a change in this line Increased presence of ammonia ions in the media could lead to leaf expansion and retention in some of the cultures This too was not very consistent as evident from the fact that only 25 per cent of the cultures had leaves after three weeks in culture The most reliable method of getting leaf expansion and preventing abnormal leaf drop was the addition of CCC into the media at concentrations of 0 75 ppm or more The most acclaimed action of CCC is its antagonism with GA however, primary action through which CCC has manifested its effect could be that it helped to retain the leaves once they are formed More work on impact of added CCC alone and mixed with GA is needed to confirm this hypothesis

A second hurdle in formulating a protocol in P. marsupium was the sudden loss of morphogenetic potential of the buds of bijasal in culture with time Making a similar observation in P. santalinus cultures Patri et al (1988) remarked that it could be due to the endogenous levels of some factors inherited with the explant, which is/are gradually diluted and is/are completely lost after some time in culture Moreover, presence of systemic bacteria in the explant is established through the study and it could again be a reason for the altered morphogenetic potential of the explants Presence of bacteria within cultured cells can result in slow growth and an altered morphogenetic potential

(de Fossard, 1977). Axillary bud proliferation could be reduced or prevented by systemic bacterial infections or bacteria occurring interstitially between cells. They often survive in the explant with out any obvious signs, nevertheless, affecting culture response. Covert non pathogenic bacteria present through out Riger bigonia plants were able to survive *in vitro* for upto 7 months and then become suddenly apparent (Hannings and Langhans, 1974). Such situations lead to misleading and inconsistent conclusions on the culture requirements. *Bacillus* is one of the commonest genus of bacteria that occur as a persistent contaminant in explants in this way (Jones *et al.*, 1979). More studies on control of this systemic bacteria need to be undertaken to draw conclusion on this.

As the protocol for organogenesis could not be fully perfected due to the problem of lack of leaf production in culture, attempts were not made for obtaining root production in culture.

SUMMARY

SUMMARY

Investigations were carried out at the College of Forestry, Vellanikkara during 1991-1993 to standardise various aspects of micropropagation technique in bijasal (Pterocarpus marsupium Roxb) The salient findings of the study are

1 Nodal segments of size 0 5 to 1 5 cm were found to be the best explants for initiating axillary bud release in culture

2 A prophylactic spray with a mixture of fungicides (Carbendazim and Mancozeb) was found to reduce culture contamination

3. A seasonal influence on both culture contamination (with peak in rainy periods) and bud break (with peak between January and August) was observed

4 Mercuric chloride (0 01 %) was found as a better surface sterilant compared to chlorine water and hydrogen peroxide Treating explants with mercuric chloride (0 01 %) for 10 min was found to be most effective

5 Systemic bacterial infection was noted to be prevalent in the primary explants The bacteria were identified as the genus Bacillus In the antibiotic sensitivity test, doxycycline and chloramphenicol were found to be the best chemicals to control these microbes

6 Considerable differences were evident between basal media compositions, with MS and MS(a) giving the best results The media MS (a) which had a lesser concentration of ammonia than MS was observed to be the only media that had leaf retained, on the regenerated shoots after three weeks in culture

7 Kinetin alone tried at various concentrations in MS and WPM media failed to induce bud break in bijasal.

8 Several combinations of auxins with kinetin in MS and WPM induced multiple shoot formation When 2 0 ppm kinetin was combined with 0 1 ppm of IAA in WPM the best results were obtained

9 Benzyl adenine supplied to MS and WPM induced bud break from axillary buds

10 Addition of auxins to the media along with BA in both MS and WPM increased the growth and shoot induction from axillary buds

11 None of the combinations involving cytokinins and auxins had leaves in regenerated shoots after three weeks in culture either due to leaf drop or lack of leaf morphogenesis

12 Gibberelic acid added to the media had negative influence on bud break from axillary buds

13 Abscissic acid blocked bud break from the explants completely

14 Silver nitrate added to the medium failed to induce leaf expansion and reduced in number of shoots per culture This compound, however did not affect number of cultures showing response

15 Cobalt chloride had no beneficial effect on leaf expansion, rate of shoot production or number of cultures showing response

16 No beneficial effect was obtained by the addition of coconut water in the media

17 Adenine sulphate supplemented to WPM and MS deliteriously affected rate of shoot production and shoot growth while having no impact on number of cultures having leaves

18 Casein hydrolysate had no beneficial effect in axillary bud culture of bijasal

19 Activated charcoal at strength of 0 3 per cent or more prevented bud release from axillary buds

20 Cycocel added to the media at a strength of 0 75 ppm or more resulted in over 80 per cent of the cultures with leaf after three weeks in culture This compound, however reduced the rate of shoot production and growth of shoot in culture

21 Sucrose at 2 0 per cent or at 3 0 per cent along with 1 0 per cent maltose was the ideal carbon source for the explants in culture

22 Physical state of the culture medium had remarkable influence on the culture Semisolid media with agar at a strength of 0 8 per cent was found to be ideal for multiplication

23 Culture of bijasal did not show variation in response to a range of pH from 5 0-6 0

24 Differences were noticed among various light treatments with an illumination of 2000 lux giving the best results

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* Originals not seen

• *PLATES*

Plate 1 a. Axillary bud in culture

Plate 1.b Shoot initiation in culture
Medium: WPM + 2 0 ppm kinetin + 0.1 ppm IAA
Culture period: 2 weeks

Plate 2 a Shoot formation in culture with out leaf morphogenesis.
Medium: MS + 2.0 ppm BA + 0.1 ppm IBA
Culture period: 2 weeks

Plate 2 b Multiple shoots in culture showing leaf drop
Medium: WPM + 2 0 ppm Kinetin + 0.1 ppm IAA.
Culture period: 3 weeks

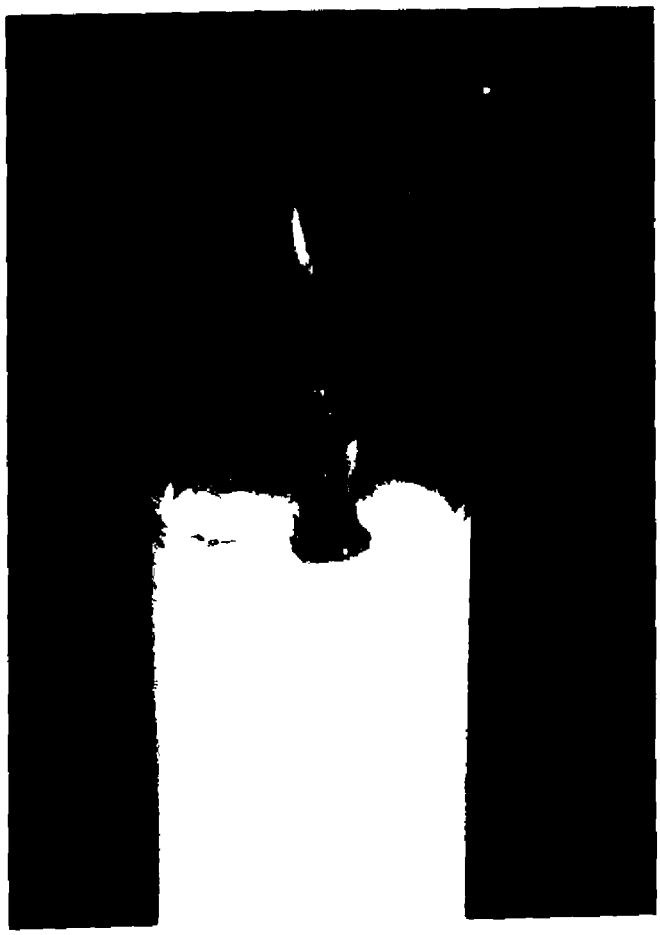


Plate 3 a Drying of leaf and terminal bud in culture
Medium: MS + 2 0 ppm BA + 0 1 ppm NAA
Culture period: 3 weeks

Plate 3 b Abscission of terminal bud of main shoot in culture
Medium· WPM + 2 0 ppm kinetin + 0 5 ppm IBA
Culture period: 3 weeks

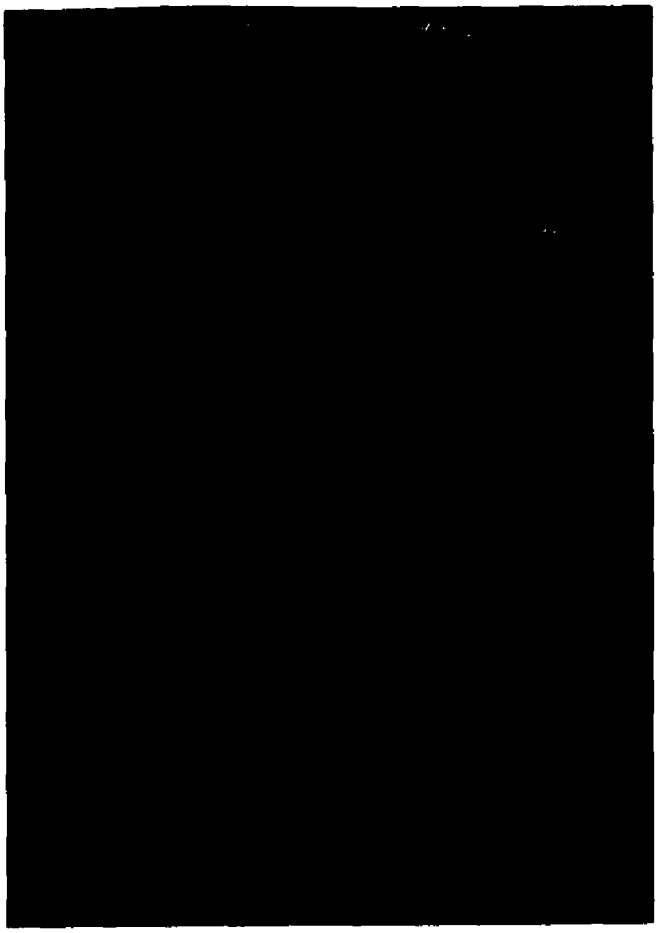
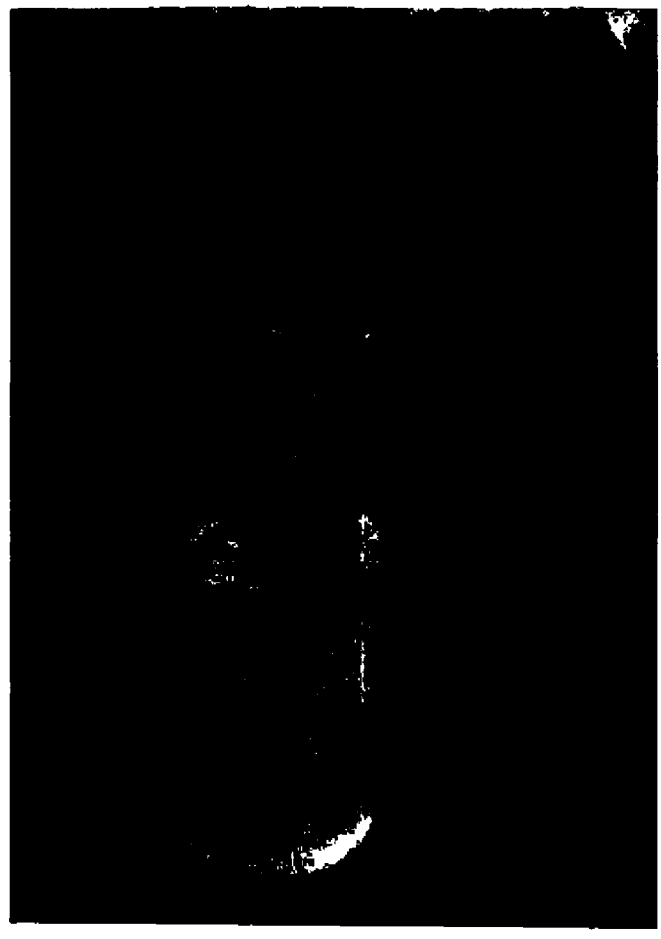


Plate 4 a Shoot showing axillary branching in culture
Medium: MS + 2 0 ppm BA + 0 1 ppm NAA
Culture period: 3 weeks

Plate 4 b Shoots showing leaf morphogenesis.
Media: WPM + 2 0 ppm kinetin + 0.1 ppm IAA + 1 0 ppm cycocel
Culture period: 3 weeks



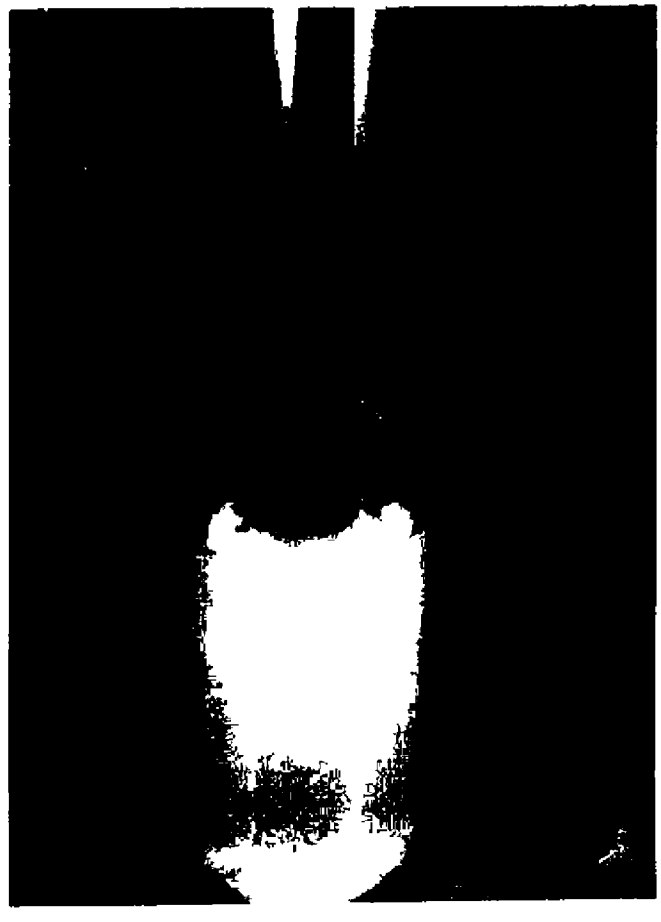
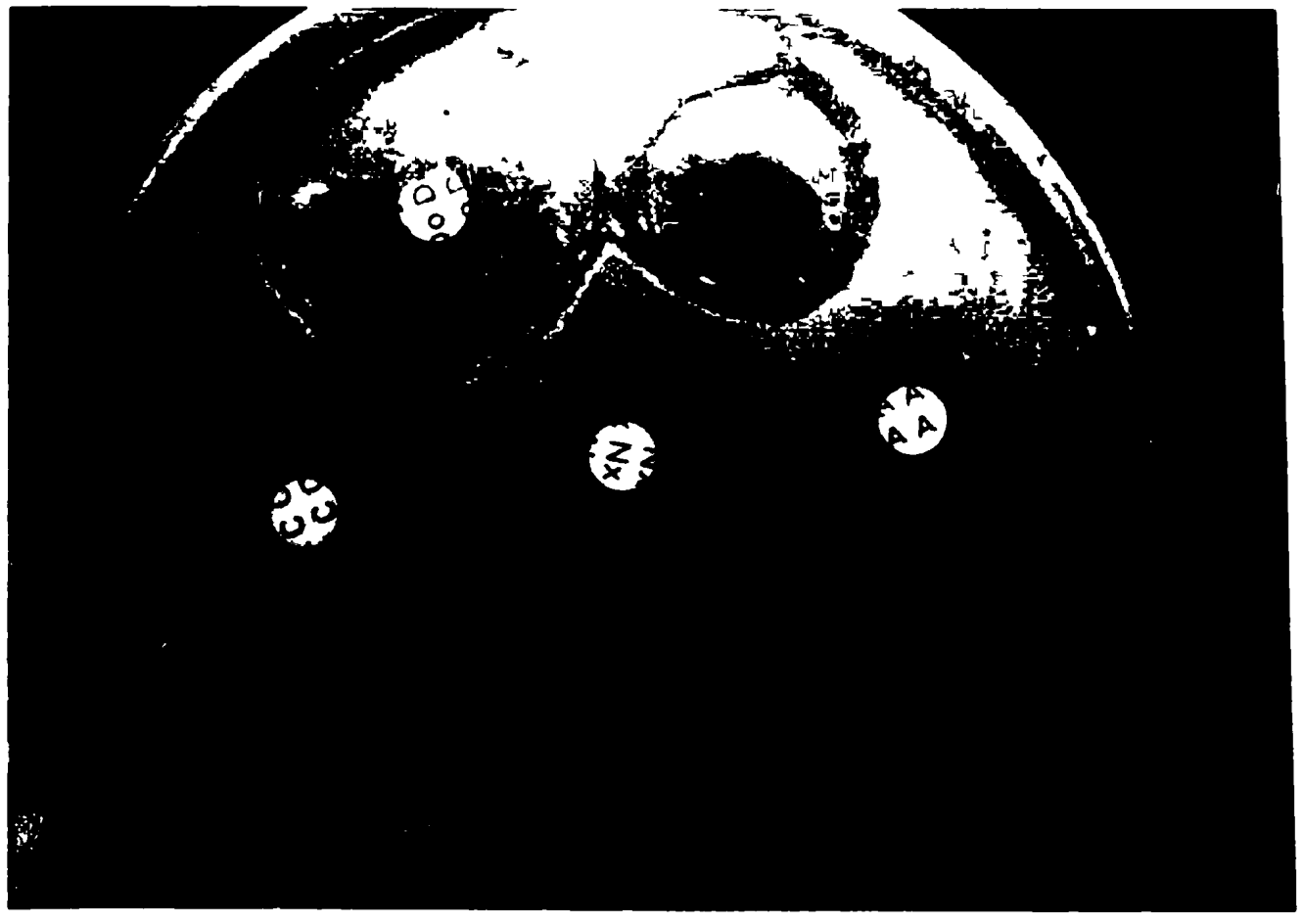


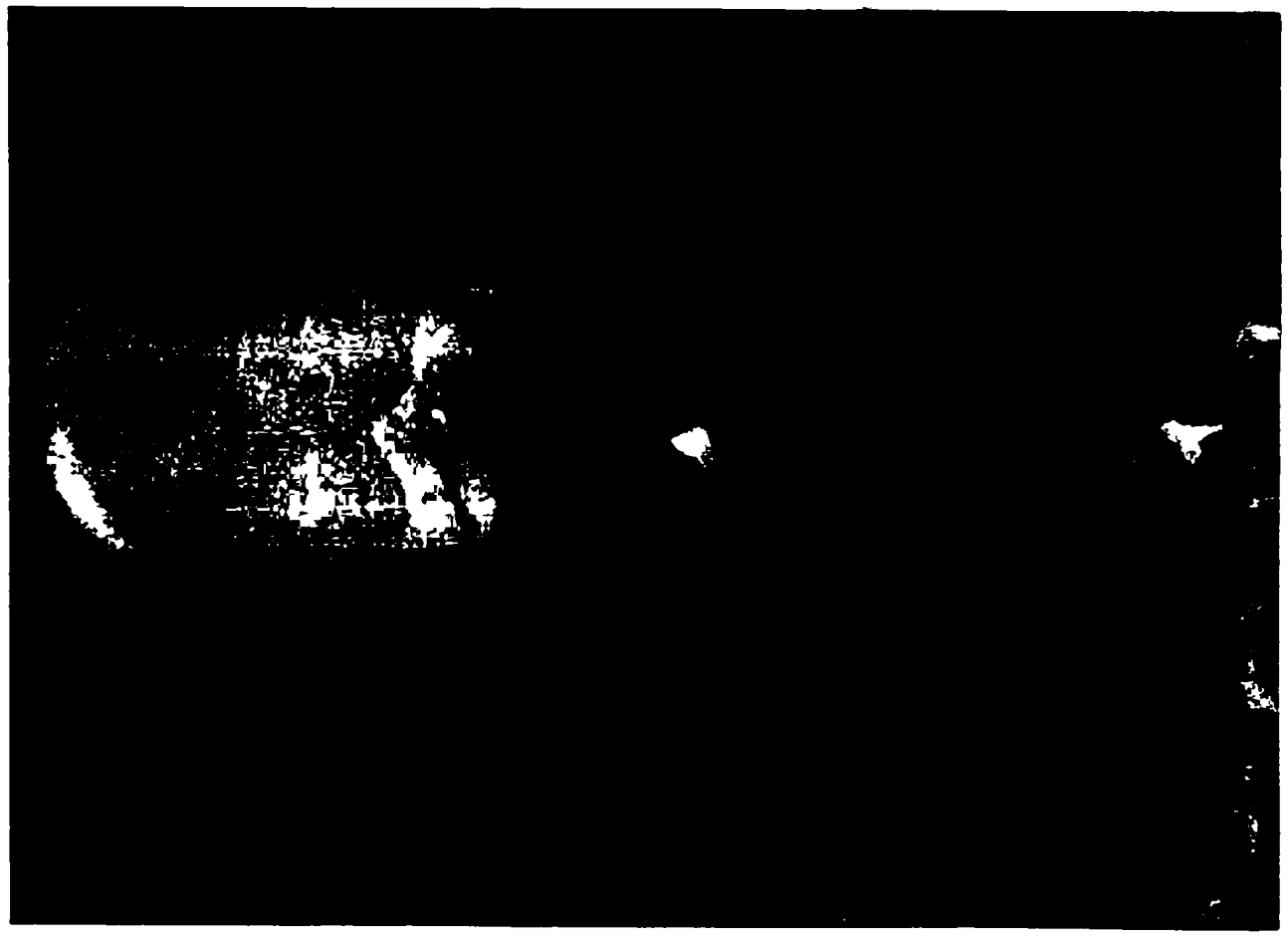
Plate 5 a Drying of shoots kept for initiation of continous culture
Medium: WPM + 2.0 ppm kinetin + 0 1 ppm IAA
Culture period: 1 week

Plate 5 b Antibiotic sensitivity test on systemic bacteria obtained from culture.

Legend

Doxycycline (D)
Chloramphenicol (C)
Norflexacin (Nx)
Oxytetracycline (O)
Pencillin (P)
Ciphalosporin (Cp)
Ampicillin (A)





In vitro Propagation of Bijasal (*Pterocarpus marsupium* Rxob.) Through Tissue Culture

SANTHOSH KUMAR A. V.

ABSTRACT OF THESIS

Submitted in partial fulfilment of the
requirement for the degree of

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FACULTY OF AGRICULTURE
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ABSTRACT

The present investigation was carried out at the College of Forestry Vellanikkara during 1991-93 with an objective of making a protocol for micropropagation of bijasal (Pterocarpus marsupium) Axillary buds obtained from mature trees were used as the explants

During the study it was found that nodal segments of size about 1 cm was ideal as the explants Prophylactic spraying of mother trees with mixture of Bavistin and Indofil M-45 coupled with surface sterilization of explants with 0 1 per cent mercuric chloride for 10 minutes could control culture contamination to the greatest extent However systemic bacterial infection in explants could not be controlled Murashige and Skoog (MS) medium was noted to be suitable for primary culture establishment Woody plant medium (WPM) supplemented with 2 0 ppm kinetin and 0 1 ppm IAA was the best for inducing multiple shoots from primary explants The various growth regulator combinations however failed to induce leaf morphogenesis in shoots Among the various media additives tested CCC had a beneficial role in leaf production in culture Casein hydrolysate adenine sulphate coconut water silver nitrate cobalt chloride and activated charcoal were the other additives tried without having any significant beneficial effect on culture of bijasal Sucrose at two per cent or three per cent sucrose with one per cent maltose were noted to be ideal carbon sources in culture Semi-solid medium having 0 8 per cent agar was found to be best for

culturing the nodal segments The culture did not show any difference in growth in a range of pH from 5 to 6 An illumination of 2000 Lux light was most ideal for incubating the cultures All attemots to establish continuous cultures failed due to the sudden loss of morphogenetic potential of cells on culture