

**MORPHOGENETIC STUDIES IN *PIPER NIGRUM* L. AND *PIPER*
COLUBRINUM LINK.**

**A THESIS
SUBMITTED TO THE
UNIVERSITY OF PUNE**

**FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY**

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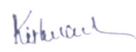
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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled ‘ Morphogenetic studies in *Piper nigrum* L. and *Piper colubrinum* Link.’ submitted by Ms. Swati Kelkar was carried out under my supervision at Plant Tissue Culture Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Pune

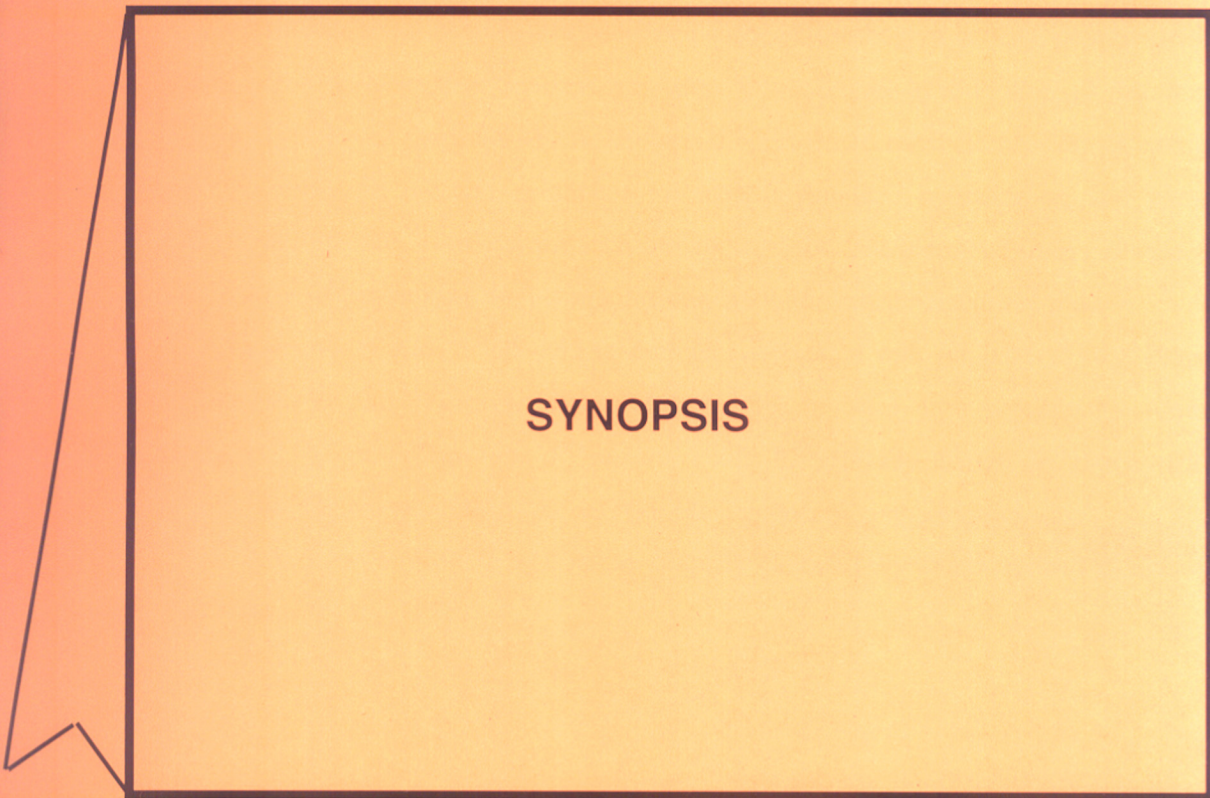

(Dr. K. V. Krishnamurthy)

Date

Research Guide

Abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
2-ip	6-(γ,γ -dimethylallylamino)purine
B5	Gamborg's Medium
BA	6-Benzyl Adenine
BD	Blayde's Medium (1966)
CH	Caesin Hydrolysate
CIM	Callus Induction Medium
CM	Coconut Milk
CPW13M	CPW salts with Mannitol 13% (w/v)
EDTA	Ethylenediaminetetraacetic acid
GA ₃	Gibberellic acid
half MS	MS medium with half strength major elements
IAA	Indole-3 acetic acid
IBA	Indole-3 butyric acid
IM	Induction Medium
MS	Murashige and Skoog
NA	Nutrient Agar
NAA	α -Naphthalene acetic acid
NB	Nutrient Broth
PDA	Potato Dextrose Agar
SBI	Shoot Bud Induction
SH	Schenk and Hildebrandt (1972)
SM	Subculture Medium
TDZ	Thidiazuron
V-47	Binding's medium (1974)
WPM	Woody Plant Medium (Lloyd and McCown 1980)
GP	Germination percentage
NR	Nodal Ring



SYNOPSIS

Black pepper, (*Piper nigrum* L.) is the most important spice crop of India and also is the largest foreign exchange earner among the plantation crops. It is also valued for its medicinal properties such as stomachic, antiperiodic, alterative, rubefacient, hypercoagulative and bio-enhancer. The major biological constraint responsible for severe crop and yield losses in black pepper is the *Phytophthora* - nematode pathogen complex causing quick wilt disease. *Piper colubrinum* alone was found to be resistant to quick wilt out of the 32 species of the genus *Piper* screened by inoculating zoospores of *Phytophthora*.

Lack of inherent potential for resistance to the wilt pathogens in black pepper, graft incompatibility with resistant species and failure in successful hybridization of the two species has prompted the use of biotechnological approach to solve the problem of quick wilt. This approach involves protoplast mediated asymmetric hybridization and plant genetic transformation. However for such studies, a reliable and reproducible *in vitro* plant regeneration system for both the parental species are the essential basic pre-requisites.

The present study is devoted to problems associated with fulfilling of such basic pre-requisites so that the protocols standardized for *in vitro* plant regeneration from explants of *P. colubrinum* and *P. nigrum* could be gainfully utilized in asymmetric hybridization studies to develop wilt resistant black pepper (*P. nigrum*) lines.

The thesis is divided into seven chapters followed by summary and a list of references.

CHAPTER 1. GENERAL INTRODUCTION:

This chapter covers the survey of literature involving studies carried out in the past on various basic and applied aspects of *Piper spp.* The development and uses of various tissue culture techniques are also described.

CHAPTER 2. MATERIALS AND METHODS:

Basic techniques of plant tissue culture used during the course of work such as preparation of glassware, plasticware and media and surface sterilization, culture of explants, and histology etc. are described in this chapter.

CHAPTER 3. ESTABLISHMENT OF CULTURES *IN VITRO*

The problems encountered during initial establishment of cultures, such as, bacterial and fungal contamination and exudation of phenolics are dealt in this chapter. Microbiological methods used to isolate and identify the contaminants are described. The efficacy of different surface sterilization techniques, physical (effect of temperature and leaching) as well as chemical (use of different antifungal and antibacterial agents) methods employed to reduce or eliminate contamination are assessed in this chapter. The use of anti-oxidants, frequent subculture and use of paraffin wax to control phenolics are also described.

CHAPTER 4. STUDY OF PLANT REGENERATION FROM DIFFERENT EXPLANTS DERIVED FROM MATURE PLANTS OF *PIPER COLUBRINUM* LINK.

Effect of different basal media, concentrations and combinations of plant growth regulators on morphogenetic response of leaf, stem, root and inflorescence explants is summarized in this chapter. The protocols standardized for direct and indirect organogenesis with leaf and stem explants and the procedure for *in vitro* clonal multiplication of apical and lateral meristems is described.

CHAPTER 5. STUDY OF ORGANOGENESIS FROM JUVENILE EXPLANTS OF *PIPER COLUBRINUM* LINK.

This chapter summarizes the different parameters studied to obtain plant regeneration from leaf, internode, petiole, root, node and shoot apex explants derived from *in vitro* grown plants. Manipulation of *in vitro* plants for protoplast isolation and *Agrobacterium* mediated transformation are also described.

CHAPTER 6. STUDIES ON REGENERATION POTENTIAL OF MATURE EXPLANTS OF THREE CULTIVARS OF *PIPER NIGRUM* L.

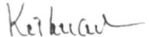
The optimization of media, culture conditions and use of plant growth regulators for induction of morphogenesis in leaf, stem, and aerial roots in three cultivars i.e. panniyur-1, karimunda and KS-27 are described in details in this chapter. A procedure for multiple shoot induction from node and shoot apex explants is also described.

CHAPTER 7. STUDIES ON IN VITRO CULTURE OF SEEDS, EMBRYOS AND JUVENILE EXPLANTS OF DIFFERENT CULTIVARS OF *PIPER NIGRUM* L.

In this chapter standardization of *in vitro* seed germination of six cultivars and the effect of different factors on seed and embryonal explants are discussed. The protocol standardized for multiple shoot formation in four cultivars of black pepper, panniyur-1, karimunda, kuthiravally, and kutching are also described.

KEY REFERENCES

1. G. J. Turner (1971) Resistance of *Piper spp.* And other plants to infection by *Phytophthora* from *Piper nigrum* Trans. Br. Mycol. Soc. 57: 61-66
2. V. H. Mathews and P. S. Rao (1984) In vitro responses of black pepper (*Piper nigrum* L.) Current Science 53: 183-186.


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Research Guide


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CHAPTER 1

GENERAL INTRODUCTION

PART A. GENERAL INTRODUCTION TO PLANT TISSUE CULTURE TECHNIQUES

Plants are the ultimate and renewable source of food and medicine for human beings. This has led man to manipulate and modify the plants so as to suit his growing requirements. Since the earliest primitive farming, man has been continuously carrying out experiments for plant improvement for the last 20-30 thousand years (Pauls, 1995). The techniques developed over the last 150 years have laid the foundations of modern science of Agriculture. The most important recent development in the field of plant improvement is that of Plant Tissue Culture techniques (Brar and Khush, 1994; Ratner, 1989).

Plant tissue culture is the maintenance and propagation of plant parts as small as a single cell in axenic culture under controlled environments (Pauls, 1995). The theoretical basis of plant tissue culture was established as early as 1838-1839 in Schwann and Schlieden's Cell Theory. Haberlandt (1902) first attempted to prove this theory experimentally using monocotyledonous plants and though his attempt was unsuccessful, he elaborated the concept of totipotency. Totipotency of plant cells refers to the potential of an individual cell to regenerate a whole plant. Since Haberlandt's time rapid progress has been made in this field in a relatively short period (Table 1.1). Several techniques have been developed which have potential uses in different areas of plant improvement as listed in Table 1.2.

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Table 1.1. Historical Account of Progress in the Field of Plant Tissue Culture
(Pierik, 1987; Brar and Khush, 1994)

Year	Important Milestones
1838	Cell theory suggesting totipotentiality of cells by Schwann and Schlieden.
1892	Plants synthesize organ forming substances, which are polarly distributed.
1902	First but unsuccessful attempt of tissue culture using monocots.
1904	First attempt in embryo culture of selected Crucifers.
1909	Fusion of plant protoplasts though the products failed to survive.
1922	Asymbiotic germination of orchid seeds. <i>In vitro</i> culture of root tips.
1925	Embryo culture for interspecific crosses in <i>Linum</i> spp.
1929	Embryo culture to avoid cross incompatibility in <i>Linum</i> spp.
1934	<i>In vitro</i> culture of cambial tissues of different trees and shrubs failed. Successful culture of tomato roots.
1936	Embryo culture of different gymnosperms.
1939	Successful continuously growing callus cultures.
1940	Culture of cambial tissue of <i>Ulmus</i> to study adventitious shoot formation.
1941	Coconut Milk used as cell division factor for <i>Datura</i> embryos. <i>In vitro</i> culture of crown gall tissues.
1944	First <i>in vitro</i> culture of tobacco used to study adventitious shoot formation.
1945	Cultivation of excised stem tips of <i>Asparagus</i> .
1946	First whole plants of <i>Lupinus</i> and <i>Tropaeollas</i> from shoot tips.
1948	Formation of adventitious shoots and roots in tobacco.
1950	Organs regenerated from callus of <i>Sequoia</i> .
1952	Virus-free <i>Dahlias</i> through meristem culture. First successful micrografts.
1953	Haploid callus of <i>Ginkgo biloba</i> from pollen grain callus.
1954	First plant from single cell.
1955	Discovery of Kinetin.
1956	Realization of growth of culture in multi-litre suspension system for secondary metabolite production.
1957	Discovery of root or shoot formation in culture depends on auxin : cytokinin ratio.
1958	Regeneration of somatic embryos from nucellus of <i>Citrus</i> ovules. Pro-embryo formation in callus clumps and cell suspension of carrot.
1960	First test tube fertilization in <i>Papaver rhoeas</i> . Enzymatic degradation of cell wall for protoplast formation. Vegetative propagation of orchids by meristem culture.
1962	Development of MS medium.
1964	First haploid plants from <i>Datura</i> androgenesis. Regeneration of roots and shoots on callus of <i>Populus tremuloides</i> .

Table 1.1 contd.

Year	Important Milestones
1965	<i>In vitro</i> flower induction in tobacco. Differentiation of tobacco plants from single isolated cells in microculture.
1967	Flower induction in <i>Lunaria annua</i> by vernalization <i>in vitro</i> . Haploid plants from pollen grains of tobacco.
1969	Protoplast isolation from suspension culture of <i>Haplopappus gracilis</i> .
1970	Selection of biochemical mutants in tobacco. Embryo culture for haploid production in Barley. Successful protoplast fusion.
1971	Plant regeneration from protoplast.
1972	Interspecific hybridization of <i>Nicotiana</i> spp. using protoplasts.
1973	Cytokinins found to be capable of breaking dormancy in Gerberas.
1974	Induction of branching by cytokinins in <i>Gerbera</i> shoot tips. Regeneration of haploid <i>Petunia</i> plants from protoplasts. Fusion of haploid protoplasts to form polyploids. Biotransformation in plant tissue culture. Ti plasmid has the tumor inducing principle in crown gall.
1975	Positive selection of maize callus culture resistant to <i>Helminthosporium maydis</i> .
1976	Shoot induction from cryo-preserved shoot tips of carnation . Protoplast fusion of <i>Petunia hybrida</i> with <i>P. parodii</i> . Octopine and Nopaline synthesis and break-down is regulated by Ti plasmid.
1977	Successful integration of T-DNA in plants.
1978	Somatic hybridization of tomato and potato.
1979	Co-cultivation procedure developed for the <i>Agrobacterium</i> mediated transformation of protoplasts.
1980	The use of immobilized cells for biotransformation of digitoxin into digoxin.
1981	Introduction of the term somaclonal variation. Isolation of auxotrophs by cell colony screening in haploid protoplasts of <i>Nicotiana plumbaginifolia</i> treated with mutagens.
1982	Naked DNA transformation of protoplasts. Electrofusion of protoplasts.
1983	Intergeneric cybrid in radish and rape.
1984	Transformation of plant cells with plasmid DNA.
1985	Infection and transformation of leaf discs with <i>Agrobacterium tumefaciens</i> and regeneration of transformed plants.
1987	Use of Microprojectile gun for transformation and recovery of individuals showing transient gene expression.
1988	Recovery of stable transformants through particle bombardment.
1996	Laser microbeam for manipulation of plant cells.

Table 1.2. Different techniques of Plant Tissue Culture and their Applications in Plant Improvement.

Tissue culture technique	Applications
Shoot apical meristem culture	Production of virus free germplasm. Mass production of desirable genotypes. Facilitates international exchange. Cryopreservation or <i>in vitro</i> conservation of germplasm. Identification of developmental switches in morphogenetic pathways.
Embryo Culture	Haploid production. Overcoming embryo abortion. Overcoming seed dormancy and self sterility of seeds. Embryo rescue in distant hybridization.
Ovary and ovule culture	Recovery of hybrid embryos overcoming embryo abortion at very early stages of development of zygote.
<i>In vitro</i> fertilization	Production of distant hybrids avoiding style and stigmatic incompatibility that inhibits pollen germination and pollen tube growth. Production of transgenics by injecting exogenous DNA in the nuclei of gametes and zygotes.
Anther and Microspore culture	Production of haploids. Production of homozygous lines through chromosome doubling and reduction in breeding cycle. Genetic transformation using microspores. Production of useful gametoclonal variations.
Cell culture and <i>in vitro</i> selection at cellular level	Production of plants and somatic embryos. Studying growth and physiological requirements of plant cells. Production of secondary metabolites. Induction and selection of useful mutants at cell level for disease resistance, stress tolerance and improved nutritional quality in less time and space.
Organ culture	Mass production of plants of elite and rare germplasm. Production of calli and roots for production of secondary metabolites.
Cryopreservation	Long-term preservation of useful germplasm. Conservation of available natural genetic variability.
Somatic Embryogenesis	Mass multiplication of elite germplasm and Production of artificial seeds. As source material for protoplast work and genetic transformation. Production of primary metabolites specific to seeds such as lipids in oil seeds.

Table 1.2 contd.

Tissue culture technique	Applications
Organogenesis	Mass multiplication of elite germplasm. As source material for protoplast work and genetic transformation and Micrografting. Conservation of endangered genotypes.
Callus cultures	Production of plantlets through somatic embryogenesis or organogenesis. Generation of useful somaclonal and gametoclonal variants such as resistance to disease, herbicides etc. Production of useful secondary metabolites. Source of protoplasts and suspension cultures. Selection of cell lines with valuable properties
Seed culture	To increase germination efficiency and germling production in seeds difficult to germinate <i>in vivo</i> . To induce multiple shoot formation.
<i>In vitro</i> flowering	Reduction in long life cycle in perennials such as Bamboo. Continuous supply of flowers and seeds irrespective of season.
Micrografting	Overcoming graft incompatibility. Rapid mass propagation of elite scions grafted on rootstocks having desirable traits like resistance to soil-borne diseases. Multiplication and survival of difficult to root species and transformants.
<i>In vitro</i> production of secondary metabolites	Production drugs, aromatic substances, pigments without destruction of mother plants. Increased productivity by manipulations of cultural parameters.
Protoplast isolation, culture and fusion	Combining distant genomes to produce somatic hybrids, asymmetric hybrids and cybrids. Production of organelle recombinants. Transfer of CMS (cytoplasmic male sterility) in elite lines. Induction of juvenile characters in otherwise mature tissue. Source material for genetic transformation.
Genetic Transformation	Introduction of foreign DNA to generate novel genetic combinations. Transfer of desirable genes such as disease and pest resistance from related or unrelated plant species into high yielding susceptible cultivars. To study structure and function of genes.
<i>In vitro</i> mutagenesis	Polyploidization for increase in biomass or yield. For introducing genetic variability, rapid selection and multiplication of useful mutants. As a tool for developmental genetics and elucidation of biochemical processes.

Plant tissue culture techniques could be especially useful for the perennials such as plantation crops which are less amenable to conventional plant improvement techniques. The slow progress of conventional techniques in plantation crops could be attributed to the following reasons :

1. Perennial life-cycles.
2. Long juvenile phases.
3. Large areas required for experimentation.
4. Subsequent large requirement of planting material for experiments.
5. Low and short seed viability in some cases.
6. Narrow genetic base resulting from constant vegetative propagation.
7. Segregation of hybrid progeny in F_1 generation making hybridization procedure more tedious and lengthy in some cases.
8. Incompatibility relationships barring hybridization.
9. Dioecy i.e. separate male and female plants.

The use of plant tissue culture techniques could provide suitable logical solutions to most of these problems. Black pepper of commerce (*Piper nigrum* L.), the most important spice crop of India and the largest foreign-exchange-earner among the plantation crops is often beset with many of the problems cited above. The low yield per vine and susceptibility to quick wilt disease caused by *Phytophthora capsici* Leonian, are the worst production constraints for black pepper economy. These problems are not yet completely resolved by conventional techniques. Hence, plant tissue culture methods may be an alternative to be used to supplement the effort of plant breeders with due consideration to the basic botanical and conventional studies carried out so far. The basic studies often provide an insight into problems faced during application of conventional and non-conventional improvement techniques. The following section deals with such basic as well as applied aspects studied so far in black pepper.

Piper colubrinum Link., a wild species with multiple resistance traits has formed the basis of improvement of pepper for disease resistance using traditional as well as modern approaches. It is resistant to the fungal pathogens *Phytophthora capsici* and

Fusarium solani causing quick and slow wilt diseases respectively; as well as to the parasitic nematodes *Radopholus similis* (Cobb.) Thorne and *Meloidogyne incognita* Chitwood. In spite of extensive literature survey, comprehensive information about this species could not be obtained as it is a wild species. The available information on the wild species *P. colubrinum*, is mainly restricted to its participation in improvement programs and is provided in the following section.

PART B. INTRODUCTION TO *PIPER* SPP.: BASIC AND APPLIED STUDIES.

Piper nigrum L. (Black pepper)

B.1. Origin and distribution:

Piper nigrum L. i.e. black pepper of commerce belongs to family Piperaceae. It is a very large family and is considered to be one of the most heterogeneous assemblages among dicots. It is pan-tropically distributed in South and Central America and South Asia. The genus *Piper* consists of about 1000 species of which 110 are of Indian origin. (Ravindran and Babu, 1994). Two independent centers of distribution are recognized in India namely, the Northern trans-Gangetic region and submountainous South-West region of Western Ghats.

Piper nigrum L. is believed to have originated in the tropical evergreen forests of Malabar region of Western Ghats of India where the greatest number of cultivars along with a number of wild accessions occur. From the Western Ghats, black pepper got dispersed eastwards in Assam, Burma and further all over the tropics. The wild occurrence of black pepper in hills of Assam and Burma might have resulted from naturalization after early introduction (Purseglove, 1974, 1981).

Ravindran (1990) suggested the hybrid origin of *P. nigrum* from the putative parents, *Piper wightii* Miq. and *Piper galeatum* Cas D.C. In morphological characters, *P. wightii* is very close to *P. nigrum* as shown by cluster analysis carried out on the basis of as many as 30 characters. (Ravindran *et al.*, 1992). However the chemical composition of the 2 species differ. Whereas chemotaxonomically *P. galeatum* shows 70% similarity to *P. nigrum* but shares only a few of its morphological characters. The presence of intermediates like *P. sugandhi* further support the possibility of *P. galeatum* being one of the progenitors of black pepper. However, these assumptions require further study to confirm the exact ancestry of black pepper (Ravindran and Babu, 1994; Ravindran *et al.*, 1997 a, b). It is proposed that *P. nigrum* arose as a natural hybrid by a chance crossing of these two species and the reproductive isolation following the chance crossing aided by vegetative propagation must have led to speciation (Ravindran *et al.*, 1992).

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B.2. Morphology:

It is a perennial woody climber (Fig. 1.1A), having adventitious feeding roots as well as supporting or climbing aerial roots. The branching pattern is pleomorphic comprised of orthotropic vegetative climbing branches, on which plagiotropic fruiting branches (Fig. 1.1B) are borne in axils of leaves and the geotropic runners which eventually act as vegetative propagation units. The aerial stems bear alternate, simple, petiolate leaves with ovate to cordate lamina and acute apices (Kumar *et al.*, 1997). The colour of shoot tip and leaf morphology often serve as distinguishing characters for cultivar classification.

The black pepper plants in wild state are dioecious but the cultivated plants are usually gynodioecious (bearing female and bisexual flowers) or trimonoecious (bearing female, male and bisexual flowers). The flowers are borne opposite to leaves on plagiotropic branches in pendant spikes. The flowers are monoecious or dioecious, minute, bracteate with 2-4 stamens and/or 3-5 stigmas but without any perianth lobes. The monoecious flowers are protogynous. Black pepper is predominantly self-pollinated plant. Autogamy or geitonogamy appears to be common in hermaphrodite flowers. But the mode of pollination is not clearly understood in unisexual plants. It is suspected to be achieved either by rain drops or wind.

The fruits are sessile, globose, single seeded drupes with pulpy mesocarp and exocarp becoming red when ripe. The seeds have little endosperm, minute embryo and copious perisperm which is nutritive in function.

B.3. Anatomy:

The genus *Piper* is characterized by anomalous vasculature in the stem. The distribution of vascular bundles in the axis is scattered and widely spaced similar to the monocots (Rahiman and Nair, 1983). However, unlike monocots, secondary thickening of stem occurs in piperaceous plants through interfascicular cambial activity. This abnormal anatomy is considered as a possible reason for graft incompatibility between *P. nigrum* and its foot-rot resistant wild root-stock *P. colubrinum* reported by Alconero *et al.*, (1972).

B.4. Embryology:

Similar to anatomy, black pepper is characterized by certain peculiar

embryological features such as prevalence of embryoless seeds and presence of perisperm. Embryolessness i.e. absence of well differentiated embryo from mature seeds is reported by Kanta (1961). She also observed abnormalities in endosperm. She attributed absence of embryos to relatively high degree of pollen sterility and early degeneration of zygotes caused by accumulation of calcium oxalate crystals in ovary wall, perisperm and embryo sac. She categorized that out of 100 seeds, 32 are embryoless but with healthy endosperm, 22 have unhealthy embryos, 6 are without both embryo and endosperm, 20 have globular and heart shaped embryos and only 20 have fully developed embryos and healthy endosperm (Kanta, 1961). The normal endosperm is persistent in the seed and forms a top-shaped structure (Kanta, 1963). The main nutritive tissue of black pepper embryo is perisperm. It is derived from the nucellus. The nucellar cells divide and enlarge and become densely cytoplasmic. They store starch and oil globules. In a recent report, West *et al.* (1994) recorded presence of electron dense globoids in black pepper perisperm. These globoids contained phosphorus, magnesium, calcium. The globoids resemble the mineral nutrient store bodies found in gymnosperm embryos. The perisperm occupies bulk (95%) of a mature seed.

B.5. Cytology:

The *Piper* species from North and South India show divergence in the basic chromosome number (x). The northern species are characterized by $x=12$ while southern species show $x=13$ (Jose and Sharma, 1985; Ravindran and Babu, 1994). Different southern species show a natural polyploid series that includes diploids ($2n = 52$), triploids ($2n=78$) and tetraploids ($2n=104$). Different accessions of *Piper nigrum* itself have been found to exhibit different levels of polyploidy ranging from diploids to pentaploids (Janaki Ammal, 1945; Mathew, 1958; Jose and Sharma, 1985; Samuel and Bavappa, 1981; Rahiman and Nair, 1986). The chromosome morphology analysis suggests autopolyploidization. (Mathew, 1958). This is further supported by normal pairing of bivalents in microspores (Martin and Gregory, 1962). The polyploid nature is important for crop improvement as it is often expressed in the form of an increase in biomass, increased fruit size or an increase in secondary metabolite content. Jose and Sharma (1985) correlated the ploidy level change among *Piper* spp. with chemical content and found that oleoresin content was inversely proportional to increase in ploidy level



Fig. 1.1 - Piper nigrum (Black Pepper) in field.
 Fig. 1.2 - Fruiting Lateral Branch of Black Pepper.
 Fig. 1.3 - Piper nigrum in greenhouse

whereas piperine content was directly proportional to the increase in ploidy level.

B.6. Chemical composition:

The active principle is the alkaloid piperine ($C_{17}H_{19}NO_3$). Other alkaloids like piperidine, pipericide, dehydropiperinaline are present in lesser quantities. The pungency of black pepper is due to the resin chavacine, most abundant in the mesocarp. The odour is due to the volatile oil containing terpenes. Black pepper contains 8-13% moisture, 2-42% starch, 5-8% piperine and 1-2.5% volatile oil. The chemical constituents of *Piper* spp. are excellently reviewed by Parmar *et al.* (1997).

B.7. Cultivation:

Black pepper is propagated vegetatively by stem cuttings, generally obtained from vegetative branches (Fig. 1.1C). Though use of 2-3 node cuttings was common earlier, rapid multiplication method /bamboo split method developed by Bavappa and Gurusinghe (1978) is now widely used to achieve a multiplication rate of 1:40 per year. The cuttings after successful rooting are transferred to the orchards and are usually trailed on coconut or arecanut palms or other living or nonliving supports. The use of plant growth regulators IAA, IBA, NAA and 2,4-D (at 30 to 5000 mg l⁻¹) for better rooting of the cuttings is also practised (Pillay *et al.*, 1982; Irulappan *et al.*, 1982; Salvi and Desai, 1989; Sridhar and Shyam Singh, 1990).

The use of fruiting laterals as planting material gives rise to Bush peppers which have a bushy rather than the climbing habit. They bear fruits within 3-4 years as against 6-8 years of climbing vines and have the advantage of easy harvesting. But they are not economical in terms of land use and the yield per vine (Kumar *et al.*, 1997).

The use of seeds as propagules is rare though not new. Since olden times the farmers of Malabar have been using seed germination to maintain vigour. However, due to recalcitrant nature of the seeds and heterogeneity of the progeny, it is not used as a means of commercial propagation.

B.8. Economic importance:

The dried fruit of *Piper nigrum* is the black pepper of commerce; one of the oldest and the most important of the spices. It was one of the first articles of commerce to be exchanged between the East and the West and the trade is still continued. Black pepper

Fig. 1.1. Morphology and propagation of black pepper *Piper nigrum* L.

A. Black pepper vines in field.

B. Fruiting lateral branch of black pepper

C. Four year old Black pepper stem cuttings maintained in Green-house.

alone brings foreign exchange worth Rs. 240 crores (Kumar *et al.*, 1997). It is widely used for flavoring curries, pickles, sauces, sausages, ketchups and also for seasoning dishes. Pepper oil is distilled from the fruits and is used in perfumery especially for scenting bouquets and in soap industry. It is also a valuable adjuvant with extensive culinary uses and is used to add flavor to certain beverages. The piperine is used as a source of synthetic heliotropine for flavoring beer and as an insect repellent (Wealth of India, 1969; Purseglove, 1987). Since ancient times white pepper is very popular among Europeans (Govindarajan, 1976; Stobart, 1983) for its delicate aroma and less pungency and hot taste. It is the black pepper fruit without epicarp and outer portion of mesocarp. The pepper waste consisting of epicarp and outer mesocarp produced during preparation of white pepper combined with common salt is called Pepper-sal and is an important by-product of black pepper used as a flavoring agent. In addition to these traditional products, different value added products such as pepper oleoresins or pepper extracts, frozen or dehydrated or freeze dried green pepper, green pepper in brine, canned tender green pepper, ground pepper, sterile pepper, organic pepper, encapsulated pepper and pepper slabs are now manufactured considering demands of the global market (Peter, 1997). There are numerous reports on antimicrobial, antifungal, insecticidal and molluscidal properties of pepper that are well reviewed by Parmar *et al.* (1997).

Besides its extensive culinary uses, black pepper is also valued for its medicinal properties. It is used in traditional Ayurvedic medicine as an aromatic stimulant, in cholera, weakness following fevers, vertigo, coma; as a stomachic in dyspepsia and flatulence, as an antiperiodic in malaria and as alterative in paraplegia, and arthritis. It is recommended for asthma, neuralgic pains, diseases of throat, piles, urinary discharges, ozoena, cardiac diseases, night blindness and diabetes. It is anthelmintic, disphoretic and carminative and hence used to cure chronic dysentery. Applied externally it is rubefascent, relaxes sore throat, and cures alopecia and other skin diseases. It is supposed to cure bald patches caused by ring worm infection. Black pepper is also considered to be hypercoagulant. If used in excess, it causes biliousness and is an abortifacient. (Wealth of India, 1969; Warriar, 1981). In the folklore medicine black pepper is used as a remedy for common coughs and colds, extract of black pepper stems is used as eardrops (Warriar, 1981). Some tribals use black pepper as an antifertility drug

(Laxmanan, 1990). Use of black pepper is also recommended in homeopathic medicine. Black pepper also has mutagenic and carcinogenic properties.(Parmar *et al.*, 1997).

The use of black pepper as bioenhancer is patented by the scientists from Regional Research Laboratory, Jammu (US patent no. US 5439891, 1995). They found that addition of piperine to the antileprosy and anti tuberculosis formulations gives desired effects at less than half the amount of antibiotic Rifampicin. This makes the treatment more cost effective and also reduces the harmful side- effects of the drugs.

B.9. The problems:

On account of extensive economic and medicinal uses of black pepper, the demand for black pepper in international market is ever increasing. It is estimated that global demand for black pepper would increase up to 1.85-2.1 lakh tons by 2000 A.D. against the current demand of 1.5 lakh tons (Peter, 1997). To meet this huge demand the yield must be increased. However, in India extremely low yield per unit area is the major problem of black pepper economy. India accounts for 54% of the total area under black pepper cultivation but its production is only 26.6%. Whereas other countries like Indonesia, Malaysia and Brazil due to higher productivity have larger share of income despite lesser area under cultivation (Kumar *et al*, 1997). The national productivity is only 290 kg/ha against 2925 kg/ha in Malaysia (Peter, 1997, 1999). Table 1.3 gives details of productivity and export of black pepper for the past few years. The biotic and abiotic stresses responsible for lower yield must be overcome to increase pepper productivity. The massive crop losses caused by these stresses have introduced another problem of scarcity of planting material. Approximately one third of the total area under black pepper plantation needs to be replanted (Nair, 1992).

Table 1.3 . Agronomic profile of productivity of black pepper in India for 1991-1996.

	1991-1992	1992-1993	1993-1994	1994-1995	1995-1996
Area (x1000 ha)	174.87	177.36	183.45	195.05	198.1
Production (x1000 t)	42.69	54.90	46.10	53.11	61.6
Export (x1000 t)	20.54	23.82	48.76	37.26	24.15
Value (%)	19.51	18.86	38.16	24.99	25.69

B.9.1. Abiotic stress: The important abiotic stresses for black pepper are inadequate light conditions and loss of soil moisture. The suboptimal light in black pepper gardens affects the yield. It does so by affecting the floral biology. In insufficient light conditions, production of female flowers increases and that of bisexual flowers decreases and it also inhibits the anther emergence (Ravindran *et al.*, 1994) which may cause lower fruit set and yield. Depletion of soil moisture due to changes in climatic conditions and deforestation is another limiting factor that affects vine survival, if not yield. To overcome these factors, cultivars tolerant to shade and water stress are required.

B.9.2. Biotic stress: The biotic stress is exerted by susceptibility of black pepper cultivars to different pests and diseases caused by microbial pathogens.

Diseases of black pepper : In India more than 17 diseases of black pepper are reported. These include nursery diseases like leaf blight caused by *Rhizoctonia solani*, basal wilt caused by *Sclerotium rolfsii* and diseases of grown up vines include foot-rot caused by *Phytophthora capsici*, slow decline caused by a complex of organisms comprising nematodes and fungi like *Fusarium* spp., *Rhizoctonia solani*, 'Pollu' disease caused by *Colletotrichum gloeosporioides*, bacterial leaf spot caused by *Xanthomonas campestris* and little leaf and phyllody diseases of uncertain origin (Nambiar and Sarma, 1985, Sarma 1993).

Of all these diseases, foot-rot is the most destructive, causing upto 15%-30% crop losses (Sarma 1993; Pillay *et al.*, 1994). It is caused by the fungus *Phytophthora capsici* Leonian. It is a soil-borne pathogen and infects leaves, stems, roots and also collar i.e. the stem at ground level. All these parts may get infected independently or the infection may

spread from one organ to other. The collar rot is fatal. *Phytophthora* root rot is found to infect black pepper all over the world and the high yielding cultivars of black pepper in all the pepper growing countries are susceptible to this fungus. It has a broad host range and out of 32 species of *Piper* screened, only one species, *P. colubrinum* Link. exhibited complete resistance to the fungus (Turner, 1971). It causes foot-rot in other economically important species like *P. betel*.

Pests of black pepper : Important pests of black pepper are pollu beetle, (*Longitarsus nigripennis*), leaf gall thrips (*Liothrips karnyi*), the scale insects and the plant parasitic nematodes, the burrowing nematode (*Radopholus similis* (Cobb.)Thorne) and root-knot nematode (*Meloidogyne incognita* Chitwood). The pollu beetle feeds on the growing points, tender shoots, tender and mature leaves, tender inflorescences and fruits. The grubs of the beetle cause extreme damage by burrowing into fruits. The leaf gall thrips infest leaves and form galls and malformations of leaves. The scale insects feed on plant sap and cause drying of the infested part. The plant parasitic nematodes are the most deleterious pests (Ramana, 1993). The root-knot nematodes inhabit roots and cause hypertrophy and hyperplasia in roots. The burrowing nematode enters through roots and feeds on tissues inside. It causes root necrosis and rotting. It is proposed that the nematodes form a complex with foot-rot fungus and act as a complex pathogen system. The *Phytophthora*-resistant Brazilian wild species *P. colubrinum* is also resistant to infection by the nematodes (Sarma, 1993).

Recently, a study carried out to assess comparative damage caused by different pests and diseases in black pepper revealed that total annual average yield loss caused by biotic stresses amounted to 33%. Of this 13% loss was stand loss i.e. death of plants and 20% was partial loss causing decline in yield. The *Phytophthora* was major contributor to the stand loss, causing 9% loss. The partial loss caused by *Phytophthora* amounted to 1% but Pollu beetle caused 12% of the partial loss (Prabhakaran, 1997).

B.10. Improvement in black pepper:

Decline in yield due to biotic and abiotic stresses is the major constraint in black pepper economy. Hence increase in yield is the prime aim in black pepper improvement and this could be achieved by acquiring resistance to different pests and diseases and tolerance to abiotic stresses. Supplementary strategies like development of varieties

suitable to different altitudes, improvement in boldness of fruits and increase in oleoresin content would ultimately contribute to increase in yield. Conventional plant breeding methods and non-conventional plant tissue culture techniques are both being attempted to bring about improvement in black pepper.

B.10.1. Conventional techniques:

The conventional breeding methods include plant introduction, selection and hybridization. Mutation breeding is a comparatively new approach in conventional breeding strategies. The cultural practices like grafting are also important means of improving vegetatively propagated crops. The chemical or biological control systems used to overcome diseases are also covered in conventional methods. The use of these methods for black pepper improvement is reviewed here.

Plant introduction method has limited scope in improvement of black pepper since *Piper nigrum* is native to India. The introduction of high yielding varieties such as Kuching from Malaysia is limited to the research stations.

Piper colubrinum is an important introduction from Brazil. The plant is introduced for its multiple resistance traits, to be used as a donor for foot-rot and nematode resistance.

Selection is the most important improvement method for vegetatively propagated crops like black pepper. All the local cultivars (nearly 100) are the result of early selection for higher yield (Pillay *et al.*, 1994; Ravindran *et al.*, 1997). Most of these cultivars are supposed to have originated as natural hybrids e.g. Kuthiravally, Karimunda which were further domesticated by selection. Early selection has resulted in two distinct groups of black pepper cultivars, one from northern part of Kerala state and other from the southern part (Ibrahim *et al.*, 1972; Ravindran *et al.*, 1997).

The selection among existing cultivars carried out for specific characters such as nematode tolerance has evolved nematode tolerant variety Ottaplackal I (Pillay *et al.*, 1994). Suitability of different varieties to different agroecological regions is also another criterion of selection as recommended by Ravindran and Babu (1988) and Pillay *et al.* (1994).

The selection within existing cultivars of black pepper (owing to considerable intravarietal variation for quantitative characters like yield) has led to the isolation of

superior clones such as Subhakara and Sreekara from cv. Karimunda.

Hybridization is practised to generate new gene combinations that contribute to increase in variability. It is one of the best procedures to widen the genetic base in vegetatively propagated crops. But in heterozygous crops like black pepper, the segregation of characters occurs in F_1 generation itself. This necessitates a very large F_1 population and hence a larger number of crossing events between the parents. Further the number of hybrid seeds produced and chances of obtaining desired gene combinations are very less until tedious and lengthy method of repeated crossing between same parents is practised. Owing to these difficulties, only two varieties i.e. Panniyur-1 and Panniyur-3 have been evolved through hybridization in black pepper. The first hybrid Panniyur-1 resulted from a cross between cv. Uthirencotta and cv. Cheriakaniakadan in 1954. Repeated crossing of the same parents gave rise to Panniyur-3 in 1973.

Attempts for interspecific hybridization between *P. nigrum* and *P. colubrinum* are being made at IISR (Indian Institute of Spices Research, Formerly NRCS; National Research Centre for Spices) Calicut (NRCS, 1990) for developing *Phytophthora* resistant black pepper but success is not yet reported. On the basis of phytochemical and chromosomal studies, hybridization between *P. nigrum* and *P. cubeba* is suggested by Jose and Sharma (1985) to combine high piperine content of *P. nigrum* with high oleoresin content of *P. cubeba*. It is also proposed that hybridization between *P. nigrum* and other wild species of *Piper* could combine desirable characters like longer spikes and bolder fruits (Rahiman *et al.*, 1979).

Induction of mutations to generate variability is another approach studied in *P. nigrum*. Chandy *et al.* (1980) tried to induce mutagenesis in vegetative buds using EMS (Ethyl Methane Sulphonate) and obtained transient phenotype. Colchicine induced chromosome doubling was carried out in Panniyur-1 (Nair and Ravindran, 1992) and the tetraploid plant selected showed increased vigor. Irullappan *et al.* (1982) induced mutagenesis in cuttings of Panniyur-1 and Kuthiravally using gamma rays but found that mutations adversely affected rooting.

The soil-borne nature of *Phytophthora capsici* has directed the use of resistant genotypes as a root-stock for susceptible cultivars of *P. nigrum*. Because of its total resistance to foot-rot fungus, *P. colubrinum* was considered as the ideal root-stock for

black pepper (Albuquerque, 1968). However the graft union developed longitudinal cracks and collapsed after 4 years (Alconero *et al.*, 1971), possibly due to anatomical incompatibility and unequal growth rates of stock and scion.

The chemical and biological controls for different pests and diseases of black pepper are given in Table 1.4 (Peter, 1997, 1999). Because of the inability of chemicals in attaining total control of diseases and growing demand in international market for black pepper grown from organic farming, it is necessary to look for alternative natural products. The efficacy of biological controls is yet to be established and the possible hazards connected with their use are yet to be analyzed.

Table 1.4. Control measures for diseases and pests of *P. nigrum*

Diseases or Pests	Chemical control	Biological control	Natural products
Foot Rot	Spray 1 % Bordeaux mixture and drench with 0.2% copper oxychloride	Vesicular Arbuscular Mycorrhizal (VAM) fungi and <i>Trichoderma</i> spp. and <i>Gliocladium</i> spp.	Extracts of <i>Chromolaena odorata</i> , <i>P. colubrinum</i>
Pollu beetle	Spray endosulfan or quinalphos (0.05%)	Not Known	Neem products
Scale insects	Spray dimethoate or monocrotophos (0.1%)	<i>Encarsia lounsburyi</i> , <i>Pseudoscymnus</i> spp., <i>Chilcorus</i> spp.	Not Known
Leaf gall Thrips	Spray monocrotophos or dimethoate(0.05%)	Insects like <i>Androthrips flaviceps</i> , <i>Montandoniola moraguesi</i> .	Not Known
Root knot nematode	Fumigation of soil with methyl bromide (500g/ 100cft soil)	Not Known	Neem products

B.10.2. Nonconventional techniques:

In black pepper, improvement in yield along with disease and pest resistance is desirable. A rapid propagation technique is also required to provide sufficient quantity of elite planting material. Even though the conventional techniques have contributed to crop improvement immensely, development of foot-rot resistant black pepper may need the intervention of innovative biotechnological methods. The supplementation of

conventional breeding techniques with novel biotechnological techniques such as *in vitro* morphogenesis, protoplast-mediated asymmetric hybridization and genetic transformation could be useful in accomplishing the goals of improvement.

At present the biotechnological work on *Piper* spp. is focused at *in vitro* cloning of economically important spp. such as *P. nigrum*, (Broome and Zimmerman, 1978; Chua, 1981; Mathews and Rao, 1984; Fitchet, 1990 a, b; Geetha *et al.*, 1990; Nazeem *et al.*, 1992; Babu *et al.*, 1994; Philip *et al.*, 1994; Bhat *et al.*, 1995 and Joseph *et al.*, 1997); *P. betel*, (Aminuddin *et al.*, 1993; Babu *et al.*, 1992 b); *P. longum* (Bhat *et al.*, 1992, 1995; Babu *et al.*, 1993) etc. and *in vitro* conservation of endangered spp. (Babu *et al.*, 1992 a, 1994). Other aspects of plant tissue culture such as protoplast culture, genetic transformation and secondary metabolite production are in the early stages of experimentation. Various reports on *in vitro* establishment of cultures of *Piper* spp including *P. nigrum* are represented in Tables 1.5 and 1.6. respectively.

Morphogenesis *in vitro*: This aspect of tissue culture has received the highest attention in *Piper* spp., similar to all other plant species in general. Morphogenetic studies include both organogenetic and embryogenetic pathways of plant regeneration. The first report on *in vitro* cultures of black pepper was by Broome and Zimmerman (1978) followed by Chua (1981) and Chua and Talib (1985). Mathews and Rao (1984) first published protocol for *in vitro* clonal multiplication of black pepper using seedling explants. In this first report on micropropagation using juvenile explants in cv. Panniyur-1, MS medium supplemented with BA and IAA, each at 1 mg l⁻¹ supported formation of multiple shoots from shoot apex and nodal explants. All the other explants like leaf, hypocotyl, stem, petiole, inflorescence and green drupes formed callus in MS medium containing auxins and cytokinins (Mathews and Rao 1984). The attempts to induce morphogenesis in mature explants resulted only in callus formation (Geetha *et al.* 1990). Nazeem *et al.* (1992) first established callus-mediated plant regeneration in mature leaf segments of black pepper using MS medium supplemented with BA and IAA (0.5-2.0 mg l⁻¹). Besides these attempts, a few other workers have reported *in vitro* clonal multiplication of black pepper (Fitchet 1990a, b; Nazeem, 1992; Philip *et al.*, 1992; Rema *et al.*, 1995; Bhat *et al.*, 1995; Philip *et al.*, 1996). However most of this work was centered around cv. Panniyur-1 and Kuching. The relatively low number of publications on *in vitro* studies in

black pepper is partly due to severe contamination problem hampering tissue culture work at the initial stage of establishment of aseptic cultures. Many authors have discussed the problem of fungal and bacterial contaminants in establishing *Piper* cultures. (Mathews and Rao, 1984; Fitchet, 1988, 1990; Nazeem *et al.*, 1992; Meyer *et al.*, 1992; Bhat *et al.*, 1992, 1995; Aminuddin *et al.*, 1993; Louis *et al.*, 1994; Philip *et al.*, 1994; Kelkar *et al.* 1996).

Somatic embryogenesis in black pepper using SH basal medium at half strength has been reported (Joseph *et al.*, 1996).

Genetic transformation studies: The beginning of genetic transformation studies in black pepper is marked by reports on kanamycin sensitivity study of black pepper cotyledon cultures and co-cultivation of cotyledon explants with *Agrobacterium* (Sasikumar and Veluthambi, 1994, 1996).

Somaclonal variation: Selection of somaclones from callus cultures tolerant to culture filtrate of *Phytophthora* and regeneration of plants has been reported (Shylaja, 1996).

Secondary metabolite production *in vitro*: A preliminary report on manipulation of callus culture technique for production of piperine *in vitro* has been published (Agrawal and Patwardhan, 1994).

***Piper colubrinum* Link. (Wild pepper)**

B.11. Origin and distribution:

The plant is of neo-tropical origin and is native of Brazil. It occurs wild in the forests of Brazil. Its distribution is limited to the same area although now it grows well in other black pepper growing countries like India where it was introduced as a donor parent in breeding programs for developing foot-rot resistant black pepper. Its classification is also controversial. In two recent taxonomic treatments (Steyermark, 1984; Tebbs, 1990), it has been classified as a synonym of *P. divaricatum* G. Meyer. Although Tebbs included it under section 'Churumayu', Callejas (1986) included it in subgenus 'Peltobryon' in his primary cladistic analysis of the genus *Piper* (Bornstein, personal communication).

B.12. Morphology:

Morphologically the two species *P. nigrum* and *P. colubrinum* differ drastically. *P. colubrinum* is a shrub (Fig. 1.2A) of medium height (5-8 feet) in contrast to the climbing habit of *P. nigrum*. It has a slender woody erect stem with long internodes and swollen nodes bearing short thick aerial roots. Since the plant does not need support, the presence of aerial roots appears to be a residual feature of its progenitors with climbing habit as reflected by the morphology of the aerial roots which appear hardly suitable for climbing purpose. The branching is dimorphic with erect aerial branches appearing in axils of leaves and underground runner stems serving the purpose of vegetative propagation. Aerial stems bear simple petiolate leaves with long, lanceolate lamina and acute apex. They are arranged in opposite phyllotaxy at each node. The sheathing leaf bases partially cover the nodes. The inflorescence is an erect spike shorter than that of *P. nigrum* and is borne directly on stems in axil of leaves. The flowers are minute, sessile and unisexual.

B.13. Importance:

Being a wild species, no economic uses are described in literature. The phytochemical estimations to find out chemical constituents might yield important information regarding presence of useful chemicals such as terpenes and alkaloids.

Fig. 1.2. Morphology of wild pepper.*Piper colubrinum* Link.

A. Two year old stem cutting of wild pepper in Green-house.

FIG 1.2



The importance of this species lies in its total resistance to the foot-rot causing fungus *Phytophthora capsici*. Out of 32 species of *Piper* screened for resistance to *Phytophthora*, *P. colubrinum* was discovered to be totally resistant (Turner, 1971). It is also reported to be resistant to *Fusarium solani*, the fungus causing slow wilt of black pepper and to the plant parasitic nematodes *Radopholus similis* and *Meloidogyne incognita*.

B.14. In vitro studies on *Piper colubrinum*:

Participation of *P. colubrinum* as the donor parent for disease and pest resistance in *P. nigrum* improvement program has been attempted. The use of *P. colubrinum* as rootstock was rendered unsuccessful due to the graft incompatibility between the two species. Sexual hybridization (IISR Annual Report, 1990) has not been reported to be successful as yet, suggesting possibility of reproductive isolation of the two species. Protoplast fusion has been described to overcome this barrier in other plants (Ochatt *et al.*, 1989) and could be the appropriate alternative for combining useful characters of *P. colubrinum* and *P. nigrum*.

As a pre-requisite for protoplast technology, *in vitro* plant regeneration has been standardized and reported in *P. colubrinum* (Kelkar and Krishnamurthy, 1993; Babu *et al.*, 1994; Kelkar *et al.*, 1996; Kelkar and Krishnamurthy, 1997, 1998).

Table 1.5 Tissue culture studies carried out so far in *Piper* spp.

Name of the spp.	Medium	Additives used (mg.l ⁻¹)	Explant	Result	Reference
<i>P. betel</i>	WPM	BA (3) + Kin (1)	Shoot tip & Stem	Multiple shoots	Babu KN <i>et al.</i> , (1992)
			Leaf + petiole & Root	Callus + shoots	
		Nil	<i>In vitro</i> shoots	Rooting	
	MS	NAA (3) + BA (0.05)	Shoot tip	Callus	Aminuddin <i>et al.</i> , (1993)
		Kin (0.9) + IAA (0.5)	Callus	Multiple shoots	
	B ₅	BA (1,2,5,10)	Nodes	Multiple shoots	Bhat SR <i>et al.</i> , (1995)
<i>P. chaba</i>	WPM	BA (3) + Kin (1)	Leaf, Stem	Shoots	Babu KN <i>et al.</i> , (1994)
		Nil	<i>In vitro</i> shoots	Rooting	
<i>P. colubrinum</i>	1/2 MS	BA (1,2) + NAA (0.5)/ 2,4-D (1)	Leaf	Callus + Shoots	Kelkar SM <i>et al.</i> , (1993, 1996)
		IAA/IBA (0, 0.5, 1)		Rooting	
	1/2 WPM	BA (3) + Kin (1)	Shoot tip, Leaf	Shoots +/- Callus	Babu KN <i>et al.</i> , (1994)
		Nil	Root	Shoots	
	1/2 MS	BA(0.2,0.5,2,5, 10)	Shoot tip, Node	Multiple shoots	Kelkar SM <i>et al.</i> , (1996)
		BA(0.2,0.5,2,5, 10)	Internode Leaf Root Petiole	<i>de novo</i> Shoots	Kelkar SM and Krishnamurthy KV, (1998)
	Soil	Nil	<i>In vitro</i> shoots	Rooting	
<i>P. longum</i>	MS	BA (0.5-2)	Nodes	Sprouting	Bhat SR <i>et al.</i> , (1992)
		BA (1,1.5,2) + IAA (0.5, 1), Kin (1,1.5)/ 2-iP (1.5) + IAA (0.5)	<i>In vitro</i> nodes	Callus & shoots	
	1/2 MS	IAA (0.2)	<i>In vitro</i> shoots	Rooting	

Table 1.5 contd.

Name of the spp.	Medium	Additives used (mg.l ⁻¹)	Explant	Result	Reference
<i>P. longum</i>		BA (2.5)	Node	Multiple shoots +/- callus	
		BA (3)	Leaf & Callus	<i>de novo</i> shoots	
		2,4-D (1,2) + BA (1)	Leaf & stem	callus	
		Nil	<i>In vitro</i> shoots	Rooting	
	B ₅	BA (1,2,5), + Kin (10)	Root	Shoots	Bhat SR <i>et al.</i> , (1995)
		BA (10)		Callus	
		Kin (1,2,5,10)		Callus + Shoots	
		BA (1,2,5)	Leaf	Shoots	
		BA (10), Kin (1,2,5,10)		Callus	
		BA (1,2,5,10)	Stem	Callus + Shoots	
		BA (1,2)	Node	Callus + Roots	
		Kin (5,10)		Callus + Shoots	
	WPM	BA(3) + Kin (3)	Leaf, root, Stem	Shoots	Babu KN, <i>et al.</i> , (1994)
		Nil	<i>In vitro</i> shoots	Rooting	

Table 1.6 Tissue culture studies reported so far in black pepper (*Piper nigrum* L.)

Sr. No	Medium	Additives & Conc. (mg.l ⁻¹)	Explant	Response	Reference
1	MS	Nil	Axillary Bud	Sprouting	Chua BK, (1981)
2	MS	BA (1) + IAA (1)	Shoot Tip Node (seedling)	Multiple shoots	Mathews H, Rao PS, (1984)
		BA (5) + IAA (5)	Leaf petiole (seedling)	Callus	
		NAA (1)	Stem (seedling)	Root	
		CM (15%) + NAA/ 2,4-D /IAA /pCPA (1)		Callus	
		NAA (1)	Hypocotyl	Root	
		BA/Kin/2-iP (0.1-1) + NAA/2,4-D IAA /p-CPA (1)	Spike	Callus	
		BA (2) + IAA (1) + AdSO ₄ (40)/NOA(1) BA (5) + 2,4-D (2), CH (1000)	Green Drupes	Callus	
3	MS	Phloroglucinol, (0.1-1) Phlorodazin (0.1-1), NAA (0.1)	Axillary sprouts	Rooting	Chua BK and Talib SS (1985)
4	MS	BA (5) + NAA (0.1) + l-tyrosine (100) + AdSO ₄ (160) + NaH ₂ PO ₄ (340)	Axillary Bud	Sprouting of explant	Fitchet M, (1988)
			Stem	Callus	
5	MS	BA (1)	Axillary Bud	Multiple shoots	Fitchet M, (1990 a)
		Nil		Sprouting	
6	MS	Kinetin (1-5) + NAA (0.5)	Leaf	Callus	Geetha <i>et al.</i> , (1990)
7	MS	BA / Kin (1-5) + IAA (0.5, 1) /NAA (0.2,0.5,1)	Axillary Bud	Sprouting	Nazeem P, <i>et al.</i> , (1992)
8	MS	BA (1.5, 3, 5, 10) +/- IBA (0.1) +/- AdSO ₄ (160)	Shoot Tip (mature plant)	Multiple shoots	Philip VJ, <i>et al.</i> , (1992)

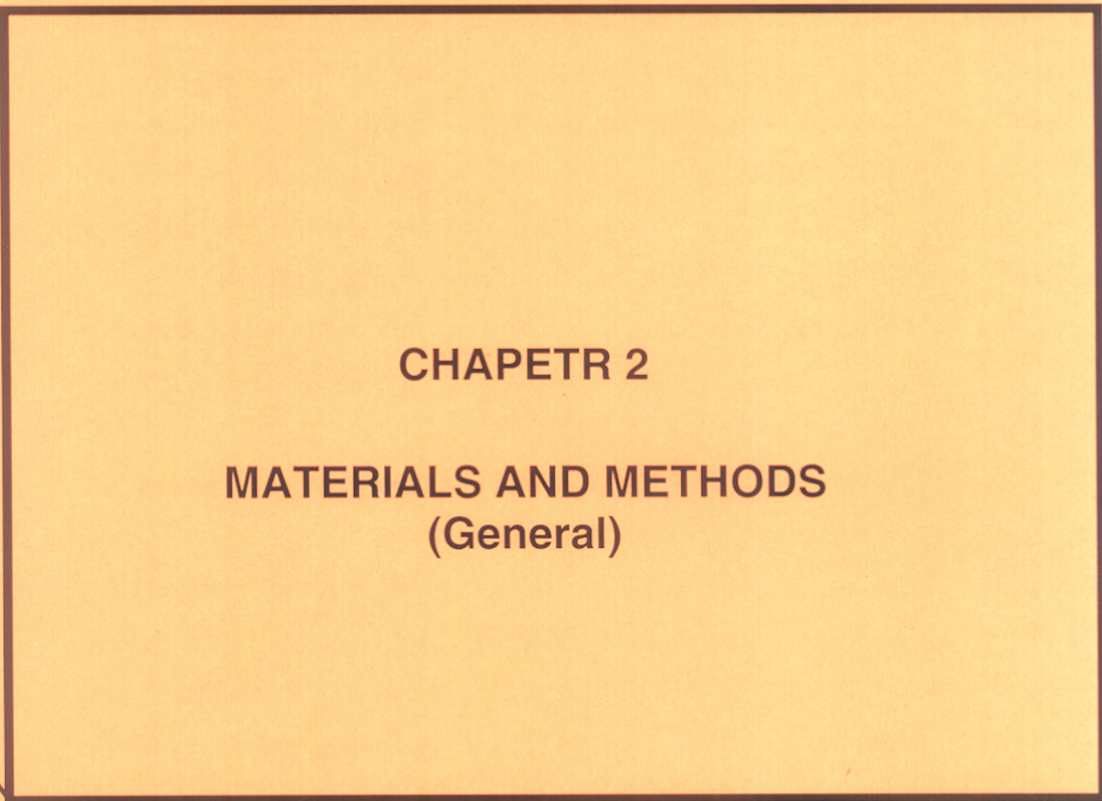
Table 1.6 contd.

Sr. No	Medium	Additives & Conc. (mg.l ⁻¹)	Explant	Response	Reference
9	MS	BA (1,2,5,10)	Node	Multiple shoots	Bhat SR, <i>et al.</i> , (1995)
		Kin (1,2,5,10)		Sprouting	
		BA (1,2,5,10)	Leaf, Root (mature)	Callus	
10	MS	BA (0.8- 1.2) + IAA (0.5- 1.2)	Stem, Leaf (mature), Epicotyl, Hypocotyl	Callus	Philip S, <i>et al.</i> , (1996)
		BA (1) + IAA (1)	Leaf (seedling)	Callus + shoots	
11	1/2 SH	Nil	Embryo	Callus + Somatic embryos	Joseph B, <i>et al.</i> , (1996)

Aims of the Thesis:

The foot-rot caused by *Phytophthora* and associated organisms is the major constraint in pepper improvement. Since the conventional methods for developing total resistance to foot-rot have not proved to be satisfactory, novel biotechnological methods could be an alternate approach. The transfer of disease resistance trait/s from wild pepper *P. colubrinum* to cultivated black pepper *Piper nigrum* varieties and /or selective transfer of disease resistance gene/s is anticipated to produce black pepper cultivars with disease resistance with high expectations. Protoplast-mediated asymmetric hybridization could be a logical proposition especially when successful sexual hybridization between these two species has not been established. Transformation of these two parents with selectable marker genes could facilitate the selection of hybrids and would also fulfill one of the pre-requisites for selective transfer of disease resistance genes to cultivars of black pepper. Developing an efficient *in vitro* regeneration system in both the parents forms an important pre-requisite. Mass multiplication protocols also can be utilized for overcoming the scarcity of planting material. For these reasons the work presented in this thesis was aimed at :

1. Characterization and identification of common microbial contaminants associated with mature explants of *Piper nigrum* and *P. colubrinum* and control of these contaminants as well as phenolic exudates and establishment of sterile cultures.
2. Development of direct and/or indirect plant regeneration protocol with mature explants of both the species.
3. Standardization of an efficient direct plant regeneration protocol with juvenile explants of both the species.
4. Initial experiments on protoplast isolation and standardization of *Agrobacterium* mediated genetic transformation procedures for at least one of the species with explants derived from *in vitro* raised plants.



CHAPETR 2

MATERIALS AND METHODS (General)

During the course of work presented in this thesis, different techniques related to plant tissue culture and microbial culture and characterization were handled. The procedures routinely followed to accomplish the *in vitro* culture work are described in this Chapter. The modifications to routine procedures and the use of specific procedures are specified in respective chapters.

2.1. Glassware and Plasticware: The glassware required for culture work and media preparation included test tubes (22 and 10 mm in diameter), conical flasks and beakers (100, 250, 500, 1000 and 2000 ml. capacity) and pipettes (1, 2, 5 and 10 ml. capacity) and was obtained from Borosil, India. The glassware used was washed with acid, soap and then with distilled water followed by dry sterilization at 150 °C for 3 h. and autoclaving at 121°C for 1h (Sagare, 1996).

The plasticware mainly comprised of disposable sterile petri-dishes (35, 55 and 85 mm in diameter) and disposable filter sterilization units from Laxbro India. The tips for micro-pipettes were obtained from Biorad, USA.

2.2. Preparation of culture media:

2.2.1. Plant tissue culture media: The chemicals used for preparation of culture media were of analytical grade. Inorganic salts, sucrose and Agar-agar were obtained from Qualigens, S. D's fine Chemicals and Hi-Media, India. All vitamins and growth regulators and the gelling agent phytigel were obtained from Sigma Chemicals Co., USA. After dissolving all constituents of media in glass distilled water, the pH was adjusted to 5.8 using 0.1 N KOH or 0.1 N HCl. For solid media the gelling agent (agar-agar or phytigel) was added as per requirement and the medium was steamed to homogenize the gelling agent. The homogenized medium was dispensed in appropriate culture vessels and autoclaved at 121°C, 100 kPa for 20 min. Heat labile ingredients such as antibiotics and some growth regulators were filter sterilized and added to the autoclaved media aseptically. The compositions of different basal media used are given in Table 2.1.

2.2.2. Microbial media: Bacterial and fungal contaminants were isolated from cultured explants at various stages of differentiation. Nutrient broth or nutrient agar prepared using dehydrated medium (Difco laboratories, USA) and Potato Dextrose Agar (PDA) were used as maintenance media for bacterial and fungal contaminants respectively. The media were prepared according to the manufacturer's instructions.

2.3. Maintenance of source plants: Rooted stem cuttings (6-8 year old) of three cultivars of black pepper, *Piper nigrum* L. namely, Panniyur-1, Karimunda and Subhakara, as well as of wild pepper, *Piper colubrinum* Link. were maintained as source plants under greenhouse conditions (Temperature, 30 ± 2^0 C; Relative humidity 70 %). The plants were watered every alternate day using roof top sprinklers. The plants were given prescribed dose of fertilizers (Purseglove, 1987) and were treated with fungicides and insecticides at regular intervals (Chapter3 of this thesis).

2.4. Pretreatments and culture of explants:

Mature as well as juvenile explants were used for tissue culture studies. The mature explants were derived from 4-6 year old greenhouse grown plants of *Piper colubrinum* and 3 cultivars of *Piper nigrum* (Panniyur-1, Karimunda and Subhakara). The juvenile explants were obtained from *in vitro* raised 50-60 days old seedlings of 4 cultivars of *Piper nigrum* (Panniyur-1, Karimunda, Kuthiravally and Kuching).

The leaf, stem, and meristem explants derived from mature plants of both the species were washed under running tap water for 5-10 min, treated with 1% (v/v) detergent (Labolene® India) for 5 min and rinsed with distilled water. The explants were agitated in 1% aqueous solution of the systemic fungicide Bavistin (BASF, India) for 30 min to 2 h (as per the explant type used) followed by surface disinfection with 70% ethanol for 5 min and thorough rinsing with distilled water. The explants were then transferred to sterile conical flasks in a laminar air flow cabinet and surface serialized with 0.1% mercuric chloride solution for 5 (leaf and shoot tip explants) or 10 min (nodes and internodes) and rinsed 5-6 times with sterile distilled water.

Seeds of seven cultivars of *P. nigrum* namely, Panniyur-1, Karimunda, Kuthiravally, Kuching, Aimpiriyam, Valiakaniakadan and Panchami were germinated *in*

vitro to obtain juvenile explants. The red, ripe fruits were obtained from pepper farms of Konkan Krishi Vidyapeeth, Dapoli; Kerala Agriculture University and Indian Institute for Spices Research, Kerala. The epicarp and fleshy mesocarp were crushed and seeds were removed. The seeds were washed under running tap water for 30-40 min and then treated with sulfuric acid (25%, v/v) for five min and thoroughly washed with water. Then 100 seeds were transferred to one 250 ml sterile conical flask in a laminar air flow cabinet and were surface sterilized with 0.1% mercuric chloride for 10 min followed by 5-6 rinses with sterile distilled water. The seeds were agitated with approximately 150 ml sterile distilled water for 16h on a gyrotatory shaker at 100 rpm in dark.

All the explants including seeds were cultured in a laminar air flow cabinet. Dissection, excision of unwanted parts and transfer of explants, was carried out on sterile filter paper using autoclaved forceps and blade holders with sterile surgical blades. The forceps and blades were dipped in alcohol and flamed after each transfer.

2.5. Culture conditions:

The explants cultured in solid media were incubated at $25 \pm 1^{\circ}\text{C}$ in dark or at light intensities of 4.6 and $38.0 \mu\text{E m}^{-2} \text{s}^{-1}$ provided by cool fluorescent tube-lights for 16 h or 24 h photoperiod. When liquid medium was used in tubes the explants were cultured on Whatman No. 1 filter paper floats and maintained stationary. When liquid medium was used in conical flasks, the cultures were incubated on a gyrotary shaker (Steelmate, India) at 100 rpm and 24 h photoperiod. Culture condition requirements differed for different explants and cultures derived therefrom at different growth stages. The details of such culture conditions of each experimental set would be provided separately.

The fungal contaminants inoculated on slants of PDA were incubated at $25 \pm 1^{\circ}\text{C}$ since that temperature supported their growth in plant tissue cultures. After profuse growth, the slants were stored at $4 \pm 1^{\circ}\text{C}$.

The bacterial isolates were cultured by inoculating single colonies in nutrient broth and placed on a shaker at 200 rpm at $27 \pm 1^{\circ}\text{C}$. Single colonies of pure bacterial isolates grown on plates of nutrient agar were used as the source of pure cultures. These cultures in log phase of growth were stored in glycerol at -70°C .

2.6. Histology:

Explants at different stages of differentiation were fixed in Formalin: Acetic acid: Alcohol (1: 1: 20) for 72 h. The fixative was decanted and the explants were dehydrated in tertiary butanol series (Sharma and Sharma, 1980). Dehydrated explants were then embedded in paraffin wax (E Merck, India Ltd. India; melting point 58 to 60⁰ C). Serial sections of 10 µm thickness were cut using a rotary microtome (Reichert Jung 2050, Supercut, Germany). The sections were heat-fixed to the slides. The slides were then passed through xylene-alcohol series (Sharma and Sharma, 1980). and stained with 1% Heidenhein's hematoxylin (Heidenhein 1892; see Gurr, 1960) with or without and counter-staining with 1% eosin and mounted in DPX mountant (Purvis *et al.*, 1964) .

2.7. Hardening of in vitro plants/shoots:

The shoots derived from different explants were rooted *in vitro* or *ex vitro*. The *in vitro* rooted shoots i.e. plantlets or unrooted shoots were carefully removed from the culture vessel and washed by gently shaking in a beaker containing water to remove agar and the medium sticking to the surface. The shoots were cut just below the last node before transferring to soil. Plantlets and cut shoots were dipped in 1% aqueous solution of the systemic fungicide bavistin (BASF, India) for 10 -15 min and then washed with fine spray of water. Treated plantlets and shoots were transferred to pots or plastic containers filled with a mixture of sand: soil or Soil: solrite in 1:1 proportion. The pots with plants were kept in moist chambers prepared by covering plastic tubs with glass sheets. Such moist chambers prevented rapid loss of humidity in the tropical environment. These moist chambers were kept in the hardening room at $27 \pm 1^{\circ}$ C for 2 weeks. The hardened plants were sprayed with a solution of MS salts at 1/10 strength twice a week for a period of two weeks. Then the hardened plants were transferred to green house. *In vitro* shoots developed adventitious roots within 3-4 weeks. Well-grown plants were shifted to bigger pots after 2-3 months.

Table 2.1: Composition of macro, micro element salts and organics in different basal media. (Owen and Miller, 1992).

INGREDIENTS (mg l ⁻¹)	MS	B ₅	WH	WPM	SH	BD	V-47
Macronutrients							
KNO ₃	1900	2500	80	-	2500	1000	1480
NH ₄ NO ₃	1650	-	-	400	-	1000	1444
CaCl ₂ · 7H ₂ O	440	150	-	221	200	-	735
Ca(NO ₃) ₂ · 4H ₂ O	-	-	300	556	-	347	-
MgSO ₄ · 7H ₂ O	370	250	750	370	400	35	984
KH ₂ PO ₄	170	-	-	170	-	300	-
KCl	-	-	65	-	-	65	-
NaH ₂ PO ₄	-	150	19	-	-	-	68
Na ₂ SO ₄	-	-	200	-	-	-	-
(NH ₄) ₂ SO ₄	-	134	-	-	-	-	-
K ₂ SO ₄	-	-	-	990	-	-	-
(NH ₄)H ₂ PO ₄	-	-	-	-	300	-	-
Micronutrients							
MnSO ₄ · 4H ₂ O	22.3	-	5	22.3	-	-	5.0
ZnSO ₄ · 7H ₂ O	8.6	2	3	-	1	1.5	1.5
H ₃ BO ₃	6.2	3	1.5	6.2	5	6.2	2.0
NiCl ₂	-	-	0.03	0.024	-	-	
KI	0.83	0.75	0.75	-	1	0.8	0.25
MoO ₃	-	-	0.001	-	-	-	
CuSO ₄ · 5H ₂ O	0.025	0.025	0.001	0.25	0.2	-	0.015

Table 2.1 contd.

INGREDIENTS (mg l ⁻¹)	MS	B ₅	WH	WPM	SH	BD	V-47
Micronutrients							
Na ₂ MoO ₄ . 2H ₂ O	0.25	0.25	-	0.25	0.1	-	0.1
CoCl ₂ .6H ₂ O	0.025	0.025	-	-	0.1	-	
Fe ₂ (SO ₄) ₃	-	-	2.5	-	-	-	
FeSO ₄ . 7H ₂ O	27.8	27.8	-	27.8	15	23.6	27.8
Na ₂ EDTA	37.3	37.3	-	37.3	20	32	37.3
Organics (mg l⁻¹)							
Thiamin. HCl	0.1	10	0.01	1	5	0.1	4.0
Pyridoxine HCl	0.5	1	0.01	0.5	0.5	0.1	0.7
Nicotinic acid	0.5	1	0.05	0.5	5	0.5	4.0
Myo-inositol	00	100	-	100	1000	-	100
Glycine	2	-	3	2	-	2.0	1.4
Biotin	-	-	-	-	-	-	
Folic acid	-	-	-	-	-	-	
Sucrose (gm)	30	20	20	20	30	30	
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8

Note. MS : Murashige and Skoog (1962); B₅ : Gamborg (1968); SH : Schenk and Hildebrandt (1972); White's : White (1963); WPM : Woody plant medium, Lloyd and McCown (1981); BD : Blayde (1966); V-47: Binding (1974)., -: not included in the medium composition.

CHAPTER 3

ESTABLISHMENT OF CULTURES *IN VITRO*

A study of characterization and control of microbial contaminants and phenolic oxidation associated with mature explants of *Piper* spp

3.1. Introduction:

Plant tissue culture involves culture of 'aseptic' cells or tissues *in vitro* (Leifert, 1994). It finds wide application in basic as well as applied aspects of plant sciences (Table 1.1 and 1.2 of this Thesis). Although the number of plants established in cultures is increasing every day, the list is predominated by herbaceous species. The earliest success in plant tissue culture was achieved in woody plants. However the rate of progress in this field was relatively slow during last 4 decades owing to the large number of bacterial and fungal contaminants associated with woody perennials and also due to autotoxic phenolic exudations produced by the woody plants (Young, *et al.*, 1984;).

3.1.1. Microbial contamination: The presence of microbial contaminants is suggested to be the major single problem in plant tissue cultures especially in tropical species (Kneifel and Leonhardt, 1992; Parkinson, *et al.*, 1996). The contaminants of plant tissue cultures include yeasts, filamentous fungi, bacteria and mycoplasma-like organisms (Leifert, *et al.*, 1992, 1994; Philip, *et al.*, 1992). However the most commonly reported contaminants are bacteria and fungi.

The bacterial and fungal contaminants infest the cultures at two different frequencies. Based on the frequency, two types of the contaminants were identified: constant and sudden. The plant tissue cultures especially woody plants show a steady relatively unchanged rate of contamination that is caused by the 'constant' contaminants. These constant contaminants are associated with the plants and introduced through explants or due to insufficient surface sterilization. The endophytic nature of several bacteria and fungi sharing different ecological associations with the plants supports this assumption (Knogge, 1996; Alfano and Collmer, 1996).

In addition there are 'sudden' outbreaks of bacterial as well as fungal contaminants. Sudden fungal contamination is often associated with mites and thrips, the arthropods that inhabit the culture rooms and act as vectors of fungal contaminants (Debergh and Maene, 1984; Blake, 1988; Leifert, *et al.*, 1991, 1994). The origin of sudden bacterial contaminants is however dubious. It has been argued that, the latent endophytic bacteria especially 'PPFM's (pink pigmented facultative methylotrophs) are responsible for sudden outbreaks of bacterial contamination (Cornu and Michel, 1987; Cassels, *et al.* 1988; Debergh, *et al.*, 1988). The sudden outbreaks of bacterial

contamination in our laboratory infested diverse plant spp. and different cultures (derived from mature, juvenile, even embryonal cultures), some of which were maintained apparently sterile for 3 years or more. So sudden bacterial contaminants may have originated from other sources as well. These sources include laboratory atmosphere and the workers.

Hazards of microbial contamination: It has been estimated that product loss due to contamination averages between 3-15% at every subculture in a majority of commercial production units and scientific plant tissue culture laboratories (Leifert, *et al.*, 1994). The commercial production units and scientific plant tissue culture laboratories both are adversely affected by the problem of microbial contamination with the same intensity. For the commercial units, contamination makes propagation of some commercially important species uneconomical, it also results in severe production losses and more often rejection of massive consignments due to quarantine regulations.

In scientific research laboratories, contamination can cause physiological and genetic changes in the explants leading to variable test results. It also renders the experiments non-reproducible and the analyses of results in such experiments may project false picture. More often it is not possible to carry out experiments at the astronomical sizes which are required to nullify the effects of contamination. The spread of contaminants not specific to hosts may lead to loss of valuable cultures especially those of endangered species. Further, the 'vitropaths' present another potential hazard. These are the bacteria that cause diseases to plants only *in vitro*. These diseases are new, nonexistent *ex vitro*. Considering the high frequency of mutations and variations in bacteria, these vitropaths could become pathogenic and cause epidemics of new diseases *ex vitro*.

Beneficial effects of contaminants: Nevertheless, the contaminants have been found to be beneficial to certain plant cultures *in vitro*. For this reason, Nowak (1996) has coined the terms 'Bacterization' and 'Biotization' of plant tissue cultures for co-existence *in vitro* with useful contaminants and suggested selective elimination of harmful contaminants. It has been reported that co-culture with contaminants associated with cultured plants could result in better growth (Pillay and Nowak, 1997), induction of morphogenesis through cytokinin production (Holland, 1997), regeneration (Saxena, *et*

al., 1996; Nowak, 1996) and rooting (Nowak, 1996), reduction in vitrification (Shetty, *et al.*, 1995, 1997) and control of other pathogens (Nowak, 1996; Wilhelm, 1996; Li, 1996). Due to close association with hosts, the contaminants might acquire useful characteristics of the host through genetic exchanges as exemplified in the *Taxus* spp. and associated fungus *Taxomyces andreanae*. Such associations could be exploited for commercial production of useful natural products (Stierle, *et al.*, 1993).

Control of contamination: Considering the multitude of problems caused by harmful contaminants, various control measures have been suggested. Physical treatments like higher temperature (Grondeau and Samson, 1994; Grum, 1996; Albers, *et al.*, 1996) and acidic pH (Leifert, *et al.*, 1994) as well as a wide array of chemicals to be used as sterilization agents such as commercial bleach and mercuric chloride, most commonly used surface sterilization agents in plant tissue cultures; hydrogen peroxide (Dirks, *et al.*, 1983), diethylpyrocarbonate (Macek, *et al.*, 1995), 8-hydroxy-quinolinol-sulfate (Machado, *et al.*, 1996), sodium dichloroisocyanurate (Parkinson, *et al.*, 1996), sulfite pads (Shure, *et al.*, 1994) and hen egg white lysozyme (Eudes, *et al.*, 1995). In addition, antifungal agents such as Bavistin, Benomyl, Amphotericin-B, Tilt (Watt, *et al.*, 1996) are reported. The use of antibiotics is the most popular therapy used against bacteria (Young, *et al.*, 1984; Falkiner, 1988; Kneifel and Leonhardt, 1992; Marino, *et al.*, 1995, Reed, *et al.*, 1995; Fellner, 1995; Fellner, *et al.*, 1996) .

Necessity of classification of contaminants: The microbe-plant interaction could be deleterious or useful. In either way, the contaminants must be identified and classified. The identification of the microbial contaminants could be useful in determining the control and prevention measures since it helps in tracing the source of contamination (Leifert, *et al.*, 1994). It is also very important in deciding the antimicrobial agent to be used for eliminating or at least controlling the contamination (Reed *et al.*, 1995). Otherwise the blind use of antimicrobial agents for control of contaminants could give rise to resistant bacteria; more difficult to control. Recently, bacteria pathogenic to human beings have been isolated from plant tissue cultures (Leifert, *et al.*, 1994) which could be easily transmitted through human contact and exchange of plants. This further intensifies the need for characterization of contaminants and careful use of antibiotics in

tissue cultures.

In case of beneficial contaminants it is essential to isolate and characterize the organisms to allow selective growth either as a pure culture or as a co-culture along with the plants for further manipulation.

3.1.2. Browning due to phenolic exudations:

Browning of explants and medium is another serious problem of woody plant cultures. It is a result of oxidation of mono- and di-phenols released from the tissue in the surrounding medium (Saxena and Gill, 1986). It is often induced by excision at the cut end of the explant that is in contact with the medium. The phenolics are produced as a wound response. The phenolic oxidation is aided by light and is auto-catalytic. The phenolics leach into the medium and being toxic to explants cause severe losses of cultures. However, the phenolics are also known to be beneficial for the growth and regeneration from explants (Laliberte and Lalonde, 1988) probably through the indirect effect on auxin synthesis. Some of the polyphenols also reduce vitrification and restore normal growth in vitrified cultures (Phan and Hegedus, 1986).

In black pepper, the problem of browning of explants has not been discussed in any of the reports except Fitchet (1990 b). However in present study it was found to be an acute problem.

In *Piper* spp., out of 16 reports on morphogenetic studies using mature material, published in last 20 years 6 reports dealt exclusively with the contamination problems (Fitchet, 1988, 1990 a, b; Aminuddin, *et al.*, (1993); Meyer, *et al.*, 1992; Nazeem, *et al.*, 1994) while 5 others have mentioned the problems of contamination (Philip, *et al.*, 1992; Bhat, *et al.* 1992, Kelkar, *et al.*, 1996; Kelkar and Krisnamurthy, 1998). All these reports except Fitchet, (1990 a, b) have discussed bacterial contamination. Aminuddin, *et al.*, (1993) while working with mature explants of *Piper betel* used a mixture of antibiotics to control bacteria and established sterile cultures. Meyer, *et al.*, (1992) and Nazeem, (1992) isolated and identified the bacterial contaminants of black pepper cultures to be *Pseudomonas* and *Bacillus* respectively. They also have suggested use of antibiotics to control bacteria. Philip, *et al.*, (1992) mentioned reduction in bacterial contamination of shoot tip explants of black pepper by use of liquid medium and filter paper floats, whereas Bhat *et al.*, (1992) in *P. longum* and Fitchet (1988) in black pepper re-sterilized

explants with 0.1% mercuric chloride to control contamination. The resterilization procedure reduced bacterial contamination in leaf explants of *P. colubrinum* (Kelkar, *et al.*, 1996). Fitchet (1990 b) carried out several experiments to control fungal and bacterial contaminants together with phenolic oxidation in *P. nigrum* cv. Kuching. However in our laboratory, in defiance of all these reported methods contamination prevailed and none of the contaminants was found to be beneficial. The fungal contamination was found to be the most serious problem that caused culture losses upto 80% or more. The bacterial contamination and phenolic oxidation occurred besides the severe fungal contamination leading to a low level of survival and growth rate of the explants *in vitro*. Thus establishment of cultures *in vitro* was an important step in tissue culture studies in *Piper* spp.

With the aim of successful establishment of cultures in *P. nigrum* and *P. colubrinum*, we carried out isolation and identification of fungi, bacteria associated with mature explants. Different control procedures were tested to combat these contaminants and also the phenolic oxidation responsible for culture losses. In this Chapter, problems encountered in establishment of pepper cultures *in vitro* and strategies followed to overcome the problems are discussed.

3.2. Materials and Methods: Contaminants were isolated from surface sterilized explants of black pepper and wild pepper. Surface sterilization was done with 0.1% solution of mercuric chloride for 5-10 minutes as per previous reports (Chua and Taleb, 1982; Mathews and Rao, 1984; Bhat, *et al.* 1992; Philip, *et al.* 1992). The same surface sterilization treatment was used as control in different experiments carried out to combat contamination.

3.2.1. Isolation, culture and identification of fungal contaminants:

The 'constant' fungal contaminants were most frequently obtained in stem explants of *P. nigrum*. Isolation of constant contaminants was carried out by 'indexing' of mother plants. For this, nodal and internodal segments (2-3 cm long) were excised from 4-6 year old green-house grown plants of 3 cultivars of *P. nigrum* (Panniyur-1, Karimunda and Subhakara). For isolation of fungi at least 10 nodal and internodal explants were collected from each plant. These explants were washed under running tap water for 5-10 min followed by wash with a detergent (Teepol, India) for 5 min. The explants were treated with 70% alcohol for 1 min and then were transferred into sterile 250 ml flasks under aseptic conditions for surface sterilization with 0.1% solution of mercuric chloride for 10-15 minutes as per previous reports (Chua and Talib, 1982; Mathews and Rao, 1984; Bhat, *et al.* 1992; Philip, *et al.*, 1992). Such explants were cultured in tubes containing MS medium and tubes containing PDA (Potato Dextrose Agar) medium (Dynamicro, India) and incubated at $25 \pm 2^\circ \text{C}$ at a light intensity of $38 \mu\text{E m}^{-2}\text{s}^{-1}$. The fungal growth occurred in 10-15 days in both media. Often the fungi occurred as mixed cultures. The fungal contaminants were separated based on morphological characters. Then the fungal growth was further cultured in slants of PDA by hyphal tip method and the cultures were stored at 4°C . Same procedure was repeated for the 'sudden' fungal contaminants that occurred in cultures belonging to different plant species. For identification, a parallel set of fungal cultures was handed over to the Mycology Department of Agharkar Institute, Pune, India.

3.2.2. Isolation, culture and identification of bacterial contaminants:

The occurrence of bacterial contaminants was found in leaf as well as stem explants of both the species of *Piper*. Therefore, leaves and stems of both the species

were used for isolation of bacteria. The stem explants included nodes as well as internodes. The explants were surface sterilized by above mentioned method and cultured in half MS medium (MS medium with major elements at half strength) supplemented with BA (3.0mg l^{-1}). The cultures were incubated at the above mentioned conditions.

The contaminant bacteria appeared after 10-15 days as a halo at the base of the explant. Bacterial growth visible in medium was transferred to nutrient broth (Difco, would be abbreviated as NB in the present work) with a sterile wire loop. These bacteria were grown in an incubator-shaker at $26 \pm 2^\circ\text{C}$ at 200 rpm until visibly turbid. A loopful of each of these suspensions was streaked onto sterile plates of nutrient agar (Difco, would be abbreviated as NA in the present work) to isolate single colonies and to check purity of isolates. The single colonies appeared within 2-3 days. Morphologically dissimilar colonies were purified by serial dilution method (Bradbury, 1988). For short-term storage the pure cultures were maintained on NA plates at 4°C whereas long term storage was achieved by preparation of glycerol stocks stored at -70°C .

For identification and classification of bacteria, several morphological (colony characterization, Gram staining and motility) and biochemical tests (oxidase, catalase, Oxidative/Fermentation metabolism, starch hydrolysis, soft rot of potato, nitrate reductase, utilization of sugars, levan formation, Methyl Red (MR) test, Vogue-Prosaukers (VP) test, citrate utilization, indol production, urease production, ability to grow at different temperatures, I-C compound utilization, NaCl tolerance) were carried out according to Bradbury, (1988) and Bergey's Manual of determinative bacteriology (1994).

3.2.3. Control of fungal and bacterial contaminants:

The preponderance of bacterial and fungal contaminants was observed in stem (nodes as well as internode) explants. Hence, stem explants were used for optimization of surface sterilization treatments and pre-treatments for minimizing contamination. The favorable treatments were further manipulated for other explants like leaf, shoot apices, roots and inflorescences.

Thermotherapy: The nodal explants derived from *Piper* spp. were washed under running tap water, wiped with 70% ethanol and blotted on filter paper. Then the explants

were incubated at 40° C and 60° C in a water bath (Newtron , India) as well as in an oven for 10 and 30 min. The explants were then surface-sterilized with 0.1% mercuric chloride for 10 min, washed with sterile distilled water 5-6 times and then cultured in petri-dishes containing half MS basal medium.

Medium acidification: Nodal explants surface sterilized with 0.1% mercuric chloride for 10 min and washed with sterile distilled water were cultured on filter paper floats in tubes containing liquid half MS medium at different pH values 3.5, 4.5, 5.6. Half MS at 5.6 served as a control for this experiment.

Running water treatment: The nodal explants were washed under running tap water overnight to remove all dirt containing the microbes sticking to the explant surfaces. Then the explants were routinely surface sterilized with 0.1% mercuric chloride and cultured in tubes containing liquid half MS medium.

Standardization of surface sterilization procedure: Nodal explants derived from green-house grown 4-5 years old plants of *Piper* spp. were washed with tap water and surface disinfected with 70% ethanol for 1 min followed by 3 rinses of distilled water. Such explants were used to standardize surface sterilization method. Three surface sterilizing agents were used to treat nodal explants: mercuric chloride (0.1, 0.5, 1.0%) with or without vacuum (at -10 kPa, using Millipore vacuum pump), commercial bleach Medisor-M (1 and 2 % solution v/v) and hydrogen peroxide (1%, Dirks *et al.*, 1986) for different durations (5, 10, 20, 30 min). Following surface sterilization and rinsing with sterile water, the explants were cultured in petridishes containing half MS medium (MS medium with major salts at half strength).

Use of fungicides: This strategy for control of fungal contaminants was operated at the level of mother plant and the explants. The mother plants grown in the green-house were periodically (every 3-4 weeks) sprayed with insecticides Endosulfan (0.05%) and monocrotophos (0.1%) for control of mites and other insects which act as vectors for fungi. The mother plants were sprayed once in a month with 2% aqueous solution of systemic fungicide Bavistin (BASF, India) and 0.2% solution of copper oxychloride. The same fungicides were used for drenching the soil.

At the explant level, the treatment with aqueous solution of fungicides Bavistin (0, 0.5, 1.0 and 5.0%, BASF, India) for 30, 60 and 120 minutes; or with Ha-mycin or

Aureofungin (50, 100mg l⁻¹, Hindustan Antibiotics, India) or 8-hydroxy-quinolinol sulfate (0.1, and 0.5 % , Sigma, USA) for 5 and 12h was carried out prior to surface sterilization with 0.1% mercuric chloride for 10 min. The explants were then cultured in half MS basal medium in petridishes.

Use of antibiotics to control bacterial contamination:

A. AntibioGramme assay: Susceptibility of the Bacterial isolates to different antibiotics was assayed by using Biodisc-12 (Pathoteq Laboratories, India) by top agar method (Bradbury, 1988). For each isolate, 50µl of 48 h grown culture in nutrient broth was used for preparing top agar petridishes. Sterile antibiotic discs (filter paper disc impregnated with a known quantity of antibiotic agents) were placed in the top-agar petri dishes which were incubated at 37⁰C for 24h in dark. The efficacy of individual antibiotic agent was deduced by measuring the diameter of clearing zone after incubation of bacteria for 24 h in dark.

B. Antibiotic assay in liquid medium: The concentration of each antibiotic used in Biodisc-12 was taken as a baseline and employed for determining the minimal bactericidal concentrations. In addition, one concentration lower and one concentration higher than the concentration used in Biodisc-12 was aseptically added to NB and the presence or absence of bacterial growth in antibiotic medium was determined by turbidity of the medium. The results of liquid antibiotic assay were confirmed by streaking on NA.

Table 3.1. Antibiotics and their concentrations given in Biodisc-12 (Pathoteq Laboratories, India).

No.	Antibiotic Agent	Symbol	Concentration
1	Ampicillin	AS	20
2	Co-Trimoxazole	BA	25
3	Cefotaxime	CF	30
4	Piperacillin	PC	100
5	Chloramphenicol	CH	30
6	Ciprofloxacin	CP	5
7	Ceftizoxime	CI	30
8	Tetracycline	TE	30
9	Ofloxacin	OF	5
10	Gentamicin	GM	10
11	Amikacin	AK	30
12	Pefloxacin	PF	10

3.2.3. Control of phenolic exudations and browning of explants and medium:

To control browning of explants caused by phenolics, nodal explants surface sterilized with 0.1% mercuric chloride were cultured in full MS medium containing anti-oxidants ascorbic acid, citric acid, cystine, MES hydrate (100, 150mg^l⁻¹ each) and polyvinyl pyrrolidone (500 and 1000mg^l⁻¹). Dark incubation of cultures, use of half strength MS medium for culture of explants, incorporation of activated charcoal (0.5%) in the medium and sealing the cut ends of nodal explants by dipping in molten paraffin wax (60⁰ C melting point) were the other strategies followed to resist browning of the explants. In yet another experiment, the explants cultured in petridishes were transferred from one position to other in the same dish every alternate day for first 10 -15 days.

3.3. Results and Discussion:

Establishment of sterile cultures from mature explants of *Piper* spp. was hampered by three major problems i.e. fungal contamination, bacterial contamination and phenolic oxidation. The magnitude of these problems showed intraspecific as well as interspecific variations. In addition, different explant types i.e. nodes, leaves and shoot apices also exhibited differences in percentages of fungal contamination, bacterial contamination and phenolic oxidation. Table 3.2 represents means of percentage of presence of constant contaminants and phenolic oxidation in 3 explant types of *P. colubrinum* and 3 cultivars of *P. nigrum* in one year.

Fungal contamination was the most serious problem in cultivars of *P. nigrum*. However in *P. colubrinum* lowest percentage of fungal contamination was found in all three explant types. Among cultivars of *P. nigrum*, Subhakara was less susceptible to fungal contamination than cv. Panniyur-1 and Karimunda. Among the explant types, nodes of all three cultivars of *P. nigrum* bore significantly higher percentage of contamination than shoot apices and leaf explants. In *P. colubrinum*, the three explant types were comparable for contamination percentage (Table 3.2).

There were no species specific differences for bacterial contamination in the nodal explants and *P. colubrinum* and three cultivars of *P. nigrum* showed comparable percentages for nodal explants. For shoot apices, the rate of contamination was highest in cv. Panniyur-1, significantly low in cv. Karimunda and intermediate in cv. Subhakara. Shoot apices of *P. colubrinum* showed bacterial contamination comparable to those of cv. Karimunda of black pepper. Leaf explants of cv. Panniyur-1 and cv. Subhakara showed significantly higher bacterial contamination than cv. Karimunda. Leaf explants of *P. colubrinum* presented lowest percentage of bacterial contamination but it was comparable to that of cv. Karimunda. Among the explants, significant differences were observed (Table 3.2) for bacterial contamination in leaf explants of cv. Subhakara and shoot apices of cv. Karimunda.

The problem of phenolic oxidation was most severe in leaf explants (Fig. 3.1A) followed by shoot apices and nodes in all genotypes. Differences among cultivars were insignificant for shoot apex and leaf explants. For nodal explants, *P. colubrinum* showed significantly low phenolic oxidation than all three cultivars of *P. nigrum* (Table 3.2).

Table 3.2. Percentage of constant contaminants and phenolic oxidation in different mature explants of *P. colubrinum* and 3 cultivars of *P. nigrum*.

Explan	Problem	% Response (Mean $\pm$ s.d.)			
		Panniyur-1	Subhakara	Karimunda	<i>P. colubrinum</i>
Node	Fungal	82.0 $\pm$ 6.0 ^{a*}	56.0 $\pm$ 9.0 ^{b*}	82.0 $\pm$ 4.0 ^{a*}	6.0 $\pm$ 2.0 ^c
	Bacterial	47.0 $\pm$ 9.0 ^A	45.0 $\pm$ 5.0 ^A	52.0 $\pm$ 8.0 ^A	35.0 $\pm$ 5.0 ^A
	Phenolics	33.3 $\pm$ 7.0 ^a	36.0 $\pm$ 5.0 ^a	35.3 $\pm$ 9.0 ^a	19.0 $\pm$ 8.0 ^a
Shoot	Fungal	37.0 $\pm$ 6.0 ^a	32.0 $\pm$ 4.0 ^a	40.0 $\pm$ 0.0 ^a	6.0 $\pm$ 2.0 ^b
Apex	Bacterial	55.0 $\pm$ 5.0 ^A	45.0 $\pm$	32.0 $\pm$ 11.0 ^B	29.0 $\pm$ 5.0 ^B
	Phenolics	53.0 $\pm$ 7.0 ^{a+}	63.0 $\pm$ 5.0 ^{b+}	60.0 $\pm$ 11.0 ^{b+}	52.0 $\pm$ 4.0 ^{a+}
Leaf	Fungal	29.0 $\pm$ 6.0 ^a	33.0 $\pm$ 5.0 ^a	38.0 $\pm$ 14.0 ^a	2.6 $\pm$ 2.0 ^b
	Bacterial	67.0 $\pm$	64.0 $\pm$ 7.0 ^{A#}	56.0 $\pm$ 5.0 ^{AB}	39.0 $\pm$ 5.6 ^B
	Phenolics	80.6 $\pm$ 9.0 ^a	72.0 $\pm$ 6.0 ^a	60.0 $\pm$ 0 ^a	76.0 $\pm$ 9.0 ^a

Superscripts in form of letters in lower case, upper case and italics show comparison among genotypes for fungal contamination, bacterial contamination and phenolics respectively.

Values followed by same in same superscripts do not show significant differences among cultivars for each problem type at 95% confidence level.

*, #, + represent significant differences among the explant types of the same genotype for fungal contamination, bacterial contamination and phenolics respectively.

3.3.1 Isolation, culture and identification of fungal contaminants:

The presence of fungal contaminants in culture was detected by growth of mycelia at cut ends of explants or in medium (Fig. 3.1B). The mycelia cultured on PDA slants were identified at the mycology department of Agharkar Research Institute, Pune, India.

A total of 6 fungi i.e. *Fusarium* sp., *Colletotrichum* sp., *Memnoniella echinata* (Riv.) Galloway, *Nigrospora sphaerica* (Sacc.) Mason. and an unidentified vegetative mycelium were found to be associated with sterilized black pepper explants. These constituted the constant contaminants of black pepper cultures. Out of these, 3 fungi, *Fusarium*, *Alternaria* and *Colletotrichum* are plant pathogenic fungi reported to be pathogenic to black pepper (Chapter 1). *Memnoniella echinata* (Riv.) Galloway and *Nigrospora sphaerica* (Sacc.) are commonly found soil fungi. Thus for majority of the fungal contaminants, probable source was the plant material itself. *Fusarium* sp.,

Alternaria and *Colletotrichum* were found in 40-50% contaminated cultures of black pepper cultivars and often occurred as mixed cultures. The other fungi caused moderate culture losses upto 10-12 %. Rare fungal contamination of *P. colubrinum* was caused by *Memnoniella echinata* (Riv.) Galloway and *Nigrospora sphaerica* (Sacc.).

Among the sudden contaminants, another species of *Fusarium*; *F. oxysporum* was found to be the most common sudden contaminant of a variety of spp. viz. *Piper* spp., *Withania somnifera*, *Taxus baccata*, *Gossypium* spp., peanut, pigeonpea and chickpea. Sudden contamination by fungi was most frequently observed in the monsoon season (May-August). Leifert, *et al.*, (1994) have reported that sudden fungal contamination was often associated with mites which act as vectors for the contaminating fungi. Presence of mites (Fig. 3.1C) alongwith *Fusarium oxysporum* in cultures was detected in this study also. The contamination with *Fusarium oxysporum* caused losses of cultures from 80-100%. It infested the cultures irrespective of species, developmental stage or explant type. The cultures incubated in same room were contaminated by this fungus at more or less same percentage frequency. In addition, *Chaetomium bostrichodes* Zopf., was found to infect cultures of both species of *Piper* and *Taxus baccata* in the monsoon season. However the percentage of contamination was less (20-30%) and slow-growing nature of this fungus restricted the losses.

Occasionally, an unidentified black mycelium and *Neurospora* spp. also occurred as sudden contaminants and caused extensive losses of the cultures.

There extent of contamination in the stem explants varied with genotypes. *Piper colubrinum* explants were free from the constant contaminant fungi as against the *P. nigrum* explants. Among the three cultivars of black pepper, cv. Subhakara was less susceptible to contamination as compared to cv. Panniyur-1 and cv. Karimunda (Table 3.2). The different explant types also showed variation in degree of fungal contamination. Shoot apices and leaf explants were less susceptible to fungal contamination than the nodal explants.

3.3.2. Isolation and Identification of Bacterial contaminants: Total seven bacterial contaminants were isolated from cultures of *Piper* spp. Two of these were

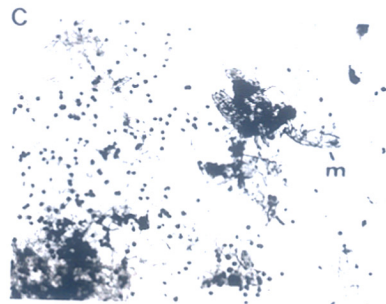
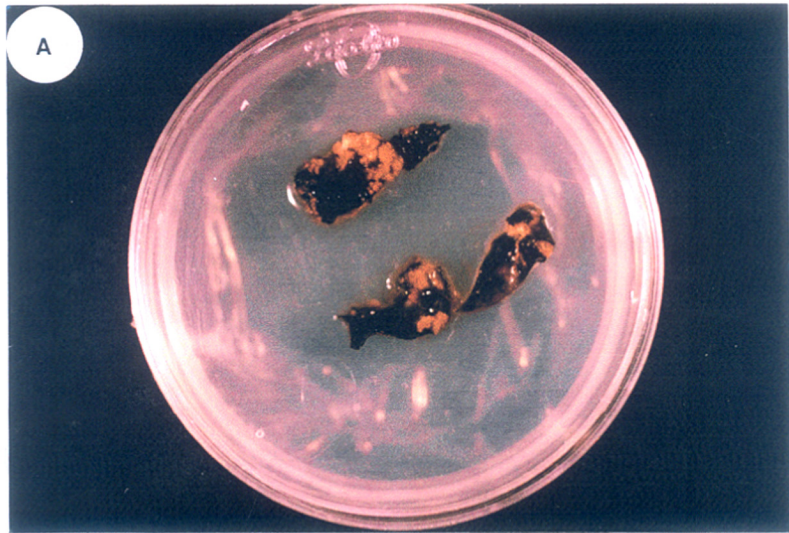
Fig. 3.1

A. Phenolic oxidation in leaf explants of wild peppr.

B. Fungal contamination in nodal explant of black pepper, cv. Panniyur-1

C. Mites (M) alongwith fungal spores.

FIG.3.1



sudden outbreak contaminants whereas 5 were persistent contaminants isolated from *Piper* spp. Sudden contamination by bacteria was not restricted to the monsoon season unlike the fungal contamination and occurred at any time during the year. The two most common sudden bacterial contaminants were pigmented bacteria; pink and intense pink in colour. The intense pink bacteria were more dangerous to cultures because their growth rate was very rapid and within two days the contaminants overgrew the cultures. The shoot meristems and callus cultures could not survive this contaminant and turned black and died. Mature axillary buds could sprout and form single bud cultures despite the contamination (Fig. 3.2A). But the sprout harbored the contaminant and died due to overgrowth of contamination when separated from the explant during subculture. It was most harmful in liquid shake-flasks and resulted in 95-100% culture loss.

The constant bacterial contaminants appeared as white or yellow growth in stem and leaf explants of both species of *Piper*. The white bacteria isolated from these two species on purification were separated as four different isolates: PW(O), PW(T), PWY(W) and PWY(Y). The first two were isolated from wild pepper whereas the latter two infested all the three cultivars of black pepper. In addition, the yellow bacterium (PY) appeared in cultures of black pepper after 2-3 weeks. The abbreviations used for the isolated denote their colony characters eg. PW(O) represents White, Opaque colonies appearing in pepper cultures.

All the contaminants were stained with Gram's staining using the double stain method (Stiner, 1988) and were found to be Gram-negative rods or coccobacilli (Figs. 3.2B-H). Out of the 7 isolates, 4 were found to be aerobes whereas 3 were facultative anaerobes as indicated by the o/f (oxidation/fermentation) test carried out in liquid medium with Durham's tubes as well as by stab inoculations (Salle, 1971; Bradbury, 1988). Based on these tests the bacteria were divided into two groups and further routine biochemical tests were carried out as per Bradbury, (1988) and Bergy's manual (ninth edition, 1989) (Table 3.4).

Based on character-correlation the bacteria were found to bear affinity to more than one species described in the Bergy's manual and could not be placed in any of the existing species. Such difficulties in allotting the plant tissue culture contaminants to specific species have been reported earlier (Reed, 1995, 1996, Leifert, 1994). The

Fig. 3.2

A. Bacterial contamination in mature nodal explant of black pepper, cv. Karimunda.

C.-I. Gram reaction of contaminant bacteria.

C, Gram-negative rods of the isolate Ppnk

D. Gram-negative rods of the isolate Ppnk-s

E. Gram-negative rods of the isolate Py

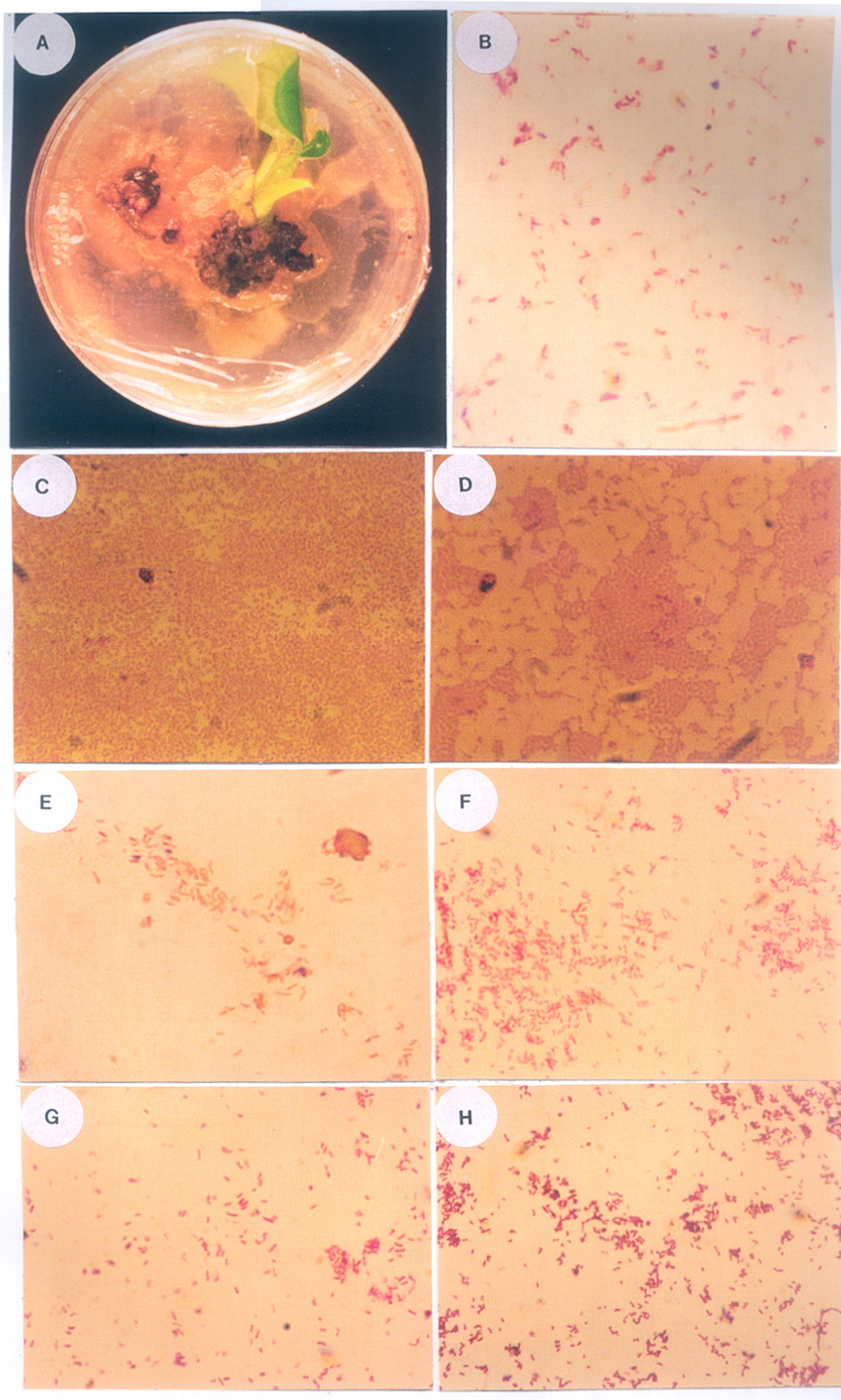
F. Gram-negative rods of the isolate PW(T)

G. Gram-negative rods of the isolate PW(O)

H. Gram-negative rods of the isolate PWY(W)

I. Gram-negative rods of the isolate PWY(Y)

FIG. 3.2



bacterial isolates were classified upto family level in facultative anaerobes and were designated to family Enterobacteriaceae. The strict aerobes were classified under Pseudomonadaceae. According to Leifert, (1994), often the explants were the chief source of contaminants belonging to Enterobacteriaceae and Pseudomonadaceae. However the pink bacteria isolated as sudden contaminants might have been introduced from laboratory environment since these two bacteria infested all the cultured species.

Table 3.3 Morphological and Biochemical characterization of facultative anaerobic contaminant bacteria infecting cultures of *Piper* spp.

No.	Tests /characters studied	P-W(T)	P-W(O)	PNK-S
1	Gram Reaction	-	-	-
2	Cell Shape	RODS	RODS	RODS
3	Motility	+	+	+
4	Cysts or cyst-like forms present	-	-	-
5	Cells Arranged in Square Tablets	-	-	-
6	Oxidase	+	+	+
7	Fluorescent pigment	-	-	-
8	Indole from Tryptophan	-	-	-
9	MR	+	+	-
10	VP	+	+	+
11	Citrate	+	+	+
12	Growth on 1-C comp.	-	-	-
13	Potato Rot	+	+	-
14	Catalase	+	+	+
15	Starch	+	-	+
16	Levan	-	+	+
17	Spore Test	-	-	-
18	Nitrate	+	+	+
19	Urease	ND	ND	ND
20	Growth at 5 ⁰ C	-	-	-
21	Growth at 27 ⁰ C	+	+	+
22	Growth at 37 ⁰ C	+	+	+
23	Growth at 60 ⁰ C	-	-	-
24	Growth in 20 % NaCl	-	-	-
25	Anaerobic Growth(stab method)	+	+	-
26	Anaerobic Growth(oil overlay method)	+	+	+
27	Growth in Ashby's Medium without N2	-	-	-
28	Gas Production in Glucose medium	+	+	-
29	Acid Production in Glucose Medium	+	+	+
30	Growth in Glucose Medium	+	+	+
31	Acid Production in Mannose medium	-	+	+
32	Growth in mannose medium	+	+	+
33	Acid production in Lactose medium	+	+	+
34	Growth in Lactose medium	+	+	+
35	Acid production in Rhamnose medium	+	+	+
36	Growth in Rhamnose medium	+	+	+
37	Acid production in Sucrose medium	+	+	+
38	Growth in Sucrose medium	+	+	+
39	Growth in Mannitol medium	+	+	+
40	Acid production in Mannitol medium	+	+	+

ND : not done

Table 3.4. Morphological and Biochemical characterization of obligate aerobic contaminant bacteria infecting cultures of *Piper* spp.

No.	Tests/Characters	P-PINK	P-Y	P-WY(Y)	P-WY(W)
1	Gram	-	-	-	-
2	Shape	RODS	CB	RODS	CB
3	Cyst or Cyst Like Forms Appear	-	-	-	-
4	Cells Arranged in Square Tablets	-	-	-	-
5	Motility	+	+	-	+
6	Cells in Gelatinous Matrices	-	-	-	-
7	Fluorescent Pigment	-	+	+	-
8	Colony Colour	Pink	Yellow	Yellow	White
9	Oxidase	-	+	WEAK +	+
10	Indole from Tryptophan	-	-	-	-
11	N ₂ Fixation	-	-	-	-
12	Denitrification to N ₂	-	-	-	-
13	Marine or Brine Bacteria	-	-	-	-
14	MR	+	-	-	-
15	VP	-	+	ND	+
16	Citrate	+	+	-	+
17	Growth on 1-C comp.	-	-	-	-
18	Potato Rot	-	+	+	+
19	Catalase	+	+	+	+
20	Starch	+	+	+	+
21	Levan	-	+	-	-
22	Spore Test	-	-	-	-
23	Nitrate	+	-	+	+
24	Urease	-	-	ND	+
25	Growth at 5 ^o C	-	-	-	-
26	Growth at 27 ^o C	+	+	+	+
27	Growth at 37 ^o C	+	+	+	+
28	Growth at 60 ^o C	-	-	-	-
29	Growth in 20 % NaCl	-	-	ND	ND
30	Anaerobic Growth(stab method)	-	-	-	-
31	Anaerobic Growth(oil overlay method)	-	-	-	-
32	Growth in Ashby's Medium Without N ₂	-	-	-	-
33	Gas Production in Glucose medium	-	-	-	-
34	Acid Production in Glucose Medium	-	-	-	-
35	Growth in Glucose Medium	-	+	-	+
36	Acid Production in Mannose medium	-	+	-	+
37	Growth in mannose medium	-	-	-	-

Table 3.4 contd.

No.	Tests/Characters	P-PINK	P-Y	P-WY(Y)	P-WY(W)
38	Acid production in Lactose medium	+	+	-	+
39	Growth in Lactose medium	+	+	-	+
40	Acid production in Rhamnose medium	-	-	-	-
41	Growth in Rhamnose medium	+	+	-	+
42	Acid production in Sucrose medium	+	-	-	-
43	Growth in Sucrose medium	+	+	-	+
44	Acid production in Mannitol medium	-	-	-	-
45	Growth in Mannitol medium	+	+	-	+

ND: not done, CB: coccobacilli.

3.3.3. Control of contaminants: To control the stable fungal and bacterial contamination, physical and chemical factors were used as control measures and applied at the level of the mother plants and explants. The physical control measures included thermotherapy i.e. higher temperatures (40⁰ C and 60⁰ C), medium acidification and overnight washing of the explants under running tap water. The chemical treatments operated at mother plant level as well as at explant level and involved use of different surface sterilization agents with or without vacuum and use of fungicides.

3.3.3.1. Physical factors: The physical factors; temperature, pH and mechanical washing were used as common control measures to restrict the growth of both fungal and bacterial contaminants.

(1) **Thermotherapy:** Use of thermotherapy has been reported for treatment of a variety of plant parts right from seeds to whole plants (Grondeau and Samson, 1994; Grum, 1996; Albers *et al.*, 1996) to control bacteria and fungi *in vitro* as well as *ex vitro*. Incubation of explants of *Piper* spp. at 40⁰ C temperature in an oven or water-bath for 10 min resulted in reduction of contamination but incubation at 40⁰C for and 30 min or at 60⁰ C for 10 Or 30 min proved fatal due to excessive phenolic oxidation (Table 3.4). The bacterial contaminants were relatively resistant to 40⁰ C because all contaminants grew well 37 ± 2⁰ C (Table 3.2. and 3.3).

Table 3.5. Effect of thermotherapy on percentage of bacterial and fungal contamination in nodal explants of 3 cultivars of *P. nigrum* L.

Temperature (°C)	Contaminati type	Duratio (min)	Explants showing contamination (%) Mean ±					
			Panniyur-1	Subhakara	Karimunda			
40 (Oven)	Fungal	10	58.3 ± 3.0 ^a	50.6 ± 1.0 ^a	56.6 ± 7.0 ^a			
	Bacterial		30.0 ± 0.0 ^Δ	21.0 ± 3.6 ^Δ	30.0 ± 0.0 ^Δ			
40 (Water	Fungal	10	46.0 ± 4.0 ^a	40.0 ± 0.0 ^a	46.5 ± 2.5 ^a			
	Bacterial		29.0 ± 1.0 ^Δ	24.0 ± 8.0 ^Δ	20.0 ± 0.0 ^Δ			
40 (Oven)	Fungal	30	All the explants showed excessive phenolics and were discarded within a week of culture.					
	Bacterial							
40 (Water	Fungal	30	All the explants showed excessive phenolics and were discarded within a week of culture.					
	Bacterial							
60 (Oven)	Fungal	10	All the explants showed excessive phenolics and were discarded within a week of culture.					
	Bacterial							
60 (Water	Fungal	10						
	Bacterial							
60 (Oven)	Fungal	30						
	Bacterial							
60 (Water	Fungal	30						
	Bacterial							

Values followed by same superscripts do not show significant differences among treatments for bacterial or fungal contamination at 95% confidence level

(2) **Acidification of medium:** Use of medium acidification to control fungi, yeasts and bacteria is described for *Delphinium* spp (Leifert *et al.*, 1994) based on studies in *Aster*, *Iris* and *Rosa*. In these plants (*Aster*, *Iris* and *Rosa*) shoot cultures reduce the pH of medium may be due to selective uptake of media components or secretion of acidic substances that eventually results in reduction in contamination (Leifert *et al.*, 1992). In *Delphinium* spp., such a phenomenon is not observed. Hence these workers carried out artificial acidification of medium and found that short term exposure to acidic pH was useful to control the accidental contamination bacteria, yeasts and fungi.

In *Piper* spp., acidified media apparently reduced fungal contamination percentage in the nodal explants of cv. Panniyur-1 and Karimunda and increased fungal contamination in cv. Subhakara. But these differences were not found to be statistically significant (Table 3.6). The percentage of bacterial contamination in acidified media was comparable to the control medium with pH at 5.6 (Table 3.6). This indicated the tolerance of contaminant fungi and bacteria to acidic pH values used (3.5, 4.5). When

the pH of control medium was checked after 3, 5, 8, 10, 12 and 15 days after culture of nodal explants, it was observed that culture of black pepper explants causes reduction of pH of the culture medium. The lowering of pH (upto 4.5 from 5.6) is drastic during first 3 days followed by gradual reduction (upto 3.5) of pH upto 10-15 days. Thus the contaminants associated with black pepper explants were habituated to low pH. Hence medium acidification could not control the contamination effectively.

(3) Running water treatment: The explants were washed under running tap water to ensure minimal presence surface contaminants before surface sterilization. This treatment did not reduce rate of contamination in any of the cultivars again suggesting endophytic origin of the contaminants (Table 3.6). These results are in agreement with those reported by Fitchet (1990 b) .

Table 3.6. Effect of medium acidification and running water treatment on contamination percentage in nodal explants of 3 cultivars of *P. nigrum* L.

Treatment	Contamination Type	Explants showing contamination (%) Mean $\pm$ s.d.		
		Panniyur-1	Subhakara	Karimunda
pH 3.5	Fungal	66.0 $\pm$ 0.0 ^b	50.0 $\pm$ 10.0 ^a	60.0 $\pm$ 10.0 ^b
	Bacterial	40.0 $\pm$ 10.0 ^A	50.0 $\pm$ 0.0 ^A	45.0 $\pm$ 5.0 ^A
pH 4.5	Fungal	60.0 $\pm$ 10.0 ^b	60.0 $\pm$ 0.0 ^a	70.0 $\pm$ 10.0 ^a
	Bacterial	40.0 $\pm$ 0.0 ^{A*}	20.0 $\pm$ 5.0 ^B	33.0 $\pm$ 3.0 ^A
pH 5.6	Fungal	82.0 $\pm$ 6.0 ^a	56.0 $\pm$ 9.0 ^{a*}	82.0 $\pm$ 4.0 ^a
(Control)	Bacterial	47.0 $\pm$ 9.0 ^A	45.0 $\pm$ 5.0 ^A	52.0 $\pm$ 8.0 ^A
Running water	Fungal	70.0 $\pm$ 8.0 ^a	52.0 $\pm$ 2.0 ^a	71.0 $\pm$ 10.0 ^a
	Bacterial	44.0 $\pm$ 5.0 ^A	32.0 $\pm$ 12.0 ^A	50.0 $\pm$ 0.0 ^A

* Differences among the cultivars for a contamination type in each treatment are significant at 95% confidence level.

Values followed by same superscript do not differ significantly among treatments within a genotype at 95% confidence level.

3.3.3.2. Chemical treatments: Diverse chemicals have been employed to combat contamination. These include the use of routinely used surface sterilization agents commercial bleach, mercuric chloride, hydrogen peroxide (Dirks *et al.*, 1983) as well as new agents like diethylpyrocarbonate (Macek *et al.*, 1995), 8-hydroxy-quinolinol-sulfate (Machado *et al.*, 1996), sodium dichloroisocyanurate (Parkinson *et al.*, 1996) and sulfite pads (Shure *et al.*, 1994). In addition antifungal agents such as bavistin, benomyl, Amphotericin-B, Tilt are also reported (Watt *et al.*, 1996).

In *Piper* spp. the control of contamination was executed at two levels, at whole plant level and at explant level (Debergh and Meane, 1988). In spite of regular insecticide and fungicide treatments of mother plants as described in materials and methods, the fungal contamination persisted indicating explants as source of contamination. To control this persistent contamination, different sterilization agents hydrogen peroxide, mercuric chloride and commercial bleach were tested.

(1) Surface sterilization treatments: For standardizing surface sterilization of mature nodal explants we tried 0.1%, 0.5% and 1% mercuric chloride in the pilot experiments, however all the combinations above 0.1% were found to be phyto-toxic. Hence in further experimentation only 0.1% mercuric chloride was used for surface sterilization. Mediclор-M the commercial bleach solution (Cristal corporation, India) was used at a concentration of 2% and 5% (v/v; 6/12 active chlorine available). Hydrogen peroxide (1% and 3%) was used as per the procedure described by Dirks *et al.*(1986).

All these agents at different durations yielded variable results (Table 3.7). Among these three agents, hydrogen peroxide resulted in uniform percentage of sterile explants among all the cultivars and for all durations (Table 3.7). At all the durations (5, 10, 20 and 30), hydrogen peroxide was comparable to mercuric chloride at 10, 20 and 30 min.

Treatment with mercuric chloride for 5 min yielded very low percentage of sterile explants in cv. Panniyur-1 and Karimunda but in cv. Subhakara it was found to be more effective. In cv. Panniyur-1 and Karimunda, percentage of sterile explants increased with increase in duration of treatments. But in cv. Subhakara, the percentage of sterile explants was less at 5 min and 20 min duration and high at 10 and 30 min.

Among the treatment durations, surface sterilization with mercuric chloride for 10 min resulted in moderate percentage of sterile explants (Table 3.7) but it was less photo-toxic and avoided blackening of the explants. Treatment with mercuric chloride for 20 min and 10 min yielded comparable results in all three cultivars. Treatment for 30 min. resulted in highest percentage of sterile explants but the differences were statistically

significant only in cv. Subhakara. Subsequently, it was phytotoxic as indicated by blackening of explants. Thus surface sterilization with mercuric chloride for 10 min was found to be optimum. For relatively tender explants like shoot apices and leaves, mercuric chloride treatment for 5 min was more useful than 10 min since it avoided blackening of explants.

To enhance the efficiency of mercuric chloride treatment and in order to offer better contact of the surface sterilant with endophytic microbes the treatment was coupled with application of vacuum provided by vacuum pump (Millipore) at -20 kPa. This facilitated better penetration of mercuric chloride inside the tissue and gave better results than mercuric chloride without vacuum (Table 3.7) for nodal explants of Panniyur-1 and Subhakara for 10 and 30 min treatment. However in Karimunda the differences between vacuum treatment and absence of vacuum were not significant and hence was not used in further work.

The use of commercial bleach led to excessive exudation of phenolics and was fatal to the explants after culture. Within a week of culture, all the explants treated with bleach had to be discarded due to phenolics. Hence its use was discontinued.

Mercuric chloride was selected for use in further experiments because it was as effective as hydrogen peroxide but was easier to use and the procedure involved was simple.

Table 3.7 Effect of different surface sterilization treatments on contamination percentage in nodal explants of 3 cultivars of *P. nigrum* L.

S.S. Agent	Conc. (%)	Duration (min)	Sterile explants (%) (Mean $\pm$ s.d.)		
			Panniyur-1	Subhakara	Karimunda
H ₂ O ₂	1.0	5	20.6 $\pm$ 8.0 ^a	18.0 $\pm$ 4.0 ^a	18.6 $\pm$ 1.0 ^a
		10	25.3 $\pm$ 4.0 ^a	26.6 $\pm$ 6.0 ^a	25.0 $\pm$ 3.0 ^a
		20	17.3 $\pm$ 7.0 ^a	27.6 $\pm$ 6.0 ^a	19.0 $\pm$ 1.0 ^a
		30	22.0 $\pm$ 3.0 ^a	30.0 $\pm$ 10.0 ^a	17.6 $\pm$ 9.0 ^a
HgCl ₂	0.1	5	9.3 $\pm$ 1.0 ^a	20.0 $\pm$ 5.0 [#]	8.6 $\pm$ 3.0 ^a
		10	20.0 $\pm$ 0.0 ^b	29.0 $\pm$ 1.0 [#]	12.0 $\pm$ 3.0 ^{ab}
		20	20.0 $\pm$ 4.0 ^b	20.0 $\pm$ 3.4 ^a	21.0 $\pm$ 2.8 ^a
		30	26.0 $\pm$ 4.0 ^b	31.0 $\pm$ 2.1 ^b	28.0 $\pm$ 2.5 ^a
HgCl ₂ (+)	0.1	5	15.6 $\pm$ 4.0 ^a	20.0 $\pm$ 0.0 ^a	13.3 $\pm$ 4.1 ^a
Vacuum		10	29.0 $\pm$ 3.0 ^b	36.0 $\pm$ 6.0 ^b	23.0 $\pm$ 2.0 ^b
		20	27.0 $\pm$ 4.0 ^b	20.0 $\pm$ 2.8 ^a	27.0 $\pm$ 1.4 ^{ab}
		30	34.0 $\pm$ 3.0 ^b	36.0 $\pm$ 5.6 ^b	ND
Mediclor-M	1.0	5	All cultures discarded due to excessive phenolic oxidation within a week of culture		
		10			
		20			
	1.0	5			
		10			
		20			

SS agent: Surface sterilization agent. Values followed by same letter indicate absence of significant differences among the treatments of each SS agent for each genotypes, # indicates differences among genotypes for a particular treatment.

(2) Use of fungicidal agents: Due to severe loss of cultures (80-90%) in the initial experiments we pre-treated the explants with aqueous solution of three broad spectrum fungicides, Bavistin (BASF, India), Ha-mycin and Aureofungin (Hindustan Antibiotics, India) and with 8-HQS, an antimycotic and antibacterial agent (Machado *et al.*, 1996) at various concentrations for different durations (Table 3.8).

Among the antifungal agents tested, Ha-mycin and Aureofungin at both the concentrations (50, 100mg l⁻¹) and at all durations yielded results comparable to the control in all the cultivars. Hence, these were not considered to be satisfactory for treating mature explants of pepper.

For the first two weeks in cultures 50-60 % of the explants treated with 8 HQS remained sterile (Table 3.8). There were no significant differences observed among the

treatments for each cultivar. Thus all the treatments (0.1 and 0.5% concentrations and 5 and 12 h durations) of 8-HQS were equally effective in controlling contamination. Among the cultivars, significantly higher percentage of sterile explants was obtained for explants of cv. Karimunda treated with 8-HQS at 0.1% for 5h.

But after 3 weeks the percentage contamination increased slowly and within 5 weeks it was found to be only slightly better than control. Thus 8-HQS was fungistatic in action and only delayed the incidence of contamination by 2-3 weeks.

Use of Bavistin at 0.5 % concentration for pre-treatment at all durations was not effective in controlling contamination in all the cultivars except for Karimunda for 2h duration. However Bavistin at 1% and 5% at all the durations considerably reduced the percentage of fungal contamination in cv. Panniyur-1 and Karimunda. In cv. Subhakara all the treatments were similar to the control except Bavistin 1.0% for 2h . Pre-treatment of the nodal explants of all the three cultivars with 1% Bavistin for 2 h was found to be the most successful in reducing the contamination (Table 3.8). The use of 5% bavistin though yielded similar results, it caused browning of explants and at the end of 3-4 weeks such explants got contaminated. This resulted in overall increased rate of contamination. The use of 0.5% Bavistin was found to be less satisfactory irrespective of time duration of treatments in all the three cultivars except in cv. Karimunda for 2h. However it was observed that use of Bavistin resulted in an increase in percentage of bacterial contamination. This could have resulted from reduction in competition for resources among the contaminating bacteria and fungi. Benlate (another trade name of Bavistin with the same active principle) induced aggravation of bacteria was also observed in Leatherleaf fern (Kremer *et al.*, 1996).

Thus the use of Bavistin (1%) for two h was optimum for all the cultivars in reducing fungal contamination and was routinely practiced in further experiments Successful use of Bavistin has been described inn Bamboo (Ramanayke and Yakandwala, 1997).

Table 3.8. Effect of pretreatment of different antimicrobial agents on the sterility of nodal explants in different cultivars of *Piper* L.

Anti-microbial Agent	Conc.	Duration	Percentage of sterile explants (Mean $\pm$ s.d.)		
			Panniyur-1	Karimunda	Subhakara
Hamycin	50 mg.l ⁻¹	(h)			
		5	ND	23.3 $\pm$ 5.7 ^a	26.0 $\pm$ 2.0 ^a
	100 mg.l ⁻¹	12	ND	21.3 $\pm$ 1.0 ^a	26.0 $\pm$ 0.0 ^a
		5	ND	26.0 $\pm$ 10.0 ^a	18.6 $\pm$ 1.0 ^a
Aureofungin	50 mg.l ⁻¹	12	ND	21.3 $\pm$ 8.0 ^a	25.3 $\pm$ 2.0 ^a
		5	8.0 $\pm$ 1.0 ^{a*}	25.6 $\pm$ 4.0 ^a	16.6 $\pm$ 4.0 ^a
	100 mg.l ⁻¹	12	18.6 $\pm$ 1.1 ^a	21.6 $\pm$ 2.8 ^a	26.0 $\pm$ 2.0 ^a
		5	17.33 $\pm$ 2.0 ^a	18.0 $\pm$ 6.0 ^a	26.6 $\pm$ 3.0 ^a
8-HQS	0.1 %	12	18.33 $\pm$ 1.0 ^a	30.0 $\pm$ 1.0 ^a	ND
		5	56.6 $\pm$ 7.0 ^b	70.0 $\pm$ 2.0 ^d	53.3 $\pm$ 11.0 ^{ab}
	0.5 %	12	60.0 $\pm$ 10.0 ^{bc}	60.0 $\pm$ 0.0 ^c	53.0 $\pm$ 2.8 ^{ab}
		5	53.3 $\pm$ 5.7 ^b	58.0 $\pm$ 2.0 ^c	48.0 $\pm$ 7.0 ^{ab}
Bavistin	0.5 %	12	40.6 $\pm$ 9.0 ^b	45.0 $\pm$ 6.0 ^c	51.0 $\pm$ 7.0 ^{ab}
		0.5	22.5 $\pm$ 3.0 ^a	21.1 $\pm$ 1.0 ^a	40.0 $\pm$ 0.0 ^{a*}
		1	27.8 $\pm$ 0.9 ^a	24.2 $\pm$ 1.0 ^a	40.0 $\pm$ 0.0 ^{a*}
		2	29.2 $\pm$ 9.0 ^a	37.2 $\pm$ 6.0 ^b	45.0 $\pm$ 2.1 ^{a*}
	1.0 %	0.5	42.2 $\pm$ 3.0 ^b	29.0 $\pm$ 5.0 ^{ab*}	40.0 $\pm$ 1.4 ^a
		1	55.0 $\pm$ 7.0 ^b	52.0 $\pm$ 3.0 ^c	55.0 $\pm$ 7.0 ^{ab}
		2	65.0 $\pm$ 7.0 ^c	70.0 $\pm$ 0.0 ^d	66.0 $\pm$ 5.0 ^b
		0.5	55.8 $\pm$ 5.0 ^b	55.0 $\pm$ 7.0 ^c	65.0 $\pm$ 7.0 ^{a*}
Control	0.0	1	47.7 $\pm$ 9.0 ^b	45.0 $\pm$ 7.0 ^c	50.0 $\pm$ 0.0 ^{ab}
		2	41.4 $\pm$ 1.0 ^b	58.0 $\pm$ 2.0 ^{c*}	47.7 $\pm$ 3.0 ^{ab}
		-	20.0 $\pm$ 0.0 ^a	12.0 $\pm$ 1.0 ^a	33.0 $\pm$ 3.0 ^{a*}

* Values significantly different at 95% confidence interval among the cultivars for each treatment.; Values followed by same letter are not significantly different at 95% confidence interval among the treatments for each genotype. ND: not done.

(3) Use of antibiotics: Use of antibiotic agents is so far the most popular method used for controlling bacterial contaminants in plant tissue cultures. The antibiotics however should be used cautiously because blind use of antibiotics could result in evolving resistant contaminants that would be even more difficult to control and could become either 'vitro-pathic' or pathogenic and could have with a broader host range.

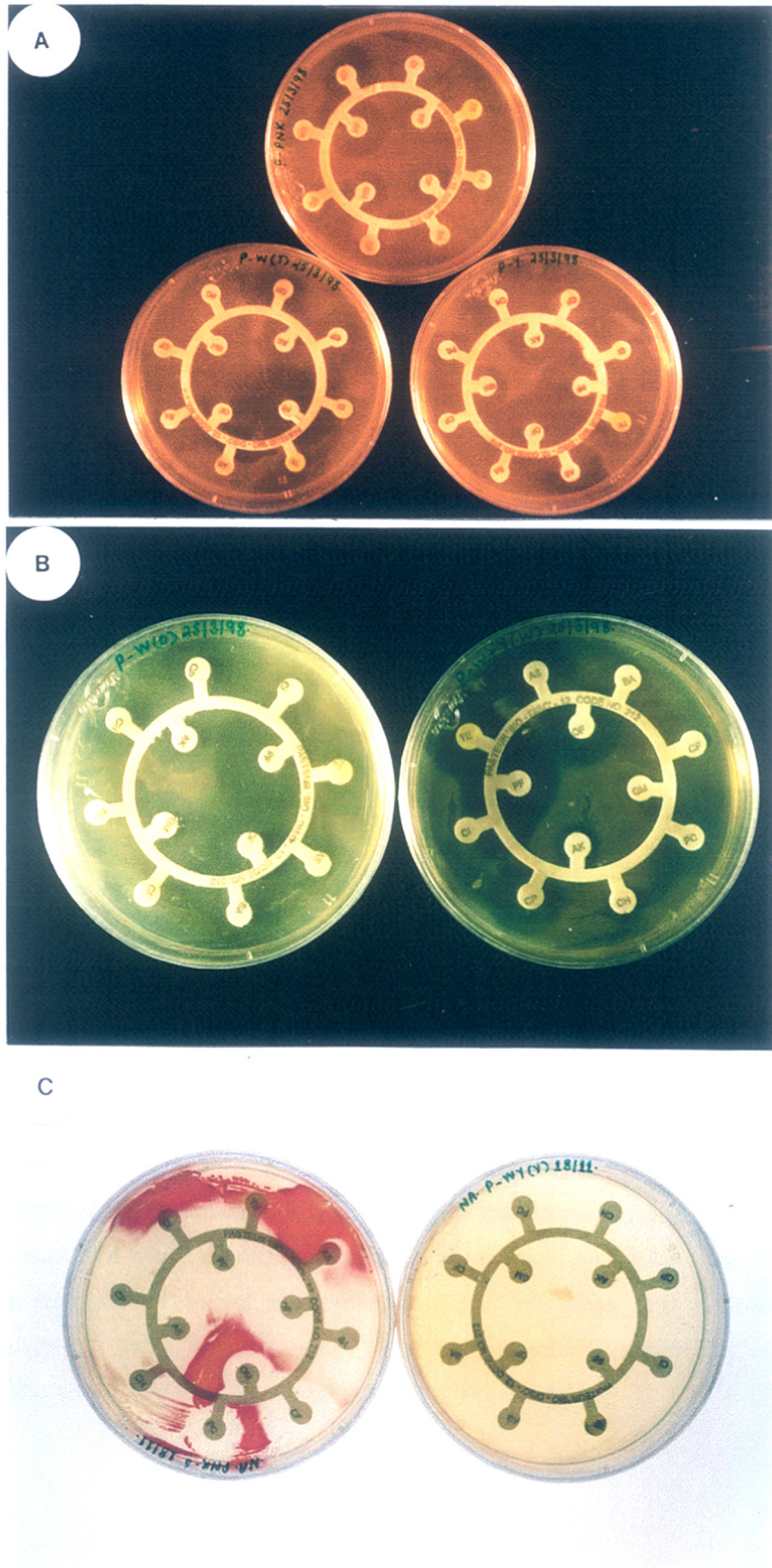
In order to identify the most potent antibiotics effective against the contaminants of *Piper* spp., an AntibioGramme assay was carried out using Biodiscs 12 (Pathoteq Labs, India) by top agar method. Sterile filter paper discs impregnated with 12 different antibiotics at given concentrations were placed on evenly grown lawn of bacterial isolates and incubated at 37⁰C for 12h. After which, the diameter of the clearing zone that described the efficacy of an antibiotic agent in controlling a particular contaminant was measured. However in an AntibioGramme assay the rate of diffusion of an antibiotic agent can not be defined and even if so, it would not be exclusive of the other agents. Further the antibiotics are known to have antagonistic as well as additive effects. Therefore, clearing zones obtained in an AntibioGramme assay might have resulted from such interactions of the antibiotics. Hence, AntibioGramme assay was used for preliminary screening of antibiotics.

In the AntibioGramme assay (Fig. 3.3 A-C) carried out for contaminants of pepper cultures, it was found that antibiotic CF was least effective among the 12 antibiotics used (Table 3.9). It was effective in controlling only one bacterial isolate and in that too, the diameter of clearing zone was very less. Antibiotics AS, PC and CL were also restricted in their efficacy and checked growth of only two isolates (Table 3.9) whereas antibiotics PF, AK and GM were effective against all the isolates of bacteria.

Fig. 3.3

Antibiogramme Assay carried out for the contaminant Bacteria exhibiting clearing zones of effective antibiotic agents.

FIG.3.3



CH, CP, TE and OF were also broad spectrum antibiotics and were effective against 6 out of 7 isolates (Table 3.9). In the clearing zones of Ofloxacin, development of small colonies of 3 bacterial isolates was observed within 24 h. These could be resistant colonies or may represent the static reaction of the antibiotic agent. Among the bacteria, Ppnk and Pwy (Y) exhibited resistance for 8 antibiotics out of 12, whereas Ppnk-S was susceptible to all antibiotics and Pw (T) was susceptible to 11 antibiotics.

Table 3.9. Results of Antibiogramme Test showing effect of different antibiotics on the contaminant bacteria.

Antibiotic	Diameter of clearing zone (cm)						
	PY	PWY(W)	PWY(Y)	PW(T)	PW(O)	P-PNK	P-
Ampicillin	-	-	-	0.2	-	-	1.0
Co-	-	-	-	0.4	0.8	-	1.2
Cefotaxime	-	-	-	-	-	-	1.4
Piperacillin	-	-	-	0.6	-	-	1.4
Chlorampheni	0.7	1.0	-	1.5	1.0	-	0.4
Ciprofloxacin	0.5	1.4	-	1.0	1.1	-	2.2
Ceftizoxime	-	-	-	0.7	0.2	-	1.0
Tetracycline	0.6	1.2	-	0.3	0.6	0.9	0.2
Ofloxacin	1.4	1.8	1.0	1.9	2.1	-	1.3
Gentamicin	1.8	1.0	0.7	0.7	0.7	0.7	0.6
Amikacin	0.4	1.0	0.8	1.1	1.1	1.0	0.9
Pefloxacin	1.8	1.9	1.2	2.1	2.0	1.0	1.3

To evaluate the efficiency of a single antibiotic agent and to confirm the results of Antibiogramme assay, antibiotic sensitivity tests were carried out in liquid medium. The concentrations used in Antibiogramme assay were further manipulated for the antibiotic sensitivity tests (Table 3.10). For the liquid assay only 6 antibiotics were used on the basis of Antibiogramme assay. Each antibiotic was aseptically added to NB at one concentration above and one concentration below the concentration used in biodisc and then inoculated with bacteria. These tests did not confirm the results of Antibiogramme assay. Amikacin, Tetracycline, Ciprofloxacin and Pefloxacin gave variable results in liquid assay. (Table 3.10). In many cases, the antibiotic concentrations used in the Biodiscs were found to be bacteriostatic and not bactericidal.

Of the 12 antibiotics tested against these isolates, only Gentamicin gave consistent results in both antibiotic assays (Table 3.10). In addition, it was reported to be less phytotoxic to pepper cultures than other antibiotics (Meyer *et al.*, 1992) and hence it was used for pre-treatment of explants. Our results also support observation made by Meyer *et al.* 1992, that, the concentrations that emerged as bactericidal in AntibioGramme or Liquid Bioassay test were not sufficient to kill the contaminants. Hence for the pre-treatment, higher concentrations (50, 100 200 and 500 mg l⁻¹) of Gentamicin were used for treating explants and contaminated cultures. The pre-treatment with the antibiotic at 200 and 500 mg l⁻¹ for 2h was found to reduce the bacterial contamination by 40-50% whereas lower concentrations resulted in bacteriostasis. The treatment However the treatment of contaminated cultures was not found to be useful in controlling contamination since the contaminants reappeared after 3-5 days.

3.3.3.3 Control of Sudden contaminants: The constant contaminants could be controlled using the antibiotics. But it was not possible to control the sudden outbreak contaminants. Since the sudden fungal contaminants were associated with mites, wiping of floor and racks with ethanol every week and fumigation of incubation rooms after removing cultures with formalin was carried out every fortnight. But this was not sufficient for controlling the contamination.

Sudden bacterial contaminants especially Ppnk-S was easily controlled by using Gentamicin. But the contaminant caused blackening and killing the tender meristems which was fatal to the cultures and the use of antibiotics thus was not practical.. The P-pnk bacterium however was difficult to control and was found to appear suddenly after 2-3 subcultures in apparently clean, Gentamicin treated cultures.

Table 3.10 Results of liquid bioassay of antibiotics determining effect of different concentrations of antibiotics on different contaminant bacteria.

Antibiotic Agent	mcg	PY	PWY (W)	PWY (Y)	PW (T)	PW (O)	P-PNK	P-PNK(S)
Amikacin	10	C	S	C	C	C	R	C
	30	C	C	C	C	C	R	C
	50	C	C	C	C	C	R	C
Tetracycline	10	C	S	C	C	C	R	R
	30	C	S	C	C	C	R	R
	50	C	C	C	C	C	R	R
Gentamicin	5	C	R	C	C	C	R	C
	10	C	R	C	C	C	R	C
	15	C	S	C	C	C	R	C
Piperacillin	50	R	R	R	R	R	R	R
	100	R	R	R	R	R	R	R
	200	R	R	R	C	S	R	R
Ciprofloxacin	2.5	C	R	C	C	C	S	C
	5	C	R	C	C	C	S	C
	10	C	R	C	C	C	C	C
Ofloxacin	2.5	C	S	C	C	C	R	C
	5	C	S	C	C	C	R	C
	10	C	C	C	C	C	S	C
Pefloxacin	5	C	S	C	R	-C	S	C
	10	C	C	C	R	C	S	C
	15	C	C	C	R	C	C	C

mcg: micrograms C: bactericidal, R: resistant, S: bacteriostatic.

3.3.4. Control of Phenolic oxidation:

For control of phenolics, sealing the cut ends with paraffin wax (60⁰ Melting temperature) was the most effective method for nodal explants. But it could not be applied to leaf explants which showed maximum browning due to phenolics. Further, when the treatment was given bacterial contaminants oozed at the point of sprouting of apical or axillary buds in the meristem explants which was fatal to the sprouts. Hence this method was not applied in further experiments.

Dark incubation was found to be very effective in lowering the number of explants showing phenolic oxidation (Table 3.11) in all explant types of all the genotypes. But dark incubation irreversibly blocked morphogenesis in *Piper colubrinum* leaf explants. Hence it was useful only for dark incubation of nodes and shoot apices in both species of pepper (Table 3.11).

The strategy of quick transfers in solid medium was also used to control phenolics. In this method leaf explants were cultured in petridish containing solid medium. As the sign of browning was apparent, the explant was transferred from one place to the other in the same dish, every alternate day. This indirectly controlled browning of cultures (Table 3.11) through prevention of autocatalysis. This method though successful was laborious.

Table 3.11. Effect of dark incubation and quick transfers on percentage of shoot apex, leaf and nodal explants showing phenolic oxidation cultured in MS medium.

Treatment	Explant	Explants showing phenolic oxidation (%) (Mean $\pm$ s.d.)			
		Panniyur-	Subhakara	Karimunda	<i>P. colubrinum</i>
Dark incubation	Node	13.0 $\pm$ 4.0 ^b	14.0 $\pm$ 2.0 ^b	16.0 $\pm$ 2.8 ^b	8.0 $\pm$ 3.0 ^b
	Leaf	18.0 $\pm$ 4.0 ^B	15.0 $\pm$ 1.0 ^B	17.0 $\pm$ 4.2 ^B	15.0 $\pm$ 0.0 ^B
	Shoot apex	35.0 $\pm$ 7.0 ^b	30.0 $\pm$ 14.0 ^b	22.5 $\pm$ 3.0 ^b	25.0 $\pm$ 7.0 ^b
Quick Transfers	Node	17.0 $\pm$ 2.5 ^b	22.0 $\pm$ 3.0 ^b	20.0 $\pm$ 6.3 ^b	25.0 $\pm$ 7.0 ^b
	Leaf	37.5 $\pm$ 3.0 ^B	40.0 $\pm$ 5.0 ^B	38.5 $\pm$ 7.0 ^B	39.0 $\pm$ 15.0 ^B
	Shoot apex	32.6 $\pm$ 6.0 ^b	40.0 $\pm$ 0.0 ^b	33.0 $\pm$ 4.2 ^b	29.0 $\pm$ 4.0 ^b
Control	Node	33.3 $\pm$ 7.0 ^a	36.0 $\pm$ 5.0 ^a	35.3 $\pm$ 9.0 ^a	19.0 $\pm$ 8.0 ^a
	Leaf	80.6 $\pm$ 9.0 ^A	72.0 $\pm$ 6.0 ^A	60.0 $\pm$ 0 ^A	76.0 $\pm$ 9.0 ^A
	Shoot apex	53.0 $\pm$ 7.0 ^a	63.0 $\pm$ 5.0 ^a	60.0 $\pm$ 11.0 ^a	52.0 $\pm$ 4.0 ^a

Differences among the treatments for node, leaf and shoot apex explants for all genotypes are denoted by letters in upper case, lower case and italics. Values followed by same superscripts are not significantly different from each other significant at 95% confidence level.

Use of liquid medium for cultures of *P. colubrinum* leaves was found to be useful in reducing phenolics (Deboo and Krishnamurthy, 1992; unpublished results). However it was found that the callus development and morphogenesis was hampered in liquid cultures. Similarly, addition of antioxidants, Polyvinyl pyrrolidone (500 and 1000mg l⁻¹), Ascorbic acid, citric acid or MES hydrate (150 and 500mg l⁻¹), in the full MS medium did not show significant reduction in percentage of phenolics. The use of Ascorbic acid at both the concentrations induced profuse rooting in the leaf explants.

Reduction in strength of macroelements of MS medium to half was the most effective solution for the problem of phenolic oxidation (Table 3.12). That not only reduced browning of leaf explants considerably but also enhanced callus formation in

leaf explants (Kelkar *et al.*, 1996). The addition of activated charcoal to Full MS medium reduced browning of leaf explants significantly but supplementation of the same to half MS did not bring out further reduction in browning (Table 3.12). The control of browning by charcoal is attributed to its strong adsorbent quality by which the phenolics adsorb to it and are not leached in the medium. However because of the same quality activated charcoal is known to withhold media components as well. Further it supported profuse root formation in leaf explants and hence was discontinued and use of half MS was practiced further to control browning due to phenolic oxidation .

Table 3.12 .Effect of manipulation of basal medium on phenolic oxidation in leaf explants of 3 cultivars of *P. nigrum* L and *P. colubrinum* Link.

Medium	Leaf explants showing phenolic oxidation (%) (Mean $\pm$ s.d.)			
	Panniyur-1	Subhakara	Karimunda	<i>P. colubrinum</i>
Half MS	24.6 $\pm$ 6.0 ^c	20.0 $\pm$ 8.0 ^b	25.0 $\pm$ 5.0 ^c	21.0 $\pm$ 6.0 ^c
Half MS + AC	15.0 $\pm$ 7.0 ^c	16.0 $\pm$ 5.0 ^b	ND	11.0 $\pm$ 1.0 ^c
Full MS + AC	45.0 $\pm$ 7.0 ^b	31.0 $\pm$ 9.0 ^b	42.0 $\pm$ 2.8 ^b	41.0 $\pm$ 1.2 ^b
Full MS	80.6 $\pm$ 9.0 ^a	72.0 $\pm$ 6.0 ^a	60.0 $\pm$ 0 ^a	76.0 $\pm$ 9.0 ^a

AC: Activated charcoal (0.5%), ND: not done,

Values followed by same superscripts do not show significantly differences among the 4 treatments within a genotype.

3.4.Conclusions:

The establishment of sterile cultures of *Piper* spp. presented many obstacles. Culture loss due to bacterial contamination, fungal contamination and phenolics were the most important problems faced. The extent of these problems showed variations specific to genotypes as well as explant types.

In order to combat contamination identification and classification of contaminant organisms and thereby determination of their source was carried out. This was helpful in developing control measures. As most of the contaminants were found to be associated with the explants, different procedures involving the treatment of source plants and explants were applied. These involved use of chemicals as well as physical factors such as temperature and pH. The chemical control of microbial contamination was found to be effective. Considerable reduction in fungal contamination of nodal explants of was achieved by pre-treatment of contaminants with aqueous solution of fungicide Bavistin (1%) for 2 h. Different antibiotics were tested against the bacterial contaminants and it was found that pre-treatment with Gentamicin reduced the bacterial contamination.

The phenolic oxidation was reduced by different means followed such as sealing cut ends with molten wax, dark incubation of cultures and addition of charcoal to the media. However reduction of strength of MS medium was found to be easiest and most effective method of controlling browning of different explants due to phenolic oxidation without hampering morphogenetic process.

The procedures standardized were useful in establishing sterile cultures of mature explants of *Piper* spp.

A part of this Chapter was accepted for Poster presentation 'Control of bacterial contamination in *in vitro* cultures of pepper (*Piper* spp.)' in the National seminar on Spices Biotechnology, held at Calicut 24-25 April 1996.

CHAPTER 4.

STUDY OF PLANT REGENERATION FROM
DIFFERENT EXPLANTS DERIVED FROM
MATURE PLANTS OF *PIPER COLUBRINUM*
LINK.

4.1. Introduction

Plant improvement involves the accumulation of economically important characters in different cultivars. Though advantageous for agronomic characters such as higher yields, better nutritional qualities, earliness etc., plant improvement also has serious undesirable consequences, the most important being the genetic erosion. This refers to the elimination of valuable land races and wild species due to domination of the cultivation area by the desirable cultivars. Since the success of any crop improvement program depends on the available genetic variability, genetic erosion adversely affects further attempts of crop improvement. These wild types are reservoirs of important traits such as resistance to diseases and pests, adaptability to diverse environments and are also useful in breaking the yield plateau reached by several improved varieties and hence the wild types have immense potential as donor parents in crop improvement programs. The conservation and multiplication of the wild species is therefore an absolute necessity. *In vitro* culture of plants could be used as an important tool in achieving this task (Snowcroft, 1977; Bhojwani *et al.*, 1977; Khush and Brar, 1994).

Piper colubrinum Link., is an example of useful wild species described above. It needs to be propagated *in vitro* for future use in biotechnology-aided breeding programs as a donor of disease and pest resistant traits with black pepper i.e. *P. nigrum* as a recipient. The useful characters of these two species could be combined by asymmetric hybridization and direct DNA transfer. For such experiments, an efficient *in vitro* morphogenetic system is an important pre-requisite.

Induction of morphogenesis *in vitro* is the most common aim of tissue culture programs because the capacity to regenerate plants from cultured tissues is the basic requirement for successful application of plant tissue culture technology to agriculture, horticulture, forestry and biotechnology. *In vitro* morphogenesis is regulated by genetic, epigenetic and physiological factors (Halperin, 1986). The epigenetic and physiological factors operate through explant whereas physiological controls operate through medium, growth regulators and culture conditions. Hence the explant (type and developmental stage of explant), nutrient media (composition of basal media, additives of media,

concentrations and combinations of growth regulators used) and culture conditions (gaseous atmosphere of culture vessel used, illumination, temperature and humidity) are the most important factors that control morphogenesis (Brown and Thorpe, 1986).

4.1.1. Explant: The explant type used, physiological phase of explant and its genetic background are known to affect efficiency of morphogenesis *in vitro* (Brown and Thorpe, 1986). In addition, the epigenetic controls of morphogenesis also operate through the explants (Halperin, 1986).

The somatic cells of plants possess all the necessary information for regenerating into whole plant (Zimmerman, 1993; Merckle, *et al.*, 1995). Hence potentially any explant i.e. a cell, a tissue layer, an organ or an embryo exhibits the totipotency i.e. capacity to form a whole plant. However, in practice, it has been observed that usually all cells are not totipotent (Tran-Thanh-van, 1981). The pre-existing shoot meristems or embryos are committed to form shoots and plants respectively and are more amenable to organogenetic shoot induction in contrast to organs like leaf, stem, root, petiole etc. which are committed otherwise (Tran-Thanh-Van, 1981).

It has been observed in most of the woody perennials that the juvenile or rejuvenated explants belonging to same plant species are relatively easy to regenerate than mature explants (Halperin, 1986; Thorpe and Kumar, 1993; Litz and Griffis, 1989). The cells of mature explants undergo nuclear changes concomitant with maturation which often results in irrevocable loss of totipotency (D'Amato, 1977). Thus, the age or physiological phase of explant is an important factor influencing the success of morphogenesis.

The genetic constitution of the mother plant is reflected as a factor controlling morphogenetic induction in the cultured explants. The differences in morphogenetic potential of explants from different species of the same genus or different varieties of the same species suggest presence of genetic control of morphogenesis as observed in *Nicotiana* spp. (Ogura and Tsuji, 1977; Maliga, 1977 and Wullems *et al.*, 1980), in Alfalfa (Bingham *et al.*, 1975; Reisch and Bingham, 1980); in *Petunia* spp. (Skvirsky *et al.*, 1982) and in *Larix* spp. (Riemenschneider *et al.*, 1988; Bonga and Pond, 1991). The effect of genetic factors on morphogenetic induction is amply proved by the fact that transformation with shooty mutants of *Agrobacterium tumefaciens* having mutant genes 1

and 2 in the T-DNA of Ti-plasmid result in shoot forming tumors in the transformed species (Akiyoshi *et al.*, 1984; Inez *et al.*, 1984; Schroeder *et al.*, 1984). All these studies have revealed presence of one or more genes governing shoot forming trait, inherited in Mendelian or non-Mendelian fashion, which controls morphogenetic competence and morphogenetic induction process.

In addition to genetic factors, epigenetic factors also affect morphogenetic capacity of explants. An epigenetic factor is defined as the one which is stable through a number of cell generations in the absence of an inducing factor (Brink, 1962). Thus, though stable, the impact of an epigenetic factor is reversible as observed in the production of Picloram-susceptible plants by second generation callus of a stable Picloram-resistant cell line (Chaleff and Parsons, 1978). Certain epigenetic determinants in the form of organ and stage specific proteins are found to persist in explants even after several subcultures (Raff *et al.*, 1979). Persistence of such proteins could be responsible for low morphogenetic potential of woody explants (Halperin, 1986). The effect of such epigenetic factors could be nullified by exogenous supply of signals triggering morphogenesis such as plant growth regulators like giberellic acid in *Hedera helix* (Miller and Goodin, 1976). The removal of epigenetic influence often causes a phase change from maturity to juvenility in woody explants making them more amenable for regeneration. Thus the genetic background and physiological phase of an explant influence the morphogenetic capacity of the explant.

In addition to these, miscellaneous factors like orientation of explants, surface area of explants that is in contact with medium also affect regeneration.

4.1.2. Medium and Culture Conditions: The explant mimics the genetic and physiological status as well as morphogenetic responses of the mother plant unless diverted by signals triggering morphogenesis. Generally these signals are provided by the nutrient medium and its supplements (Halperin, 1986; Thorpe and Kumar, 1993; Preece, 1995).

The manipulation of nutrient medium and culture environment allows competent cells to express their potential for morphogenesis which ultimately results from selective gene activation (Brown and Thorpe, 1986). The nutrient medium usually comprises of inorganic salts (macro-nutrients and micro-nutrients), organic compounds (vitamins and

amino acids), carbohydrate source and is supplemented with plant growth regulators. The interplay of the nutrients and plant growth regulators with the competent cells of the explant results in organized development (Preece, 1995). The intensity of this interplay is governed by culture conditions like light, humidity temperature and factors like medium pH, culture vessels, types of closures and gas exchange.

Regulation of morphogenesis by plant growth regulators has enjoyed attention of most of the researchers for the last three decades. However the mechanism of action of the plant growth regulators still remains obscure (Minocha, 1987). Different mechanisms are suggested to explain the mode of action of growth regulators: (a) through relative and absolute concentration of two or more plant growth regulators inside cell (Skoog and Miller, 1957), (b) action through metabolism of plant growth regulators (Tuli and Moyed, 1969), (c) action through sensitivity of cells operating through specific receptors (Trewavas, 1982; Weyers *et al.* 1995) and (d) through indirect effects (Giladi *et al.*, 1971).

Since the discovery of Auxin:Cytokinin ratio as a determining factor in organ formation (Skoog and Miller, 1957), extensive work is being carried out on the effect of plant growth regulators on morphogenesis though deviations from the original dogma are not rare (Halperin, 1986). The concept of endogenous plant growth regulator ratio has been proposed as an explanation for these deviations. Recently the shooty mutant strain of *Agrobacterium tumefaciens* has provided molecular support to the Auxin:Cytokinin balance theory. However the effect of media components and culture conditions on uptake and action of plant growth regulators and vice-versa also must be considered while analyzing the morphogenetic responses of the cultured explants as all these factors interact to trigger the morphogenetic response.

The effects of light, humidity, temperature, culture vessels and closures and particularly growth regulators have been extensively studied, but the effects of mineral nutrients on morphogenesis are rarely studied and poorly understood (Leifert *et al.*, 1995) although in many species use of more favorable medium promotes explant survival, growth and morphogenesis as reviewed by Preece (1995).

The medium composition affects morphogenesis and growth of cultured explants. MS medium is the most frequently used formulation so far for plant tissue cultures

(George *et al.*, 1987, 1988). It is categorized as a high salt medium. Use of such media often results in increased phenolic exudation and darkening of the media that reduces the efficiency of *in vitro* growth and morphogenesis in woody plants. Such problems could be overcome by reduction in the strength of the media. The strength of medium is a very important and highly species specific factor that decides the efficiency of morphogenesis if not morphogenetic induction itself (De Fossard *et al.*, 1974, 1995; Joseph *et al.*, 1996; Mencuccini and Rugini, 1993; Preece *et al.*, 1995; Rugini *et al.*, 1998; Li *et al.*, 1998; Kaneda *et al.*, 1998).

The effects of nitrogen and carbohydrate source on induction of somatic embryogenesis and organogenesis are relatively better studied (Leifert *et al.*, 1995; Preece, 1995). The ionic form of nitrogen i.e. ammonium or nitrate as well as the ratio of ammonium to nitrate ions have been found to govern the induction and efficiency of morphogenesis (Kirby *et al.*, 1987; Grimes and Hodges, 1990; Niedz, 1994; Matsubayashi and Sakagami, 1998).

The carbohydrates serve as the chief energy source for the explants, act as an osmoticum and also sometimes have morphogenetic effects. The carbohydrate requirement is often stage-specific e.g. they promote formation of pro-meristemoids but inhibit further progress of morphogenesis. They also have an effect on rooting. The requirement of carbohydrates for morphogenesis is also species specific. Some species (e.g. carrot, peanut) prefer higher concentrations of carbohydrates for induction and maturation of somatic embryos (Kurata *et al.*, 1992) whereas in other species (e.g. *P. nigrum*) low concentrations of carbohydrates stimulate somatic embryogenesis (Joseph *et al.*, 1997). The concentration and type of carbohydrate used can also regulate the morphogenic pathway as in *Linum usitatissimum* (Millam *et al.*, 1992), *Helianthus annuus* (Bronner *et al.*, 1994) and *Citrus deliciosa* (Cabasson *et al.*, 1995).

Besides nitrogen and carbohydrate source, calcium, boron, copper and cobalt are also reported to control morphogenetic induction (Herman, 1995). Other factors such as the physical form and pH of the medium, gelling agents used, wavelength and intensity of the light during incubation, culture vessel and closure dependent ethylene production also affect induction and efficiency of morphogenesis (Brown and Thorpe, 1986; Herman, 1995).

In this chapter the effects of different factors such as explant types, media and culture conditions and plant growth regulators on morphogenesis in *P. colubrinum* are discussed.

4.2. Materials and Methods:

4.2.1. Plant Material: Leaf, internode, aerial root, inflorescence, node and shoot apical explants were derived from 4-7 year old plants of *P. colubrinum* Link maintained in green-house. The plants were raised from stem cuttings. All explants were excised from first 3-4 nodes of the mother plant.

4.2.2. Surface Sterilization : All explants were washed under running tap water for 20-30 minutes and then treated with a detergent (Teepol) for five minutes. The explants were then surface-disinfected with 70% ethanol for 1 min and washed thoroughly with water. The explants were then transferred to sterile 250 ml flasks in a Laminar Air Flow cabinet, each sterile flask containing 2-3 pieces of a leaf or inflorescence cut horizontally or about 20 nodes or shoot apices or aerial roots or internode pieces. The leaf, inflorescence and shoot apex explants were treated with 0.1% mercuric chloride for 5 min whereas node, root and internodes were treated for 10 min and rinsed with sterile distilled water 5-6 times. Using autoclaved forceps, the explants were transferred to a stack of sterile filter papers and were cut into smaller pieces of appropriate size with a sterile surgical blade (leaf 0.5 cm², inflorescence and internodes 1 cm long, roots, nodes 0.5-0.7 cm long and shoot apices 0.3-0.5 cm long with basal stem portion 0.5-1 cm long) and were then cultured onto respective media.

4.2.3. Induction of morphogenesis in root, inflorescence, leaf and internode explants.

4.2.3.1. Effect of plant growth regulators on morphogenetic induction in root and inflorescence explants: Inflorescence and root explants were cultured in MS medium (Murashige and Skoog, 1962) supplemented with various plant growth regulators (BA, Kinetin, 2,4-D, NAA, Dicamba, Picloram) at different concentrations (0.1-10 mg l⁻¹) and combinations and were subcultured in the same medium at monthly intervals for 5-6 months.

4.2.3.2. Effect of plant growth regulator combinations on morphogenetic induction in leaf and internode explants: Leaf and internode explants were cultured in MS medium (Murashige and Skoog, 1962) supplemented with various plant growth regulators (BA, Kinetin, TDZ, 2,4-D, NAA, Dicamba, Picloram, IAA) at different concentrations (0.5-10 mg l⁻¹) and combinations. The explants formed calli in various

media combinations and were subcultured at monthly intervals in same media combinations or in different subculture media (SMs) with or without auxins or cytokinins.

4.2.3.3. Effect of various basal media formulations on morphogenetic induction in leaf and internode explants: In a mini-factorial experiment, leaf and internode explants were cultured in 6 different basal media (Blayde's medium, B-5, MS, half MS, SH and WPM) supplemented with auxins NAA or 2,4-D (0.5, 1.0 mg l⁻¹) in combination with a cytokinin BA (0.5, 2.0, 3.0 and 5.0 mg l⁻¹).

4.2.3.4. Effect of dark incubation and culture vessels on morphogenetic induction in leaf and internode explants: Leaf and internode explants were cultured in half MS medium supplemented with BA (0.5, 2.0, 3.0 and 5.0 mg l⁻¹) and NAA or 2,4-D (0.5, 1.0 mg l⁻¹) contained in culture tubes (22 mm diameter, Borosil) as well as in disposable petri-dishes (55 mm diameter, Laxbro, India) to assess effect of culture vessels on morphogenetic response. The cultures were incubated in dark as well as at 35 $\mu\text{E.m}^{-2}\text{s}^{-1}$ at 16 h photoperiod till visible callus formation occurred.

4.2.3.5. Callus-mediated shoot induction in leaf explants: Leaf pieces cultured on MS medium with macronutrients at half strength (hereafter referred to as half MS) supplemented with different growth regulators such as BA (1.0, 2.0 mg l⁻¹) with NAA (0.5 mg l⁻¹) or 2,4-D (0.5 and 1.0 mg l⁻¹) or TDZ alone (0.5 mg l⁻¹, 1.0 mg l⁻¹) formed green nodular callus in 30 days. These calli were subcultured in different subculture media after every 3 weeks. After 2-3 passages the calli formed shoot buds which elongated after 2-3 subcultures on the same media in 100 and 250 ml flasks.

4.2.3.6. Organogenetic shoot induction in internode explants: The internode explants cultured in half MS medium supplemented with BA at 5.0 mg l⁻¹ formed nodular organogenetic calli within 30 days. To avoid the harmful effects of long term exposure of BA the explants were given a short duration (10, 60 and 120 min) pulse of high BA by agitating in liquid half MS medium containing high BA (5.0, 10.0, 20.0 mg l⁻¹). The explants were then blotted on sterile filter papers and transferred to semisolid half MS basal medium. The explants formed shoot buds within 45-50 days, which elongated into shoots on the same medium contained in 100 and 250 ml flasks after 3-4 subcultures.

4.2.3.7. Effect of different basal media on sprouting of pre-existing shoot meristems: The shoot apices and nodes were cultured on different basal media used for leaf and internode explants without any plant growth regulators to assess the effect of basal media on sprouting. Sprouted meristems were subcultured in the same medium at monthly intervals.

4.2.3.8. Multiple shoot induction in pre-existing shoot meristems: The shoot apices and nodal explants were cultured in half MS basal medium supplemented with BA (0.5, 2.0, 5.0 mg l⁻¹) The shoot apices formed calli on media supplemented with BA at 2.0 and 5.0 mg l⁻¹ within 2-3 weeks which on subculture on same media formed shoot buds that elongated into shoots within a period of 2 months.

The nodal explants cultured for 35-40 days in medium containing BA (0.5, 2.0 and 5.0 mg l⁻¹) formed shoot buds from the nodal portion directly without any callus formation. The shoot buds elongated into shoots in same medium contained in 100 and 250 ml flasks after 3 subcultures.

4.2.4. Rooting of shoots derived from leaf calli, shoot apex calli and nodes: Shoots derived from leaf calli were rooted *in vitro* in half MS basal medium with or without IAA or IBA. The shoots derived from axillary and apical shoot meristems were rooted *ex vitro* in the soil.

4.2.5. Media and culture conditions: The explants were cultured in disposable petridishes or in tubes. The shoots were elongated in tubes and in conical flasks. The cultures were incubated at $25 \pm 2^{\circ}\text{C}$ either in dark or at the light intensity of $35 \mu\text{E m}^{-2}\text{s}^{-1}$ for 16 h photoperiod. Each treatment contained at least 10 explants and every experiment was repeated at least twice.

4.2.6. Acclimatization: The plantlets derived from leaf calli and shoots derived from apical and axillary meristems were acclimatized as described in Chapter 2.

4.2.7. Histology: Leaf, internode and nodal explants responding for *in vitro* shoot bud formation were fixed, sectioned serially and stained as described in Chapter 2 of this thesis.

4.3. Results and Discussion:

As described earlier, morphogenesis *in vitro* depends on action and interaction of various biological, chemical and physical factors. In order to achieve morphogenesis in *P. colubrinum*, different parameters were studied. These include explant types, growth regulators at different concentrations and combinations, duration of growth regulator treatment, basal media as well as dark and light incubation of cultured explants.

4.3.1. Effect of different explant types on morphogenetic induction:

Differential morphogenetic potentiality was exhibited by the root, internode, leaf, inflorescence and meristem explants. The leaf explants were most amenable to callus formation followed by inflorescence, internode and root explants (Tables 4.1, 4.2). The leaf explant showed four different callus morphotypes namely green nodular, white friable, whitish green friable and brown friable (Figs. 4.1 A) while internode explants gave rise to greenish white powdery calli (Fig. 4.1B), green nodular calli (Fig 4.1C) and creamish white nodular calli (Fig. 4.1D) which further proliferated into white friable calli. Root explants produced brown or white calli and the inflorescence explants always formed white friable callus. Organogenetic response in the form of rhizogenesis (Fig. 4.1E) was obtained in media combinations 15, 16, 19-22 (Tables 4.1, 4.2) in leaf, root and internode explants. Production of green nodular callus capable of shoot formation in media combinations 5, 40, 41, 48, 49, 63, 66-68 (Table 4.2) was observed only in leaf and internode explants. The shoot apices formed multiple shoots via caulogenesis whereas the nodal explants formed shoot buds directly without any callus formation. (Table 4.2).

4.3.2. Effect of different growth regulators on morphogenetic induction in root and inflorescence explants:

The root and inflorescence explants were cultured on MS medium supplemented with various growth regulators at different concentrations and combinations in order to assess their morphogenetic potential. The choice of medium was based on previous reports in *Piper* spp. (Chua, 1981; Chua and Talib, 1982; Mathews and Rao, 1984; Fitchet, 1988, 1990). Callus formation was found to be the most common morphogenetic response besides rhizogenesis from root explants in auxin containing media. Callus formation in inflorescence explants occurred within 3-4 weeks only at cut ends. In root explants callus was initiated after 5-6 weeks at the cut ends as

Fig. 4.1

A. Callus morphotypes in leaf explants of wild pepper.

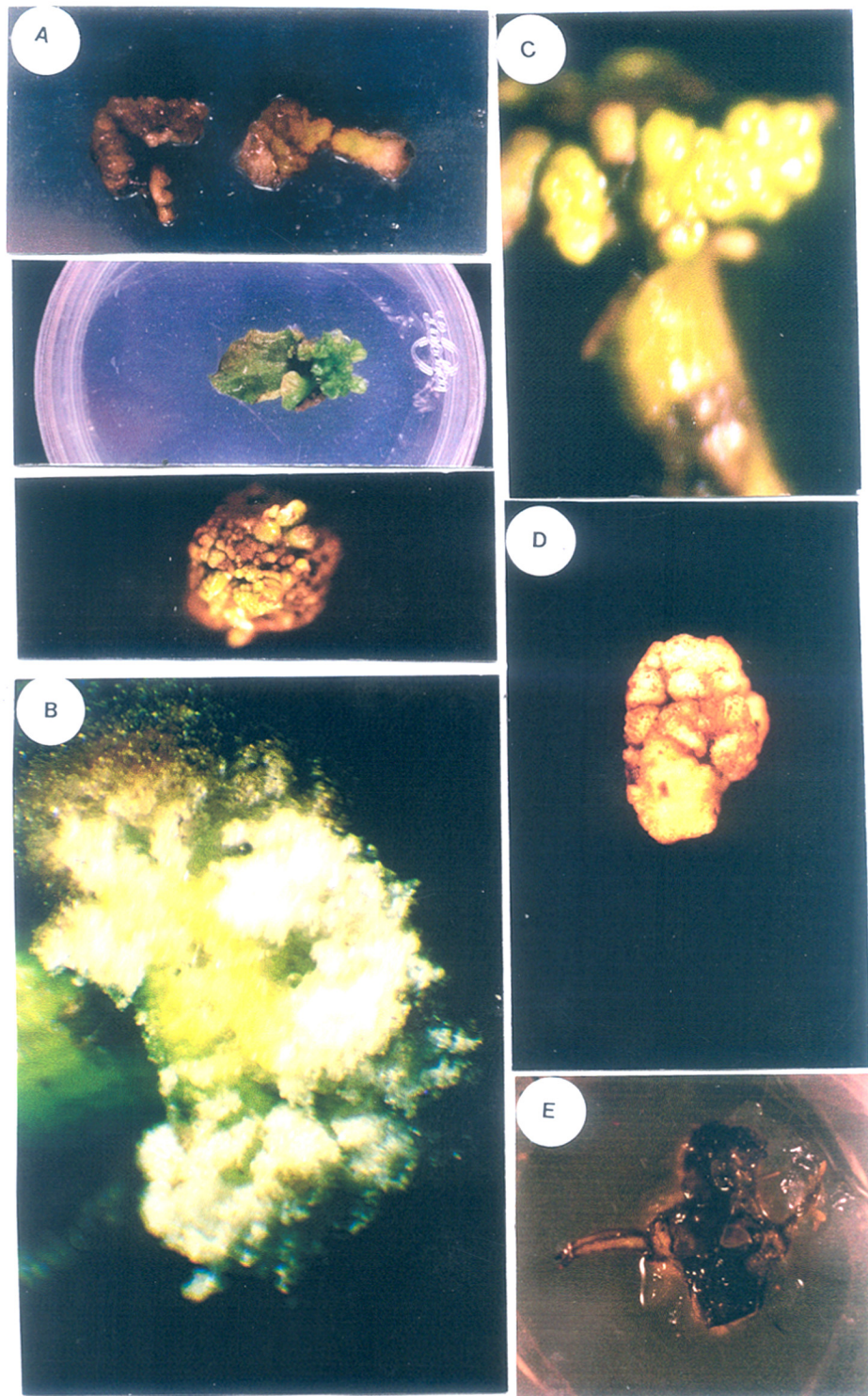
B. Powdery callus in mature internode explants of wild pepper in half MS with BA 1 mg l^{-1} .

C. Nodular callus in mature internode explants of wild pepper in half MS with BA 2 mg l^{-1} NAA 1 mg l^{-1}

D. Creamy-White callus in mature internode explants of wild pepper in half MS with Kinetin 1.0 mg l^{-1}

E. Rhizogenesis in leaf explants of wild pepper in half MS with NAA 0.5 mg l^{-1}

FIG. 4.1



well as at the median portion. In root and inflorescence explants brown and white friable callus was formed respectively. Cytokinins at high concentrations caused blackening of inflorescence explants. This type of callus did not undergo morphogenesis on subculture (Table 4.1) and degenerated after 5-6 subcultures in same media combinations or in basal medium.

4.3.3. Effect of different growth regulators on morphogenetic induction in leaf and internode explants: Different callus morphotypes were observed in leaf and internode explants (Table 4.2). Among the callus morphotypes, only the nodular green callus induced in leaf and internode explants in media containing BA or TDZ (with or without auxins) could give rise to shoot buds on further subcultures. Occasionally direct or callus mediated rhizogenesis was induced in auxin containing media in leaf and internode explants but direct shoot bud formation could not be induced from either explant (Table 4.2). High concentrations of BA and kinetin (5.0 mg l^{-1}) were detrimental to callus formation in both leaf and internode explants and caused darkening of explants as well as the callus. These results support the observations of Mathews and Rao (1984) in *Piper nigrum* L. In media containing BA and TDZ, leaf explant was swollen and entire explant underwent callusing whereas in all the other plant growth regulators callus formed only at cut ends similar to internode. The callus induction in leaf occurred within 3-4 weeks whereas in internode explants it took 5-6 weeks.

Table 4.1. Effect of different plant growth regulators on morphogenesis in inflorescence and root explants derived from mature plants of *P. colubrinum* Link.

No.	PGR/s	Conc. (mg l ⁻¹)	Root	Inflorescence
1	BA	0.1	-	-
2		0.5	-	-
3		1.0	-	+W
4		2.0	-	+W
5		5.0	+G	-
6	Kinetin	0.1	-	-
7		0.5	-	+W
8		1.0	+W	+w
9		2.0	+W	+W
10		5.0	+W	-D
11	2,4-D	0.1	+W	-
12		0.5	+W	-
13		1.0	-	+W
14		3.0	-	+W
15		5.0	-R	+W
16	NAA	10.0	-R	+W
17		0.1	-	-
18		0.5	+W	+W
19		1.0	+W; R	+W
20		3.0	+W; R	+W
21	2,4,5-T	5.0	+W; R	+W
22		10.0	+W; R	-
23		0.1	-	-
24		0.5	-	-
25		1.0	-	-
26	Dicamba	3.0	-	+W
27		5.0	-	+W
28		10.0	-	-
29		0.1	-	-
30		0.5	-	+W
31	Picloram	1.0	-	+W
32		3.0	-	+W
33		5.0	-	-
34		10.0	ND	ND
35		0.05	-	-
36		0.1	-	-
37		0.25	-	-
38		0.5	+W	-
39		1.0	ND	+W

PGR: Plant growth regulator; Conc.: Concentration; W: White callus; G: Green callus; -: No callus formation; + : callus formation; R: Root induction; D: Darkening of explant / callus; ND: Not done.

Table 4.2. Effect of different plant growth regulators on morphogenesis of mature leaf and internode explants derived of *P. colubrinum* .

No.	PGR/s	Conc.(mg l ⁻¹)	Leaf	Internode
1	BA	0.1	-	-
2		0.5	+GW	-
3		1.0	+G	+W
4		2.0	+G;D	+W
5		5.0	+D	+GN;D
6	Kinetin	0.1	-	-
7		0.5	+GW	-
8		1.0	+GW	+W
9		2.0	+W;D	+W;D
10		5.0	+W;D	+W;D
11	2,4-D	0.1	-	-
12		0.5	-	-
13		1.0	+ W	-
14		3.0	+ W;	+W
15		5.0	+ W;	+W; R
16		10.0	+ W; R	+W; R
17	NAA	0.1	-	+W
18		0.5	+ W	+W
19		1.0	+ W	+W
20		3.0	+ W; R	+W
21		5.0	+ W; R	+W
22		10.0	+ W; R	+W; R
23	2,4,5-T	0.1	-	-
24		0.5	-	-
25		1.0	+ GT	-
26		3.0	+ GT	-
27		5.0	+ GT	+W
28		10.0	+ GT	+W
29	Dicamba	0.1	+ W	-
30		0.5	+ W	-
31		1.0	+ W	-
32		3.0	+ W	-
33		5.0	+ W	+GW
34		10.0	ND	+GW
35	Picloram	0.05	+ W	-
36		0.1	+ W	-
37		0.25	+ W	-
38		0.5	+ W	+GW
39		1.0	+ W	+GW

Table 4.2 contd.

No.	PGR/s	Conc.(mg l ⁻¹)	Leaf	Internode
40	TDZ	0.5	+GN	+G
41		1.0	+GN	-
42		3.0	+GW	+GN
43		5.0	+GW	-
44		10.0	+GW; R	ND
45	BA + 2,4-D	0.1 + 0.5	+ W	-
46		0.1 + 1.0	+ W	-
47		0.1 + 2.0	+ W; R	-
48		1.0 + 0.5	+GN	+G
49		1.0 + 1.0	+GN	+G
50		1.0 + 2.0	+G; R	+W
51		2.0 + 0.5	-	+G
52		2.0 + 1.0	+W	+G
53		2.0 + 2.0	+W	+G
54		5.0 + 0.5	-	-D
55		5.0 + 1.0	-	-D
56		5.0 + 2.0	-	+G
57	BA+ NAA	0.1 + 0.5	-	-
58		0.1 + 1.0	-	-
59		0.1 + 2.0	+W	-
60		1.0 + 0.5	+G	-
61		1.0 + 1.0	+G	-
62		1.0 + 2.0	+GW	-
63		2.0 + 0.5	+GN	+G
64		2.0 + 1.0	ND	+G
65		2.0 + 2.0	+GW	+G
66		5.0 + 0.5	+	+GN; D
67		5.0 + 1.0	+GN	+GN; D

Table 4.2 contd.

No.	PGR/s	Conc.(mg l ⁻¹)	Leaf	Internode
68	BA+ NAA	5.0 + 2.0	+GN	+GN; D
69	Kinetin + 2,4-D	0.1+ 0.5	-	-
70		0.1 + 1.0	+W	-
71		0.1 + 2.0	+W; R	-
72		1.0 + 0.5	+W	-
73		1.0 + 1.0	+W	-
74		1.0 + 2.0	+W	-
75		2.0 + 0.5	+W	-
76		2.0 + 1.0	+W	-
77		2.0 + 2.0	+W	+W
78		5.0 + 0.5	-	-D
79		5.0 + 1.0	-	-D
80		5.0 + 2.0	-	++W
81	Kinetin + NAA	0.1 + 0.5	-	-
82		0.1 + 1.0	-	-
83		0.1 + 2.0	+W	-
84		1.0 + 0.5	+G	-
85		1.0 + 1.0	-	+G
86	Kinetin + NAA	1.0 + 2.0	+W	+W
87		2.0 + 0.5	+G	+G
88		2.0 + 1.0	+W	-
89		2.0 + 2.0	-	+W
90		5.0 + 0.5	-	-D
91		5.0 + 1.0	-	-D
92		5.0 + 2.0	-	-D

Table 4.2 contd

No.	PGR/s	Conc.(mg l ⁻¹)	Leaf	Internode
93	BA + IAA	1.0 + 1.0	+GW	-
94		1.0 + 2.0	+GW	-
95		1.0 + 5.0	-	R
96		2.0 + 1.0	+GW	-
97		2.0 + 2.0	+GW	-
98		2.0 + 5.0	+GW	-
99	BA + NAA + 2,4-D	0.5 + 1.0 + 1.0	+W	+W
100		2.0 + 1.0 + 1.0	+W	-
101	Kinetin + NAA + 2,4-D	0.5 + 1.0 + 1.0	+GW	+W
102		2.0 + 1.0 + 1.0	+GW	+W

PGR: Plant growth regulator; Conc.: Concentration; W: White callus; G: Green callus;

GN: Green nodular callus; GT: Green translucent callus GW: greenish White callus;

-: No callus formation; + callus formation; R: Root induction; D: Darkening of explant / callus; ND: Not done.

4.3.4. Effect of different basal media on *in vitro* response of leaf and internode explants: The effect of basal media on explant survival and morphogenesis is profound. The media components and plant growth regulators are considered to be capable of replacing each other or at least their interactions could be manipulated to minimize harmful effects of each other (Preece, 1995). A small factorial experiment involving 6 basal media and 12 auxin-cytokinin combinations (found to induce nodular callus in previous experiment) was carried out to test the effect of basal media on morphogenetic response of leaf and internode explants.

Among the basal media tested, we found that half MS was most suitable for callus induction in both leaf and internode explants, followed by WPM and B5; whereas Blayde's, SH and MS media at full strength supported poor callus formation at all the growth regulator combinations tested. This could have resulted from low-salt composition of half MS medium which effectively controlled f medium. On the basis of

these results, half MS medium was used in further studies.

Table 4.3. Effect of different basal media and hormone concentrations on the percentage of callus formation in leaf and internode explants of *P. colubrinum* Link.

PGR	Conc. (mg l ⁻¹)	MS		Half MS		SH		WPM		BD		B5	
		L	I	L	I	L	I	L	I	L	I	L	I
B + N	2.0 + 0.5	14	10	100	35	20	10	60	30	25	12	45	30
	3.0 + 0.5	26	12	80	48	25	10	50	52	20	10	50	28
	5.0 + 0.5	12	10	35	30	10	6	30	21	10	5	10	8
	2.0 + 1.0	17	9	50	40	25	25	56	25	8	8	35	10
	3.0 + 1.0	22	15	80	30	15	18	45	30	20	20	42	20
	5.0 + 1.0	15	12	30	20	10	5	32	15	22	16	26	12
B + D	2.0 + 0.5	20	22	100	40	22	20	50	55	32	15	40	21
	3.0 + 0.5	20	20	47	55	18	12	45	42	30	22	37	17
	5.0 + 0.5	11	6	33	20	7	5	33	28	17	9	28	15
	2.0 + 1.0	25	10	73	36	9	6	40	33	33	12	25	12
	3.0 + 1.0	30	14	100	35	16	15	41	27	24	10	20	10
	5.0 + 1.0	15	5	40	20	12	4	20	20	20	10	15	12

PGR: Plant growth regulator; Conc.: Concentration; B: BA; N: NAA; D: 2,4-D; MS: Murashige and Skoog's medium (1962); Half MS: Ms medium with macronutrients at half strength; SH: Schenk and Hildebrandt's medium (1972); WPM: Woody plant medium, Lloyd and McCown (1981); BD: Blayde's medium (1966); B₅: Gamborg's medium (1968); L: Leaf; I: Internode.

4.3.5. Effect of Light during incubation on *in vitro* response of leaf and internode explants: The leaf and internode explants cultured in half MS medium containing BA (2.0, 3.0 and 5.0 mg l⁻¹) with NAA (0.5, 1.0 mg l⁻¹) or 2,4-D (0.5, 1.0 mg l⁻¹) were incubated under dark conditions as well as at light intensity of 38 µE.m⁻².s⁻¹ for 16 h photoperiod. In dark the responses for callus formation were similar to those in light but all the calli were white whether nodular or friable in nature. The percentage of browning in dark conditions was less (Chapter 3). But none of these calli could be regenerated on subculture. This proves essentiality of light for successful regeneration.

4.3.6. Effect of culture vessels on *in vitro* response of leaf and internode

explants: The leaf and internode explants were cultured in half MS medium supplemented with BA (2.0, 3.0 and 5.0 mg l⁻¹) and NAA or 2,4-D (0.5, 1.0 mg l⁻¹) contained in disposable petri-dishes as well as in tubes. The morphology and percentage response of the explants for callus formation did not differ in these two culture vessels; however use of petridishes facilitated culture of more explants per replicate (Table 4.4).

Table 4.4. Effect of culture vessels on callus formation in Internode and Leaf explants in Half MS with BA and NAA or 2,4-D.

PGR (mg l ⁻¹)	Explants forming callus (%)			
	Petridish (55 x 15 mm)		Culture Tube (22 x 140 mm)	
	Leaf	Int	Leaf	Int
B 2.0 + N 0.5	100	35	88	40
B 3.0 + N 0.5	80	48	90	44
B 5.0 + N 0.5	35	30	40	40
B 2.0 + N 1.0	50	40	46	30
B 3.0 + N 1.0	80	30	70	40
B 5.0 + N 1.0	30	20	30	30
B 2.0 + D 0.5	100	40	90	50
B 3.0 + D 0.5	47	55	50	60
B 5.0 + D 0.5	33	20	40	38
B 2.0 + D 1.0	73	36	70	30
B 3.0 + D 1.0	100	35	100	20
B 5.0 + D 1.0	40	20	30	25

PGR: Plant growth regulator, Int : Internode, B: BA, N: NAA

4. 3.7. Indirect organogenesis in leaf explants:

4.3.7.1. Effect of callus initiation medium (CIM) on induction of

organogenesis: Leaf explants cultured in half MS medium containing TDZ (0.5, 1.0, 5.0 mg l⁻¹) alone or in half MS with BA (0.5, 1.0, 2.0 mg l⁻¹) with NAA or 2,4-D (0.5, 1.0 mg l⁻¹) formed callus in 3-4 weeks. These calli were subcultured on respective media at regular intervals of 3 weeks. Only the intense green nodular calli (Fig.4.2 A-1) formed on media supplemented with BA 2.0 + NAA 0.5; BA 1.0 + 2,4-D 0.5; BA 1.0 + 2,4-D 1.0; BA 2.0 + 2,4-D 0.5; TDZ 0.5; TDZ 1.0 mg l⁻¹ developed shoot buds after 2-3 subcultures

(Figs. 4.2 A-2, 4.2 B; Table 4.5).

Exogenous supply of both a cytokinin and an auxin was required for induction of organogenetic calli in leaf explants. However TDZ alone could evoke organogenetic response in the leaf explants. This supports the possible dual nature of TDZ as suggested earlier (Mok *et al.*, 1987).

4 3.7.2. Effect of subculture medium (SM) on efficiency of organogenesis:

Out of the 6 media combinations in which organogenetic green nodular calli developed, 5 could support shoot bud formation on 2-3 subcultures (Table 4.5, Fig. 4.2C). These shoot buds elongated to form shoots after 4 subcultures in the same medium. The shoot buds induced in half MS medium containing BA 1.0 + 2,4-D 1.0 and BA 2.0 + 2,4-D 0.5 failed to elongate even after 7-8 subcultures but de-differentiated into white friable callus. Such callus proliferated for another two subcultures, then turned brown and eventually died.

To increase the efficiency of shoot formation, the calli were subcultured on individual cytokinin or auxin media and also on media containing reduced levels of cytokinins BA or TDZ (0.0, 0.5 mg l⁻¹) (Table 4.5). It was observed that intense green calli cultured on media containing auxins alone, reverted to de-differentiation irreversibly and formed white friable calli which could not be regenerated after further subcultures on cytokinin containing media. The maintenance of an auxin (NAA or 2,4-D) individually in SM triggered further de-differentiation nullifying the effect of cytokinin in CIM (Table 4.5). Thus the requirement of an auxin was found to be stage-specific i.e. auxin was needed for induction of organogenesis, it became irreversibly inhibitory in the later stages of the same process indicating that the organogenetic process has distinct 'phases' with different requirements of presence and ratio of the growth regulators supplied exogenously (Christianson and Warnick 1983, 1985).

Shoot formation was not inhibited by BA at 1.0 and 2.0 mg l⁻¹ in SM but resulted in reduced number of shoots per explant. The highest number of shoots per explant was obtained in SM containing BA at 0.5 mg l⁻¹. Continued presence of BA at a lower concentration in the SM boosted the shoot formation as is evident from Table 4.5.

In SM, TDZ at the concentration of 1.0 mg l⁻¹ resulted in further proliferation of the intense green callus and profuse rooting. TDZ at 0.5 mg l⁻¹ supported shoot formation

with lower efficiency (Table 4.5) than BA at 0.5 mg l⁻¹ as well as rooting (Fig. 4.2C).

Table 4.5. Effect of growth regulators on shoot bud induction and elongation in leaf derived callus cultures of *P. colubrinum* Link.

CIM (mg l ⁻¹)	SM (mg l ⁻¹)	S B I (%)	Shoots per explant Mean $\pm$ s. d.
B 2.0 + N 0.5	B 2.0 + N 0.5	71.6 $\pm$ 1.6	7.2 $\pm$ 2.8 ^a
	N0.5	0*	0 ^b
	B 2.0	50.0 $\pm$ 0.0	3.8 $\pm$ 0.7 ^c
	B 0.5	70 $\pm$ 10.0	9.2 $\pm$ 2.7 ^a
	Half MS basal	25.0 $\pm$ 5.0	3.4 $\pm$ 1.1 ^c
B 1.0 + D 0.5	B 1.0 + D 0.5	90.0 $\pm$ 0.0	4.6 $\pm$ 1.7 ^c
	D 0.5	0*	0 ^b
	B 1.0	59.4 $\pm$ 4.9	4.5 $\pm$ 1.1 ^d
	B 0.5	80.0 $\pm$ 0.0	5.9 $\pm$ 1.7 ^{ac}
	Half MS basal	20.0 $\pm$ 0.0	2.2 $\pm$ 0.5 ^{bc}
B 1.0 + D 1.0	B 1.0 + D 1.0	48.9 $\pm$ 5.5	0 ^b
	D 1.0	0*	0 ^b
	B 1.0	20.0 $\pm$ 0.0	1.8 $\pm$ 0.7 ^{bc}
	B 0.5	49.9 $\pm$ 5.5	3.4 $\pm$ 1.3 ^{cd}
	Half MS basal	0*	0 ^b
B 2.0 + D 0.5	B 2.0 + D 0.5	53.3 $\pm$ 6.7	0 ^b
	D 0.5	0*	0 ^b
	B 2.0	60.0 $\pm$ 10.0	3.2 $\pm$ 1.4 ^d
	B 0.5	66.6 $\pm$ 6.5	3.5 $\pm$ 1.4 ^d
	Half MS basal	21.0 $\pm$ 0	2.0 $\pm$ 0.0 ^{bc}
TDZ 0.5	TDZ 0.5	33.3 $\pm$ 6.7	5.6 $\pm$ 1.7 ^{ad}
	BA 0.5	45.0 $\pm$ 5.0	7.8 $\pm$ 2.4 ^{ad}
	Half MS basal	30.0 $\pm$ 0.0	3.0 $\pm$ 1.0 ^{bd}
TDZ 1.0	TDZ 1.0	0**	0 ^b
	TDZ 0.5	20.0 $\pm$ 0.0	4.7 $\pm$ 0.9 ^d
	BA 0.5	40 $\pm$ 0.0	5.1 $\pm$ 2.0 ^d
	Half MS basal	25.0 $\pm$ 0.0	2.0 $\pm$ 1.0 ^{bd}

CIM: callus induction medium, SM: subculture medium, SBI: shoot bud induction;

*: Callus formation ; **: Callus and Root formation

Values followed by same letter do not differ significantly at 95% confidence level.

The preservation of organogenetic potential of the leaf calli subcultured on half MS basal medium could be due to the 'lingering effect of BA' (Bonga and Aderkas, 1992) as observed in *Alnus* spp. (Evers, *et al.*, 1988) which could have resulted due to

Fig. 4.2

A1. Green nodular callus in half MS with BA 2.0 and NAA 0.5 mg l⁻¹ in leaf explants of wild pepper.

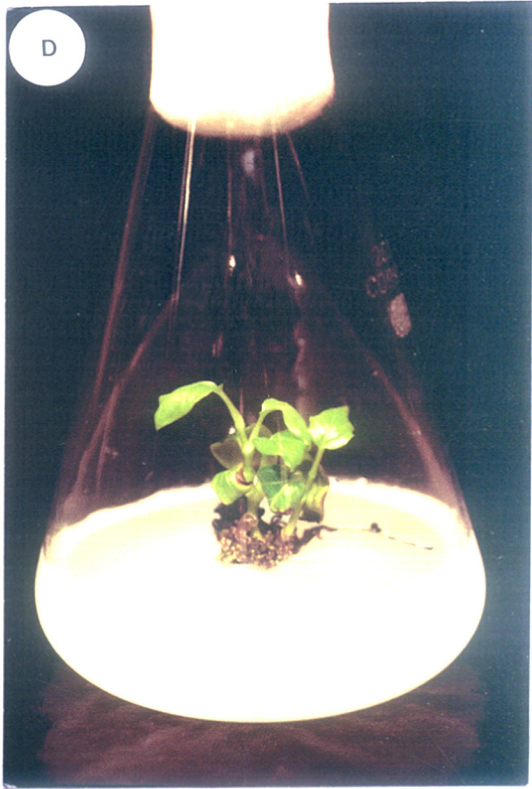
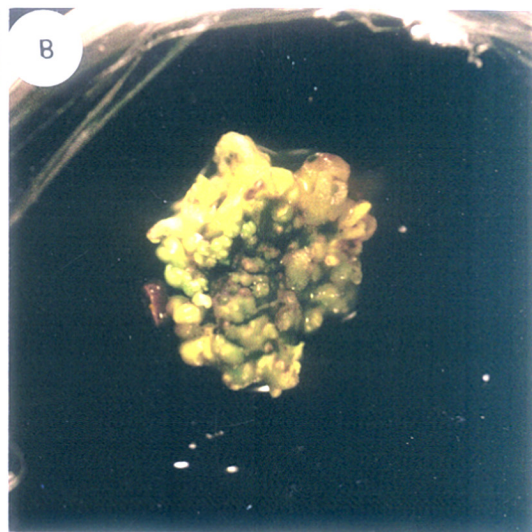
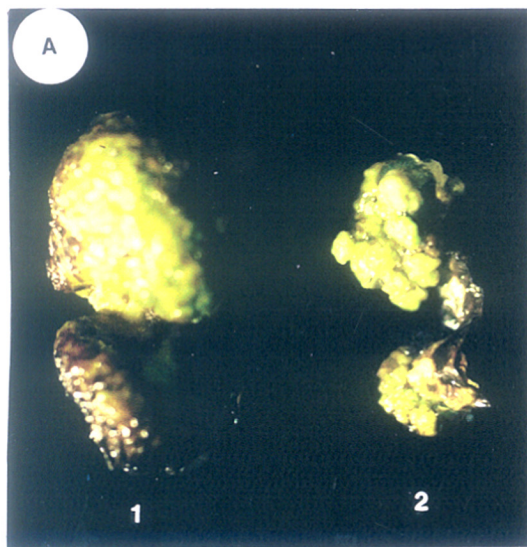
A2. Shoot bud induction in green nodular callus in half MS with BA 2.0 and NAA 0.5 mg l⁻¹ in leaf explants of wild pepper.

B. Shoot bud induction in leaf explants of wild pepper cultured in half MS with BA 2.0 mg l⁻¹ and 2,4-D 1.0 mg l⁻¹

C. Shoot bud induction alongwith root formation in leaf explants of wild pepper cultured in half MS with TDZ 0.5 mg l⁻¹.

D. Elongation of shoots developed from leaf explants of wild pepper in half MS with BA 0.5 mg l⁻¹

FIG.4.2



either the retention of BA or BA-induced irreversible commitment of the tissue for organogenesis.

4.3.7.3. *In vitro* rooting of the leaf callus derived shoots: The elongated shoots (Fig. 4.2D) were separated from callus and transferred to half MS basal medium with or without IAA (0.1-1.0 mg l⁻¹) or IBA (0.1-1.0 mg l⁻¹). Both IAA and IBA induced rooting at all the concentrations tested (Table 4.6) at varying frequency. However, callus formation (Fig. 4.3A and 4.3B) occurred at the base of shoots making the origin of roots obscure. Half MS was found to be optimum with 100% rooting without any callusing (Fig. 4.3C).

Table 4.6. Effect of plant growth regulators on rooting response of *in vitro* shoots of *Piper colubrinum* Link.

Auxin	Conc. (mg l ⁻¹)	% Rooting	Callus
IBA	0.1	10.0	+
	1.0	60.0	+
	5.0	40.0	+
	10.0	60.0	+
IAA	0.1	20.0	+
	1.0	100.0	+
	5.0	80.0	+
	10.0	20.0	+
None	-	100.0	-

4.3.7.4. Acclimatization: The rooted plantlets were transferred to pots containing soil: vermiculite mixture. A total of 60 hardened plants were successfully transferred to the greenhouse. The survival rate of rooted plantlets after hardening was 100% (Fig. 4.3D).

4.3.7.5. Histology: The origin of shoot buds from callus was examined by histological preparations. The calli showed asynchronous development of shoot buds i.e. shoot buds at more than one stage were observed in one and the same callus. Meristematic pockets of small cells with dense cytoplasm and darkly stained nuclei (Fig. 4.4A) were scattered in callus tissue indicating initial stage of differentiation. Presence of small protrusions (Fig. 4.4B) of meristematic cells suggested the initiation of shoot buds. The anatomy of differentiated shoot buds (Fig. 4.4C) alongwith leaves from callus confirmed the

Fig. 4.3

A. Rooting of shoots with callus formation in wild pepper derived from leaf callus in IAA 1.0 mg l^{-1}

B. Rooting of shoots with callus formation in wild pepper derived from leaf callus in IBA 0.5 mg l^{-1}

C Rooting of shoots without callus formation in wild pepper derived from leaf callus in
half MS basal medium

D.Acclimatization of wild pepper plantlet.

FIG.4.3



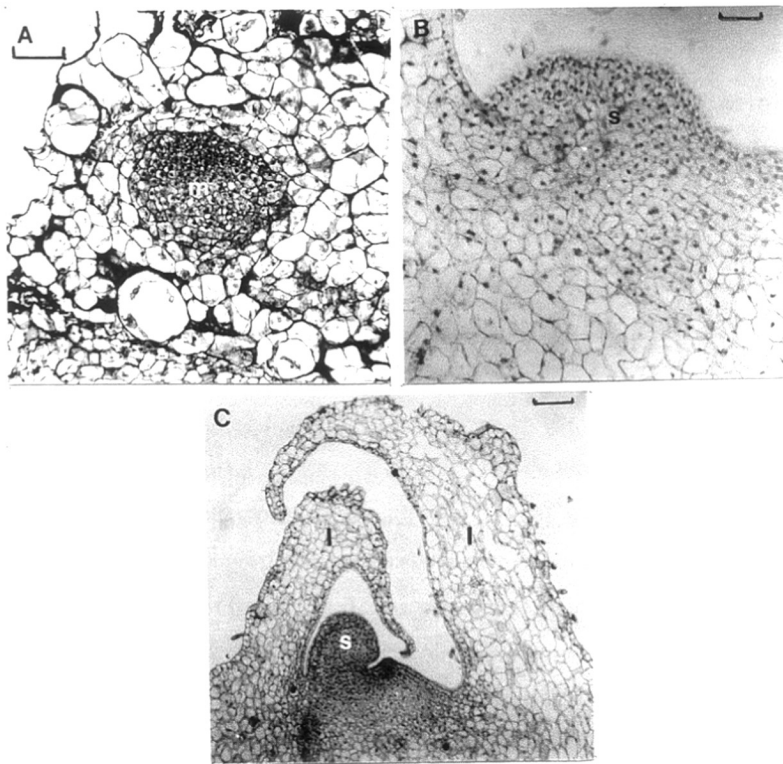
Fig. 4.4

A. Morphogenetic patch formation alongwith surrounding callus cells in leaf explant of wild pepper. Bar = 0.1 mm

B. Formation of morphogenetic dome prior to shoot bud foration in leaf explant of wild pepper. Bar = 0.1 mm

C. Shoot bud formation in leaf explant of wild pepper. Bar = 0.2 mm

FIG.4.4



organogenetic pathway of differentiation.

4.3.8. Organogenesis in internode explants:

Two patterns of organized development are recognized in the explants responding for morphogenesis:

1. Indirect pattern where de-differentiation of explants into callus is followed by re-differentiation into organs.
2. Direct pattern where explants form organs without intervening callus phase (Hicks, 1980).

4.3.8. 1. Effect of continuous exposure of BA: Both the patterns were found to occur in the internode explants. The media containing BA at 5.0 mg l^{-1} with or without NAA (Table 4.2) supported production of intense green nodular calli in 5-6 weeks from internode explants. However the calli turned brown and died. Further in media containing high BA (5.0 mg l^{-1}) the percentage of callus production was low and explant browning was high. Thus though high dose of BA was essential for organogenetic induction in internode explants, it adversely affected the process over a long period of time (5-6 weeks).

4.3.8.2. Effect of pulse treatment of BA: The relationship of the uptake of BA and shoot regeneration is very critical. Biondi *et al.* (1984) also found that the rate of uptake of BA was directly proportional to the efficiency of organogenesis and the uptake was most rapid in the first 60 minutes. It has also been observed that in many plants, BA is required only during a short meristematic induction period (Biondy and Thorpe, 1982; von Arnold and Eriksson, 1985; von Arnold and Hakman, 1988). Such short term treatments (Fukuda and Komamine, 1985) also reduced the harmful effects of BA such as excessive formation of buds, stunting of shoots and difficulties in rooting of the shoots. Hence to reduce browning of explants and calli and to obtain shoots from internode explants we carried out experiments in which the internode explants were given a pulse of BA at high concentrations (5.0, 10.0, 20.0) for 10, 60 and 120 min and then the explants were transferred to half MS basal medium that also served as the control treatment.

On incubation in half MS basal medium for 5-6 weeks, the pulse treated explants exhibited induction of small structures (Fig 4.5A, 4.5B) initially at peripheral and later in the central parts. These structures after 2-3 subcultures in the same medium elongated into shoot buds which further elongated into shoots after 6-8 weeks (Table 4.7). Sometimes shoot bud induction was accompanied by callus formation at the cut ends of the responding explants. The explants cultured on half MS basal medium, i.e. control, remained unchanged even after 3-4 subcultures, suggesting the essential role of BA in shoot-bud induction.

Table 4.7. Effect of pulse treatments of BA on internode explants of *Piper colubrinum* Link.

BA (mg l ⁻¹)	Pulse (min.)	Shoot Bud Induction (%)	Shoots/ explant Mean $\pm$ s.d.
5.0	10	0 ^a	0 ^a
	60	50.0 $\pm$ 0.0 ^b	4.1 $\pm$ 1.2 ^b
	120	53.1 $\pm$ 6.9 ^b	4.0 $\pm$ 0.5 ^b
10.0	10	0 ^a	0 ^a
	60	37.5 $\pm$ 2.5 ^c	4.0 $\pm$ 1.0 ^b
	120	45.0 $\pm$ 5.0 ^c	3.8 $\pm$ 1.0 ^b
20.0	10	0 ^a	0 ^a
	60	22.5 $\pm$ 2.5 ^d	4.2 $\pm$ 0.7 ^b
	120	36.6 $\pm$ 3.3 ^c	3.3 $\pm$ 0.8 ^b

Values followed by same letter do not differ significantly at 95% confidence level.

Unlike the leaf explants, for internode explants, BA alone was sufficient for organogenesis. The pulse of BA for 10 min could not induce shoot buds at any concentration tried. Hence, exposure of the explants for more than 10 min was required to induce shoot buds in the explants. Shoot bud formation was induced by pulse of 60 and 120 min in the internode explants. Both the treatments yielded similar response in the number of shoots formed per explant for all three concentrations of BA. The percentage of explants forming shoot buds also did not show statistically significant differences except for BA 20.0 mg l⁻¹ (Table 4.7).

Thus the increase in concentration of BA compensated the decrease in duration of treatment and this combination also could overcome the inhibition of organogenesis

caused by higher concentrations of BA. The use of liquid medium for pulse treatment also must have facilitated faster uptake of BA in agreement with earlier reports (Bornman and Vogelman, 1984)

The pulse treatment resulted in change of the organogenetic pattern. In the continuous presence of BA 5.0 mg l^{-1} the internode explants formed organogenetic callus (Table 4.1) as opposed to the *de novo* shoot bud induction in the pulse experiment.

4.3.8.3. Elongation and rooting of shoots: The shoots maintained in half MS Basal medium for 2-3 subcultures elongated and rooted in the same medium with 100% efficiency (4.5C). These shoots were not hardened and were used for further in vitro multiplication.

This protocol of plant regeneration involves direct pattern of shoot regeneration which is preferred for genetic manipulation studies. The shoot regeneration efficiency of this method is comparable to that of leaf callus mediated method. In addition the short exposure to growth regulators is also desirable considering their toxic effects at higher concentrations.

4.3.8.4. Histology: In the internode explants treated with BA pulse, induction of organogenesis was initiated in the epidermal layer (Fig. 4.5-IIIA). Certain cells of epidermis became meristematic and by repeated cell divisions, gave rise to small groups of densely stained, smaller isodiametric cells, in the subepidermal region. These Small groups upon further cell divisions formed meristematic patches and domes. The separation of the meristematic pockets from mother tissue by development of separate epidermis was however not observed as against the structures in leaf calli (Fig. 4.4A). Distinction of separate epidermis was observed only in the later stages of shoot formation (Fig. 4.5-IIIC, D). Absence of callus cells confirmed the direct origin of shoot buds.

Fig. 4.5

A. Shoot bud induction in mature internode explant of wild pepper in half MS pulse treated with BA 20.0 mg.l⁻¹ for 60 min.

B. Shoot bud induction in mature internode explant of wild pepper in half MS pulse treated with BA 5.0 mg.l⁻¹ for 120 min.

C. Elongation and rooting of shoot derived from BA pulse treated internode of wild pepper in half MS basal medium.

FIG. 4.5

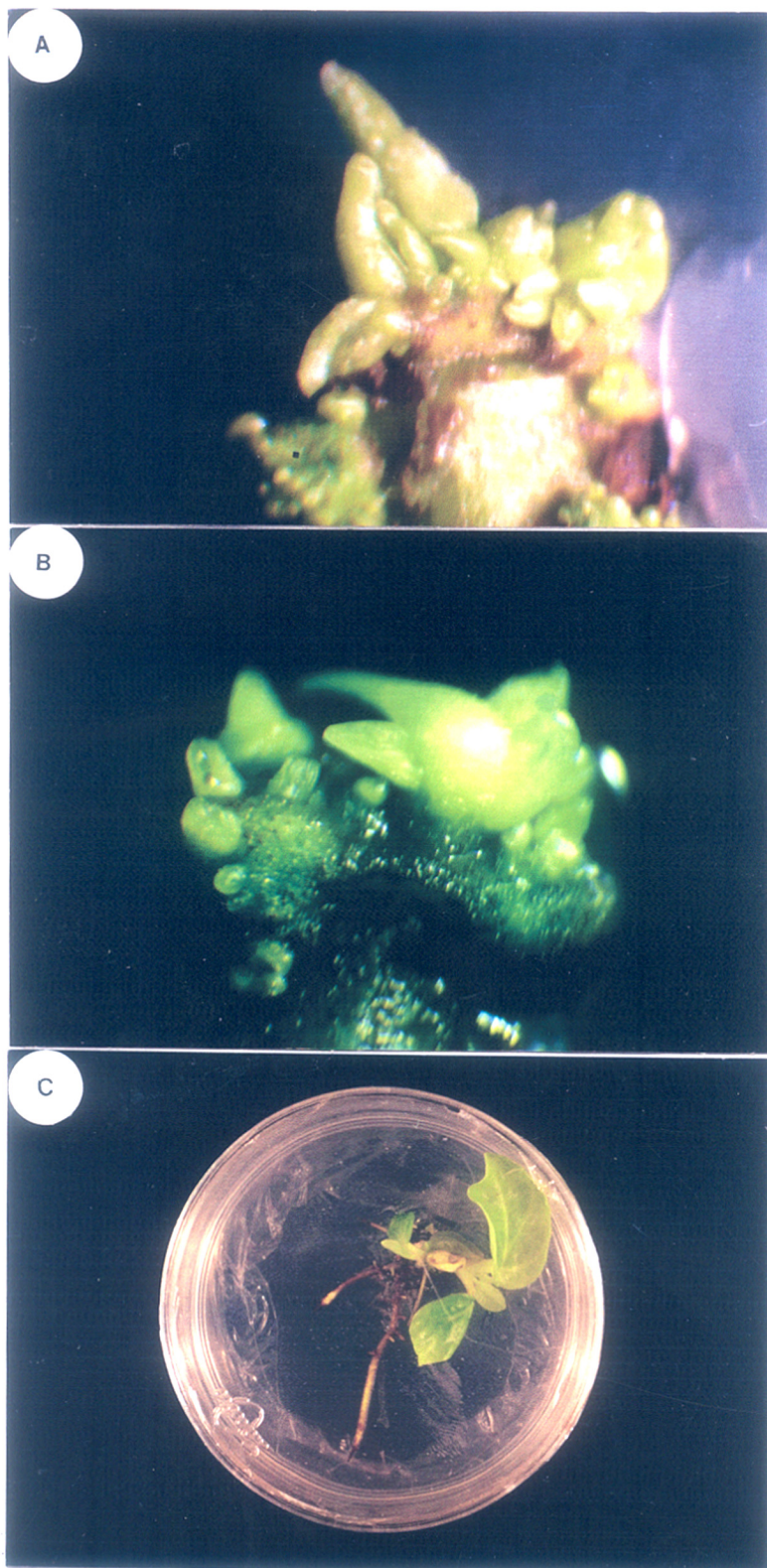
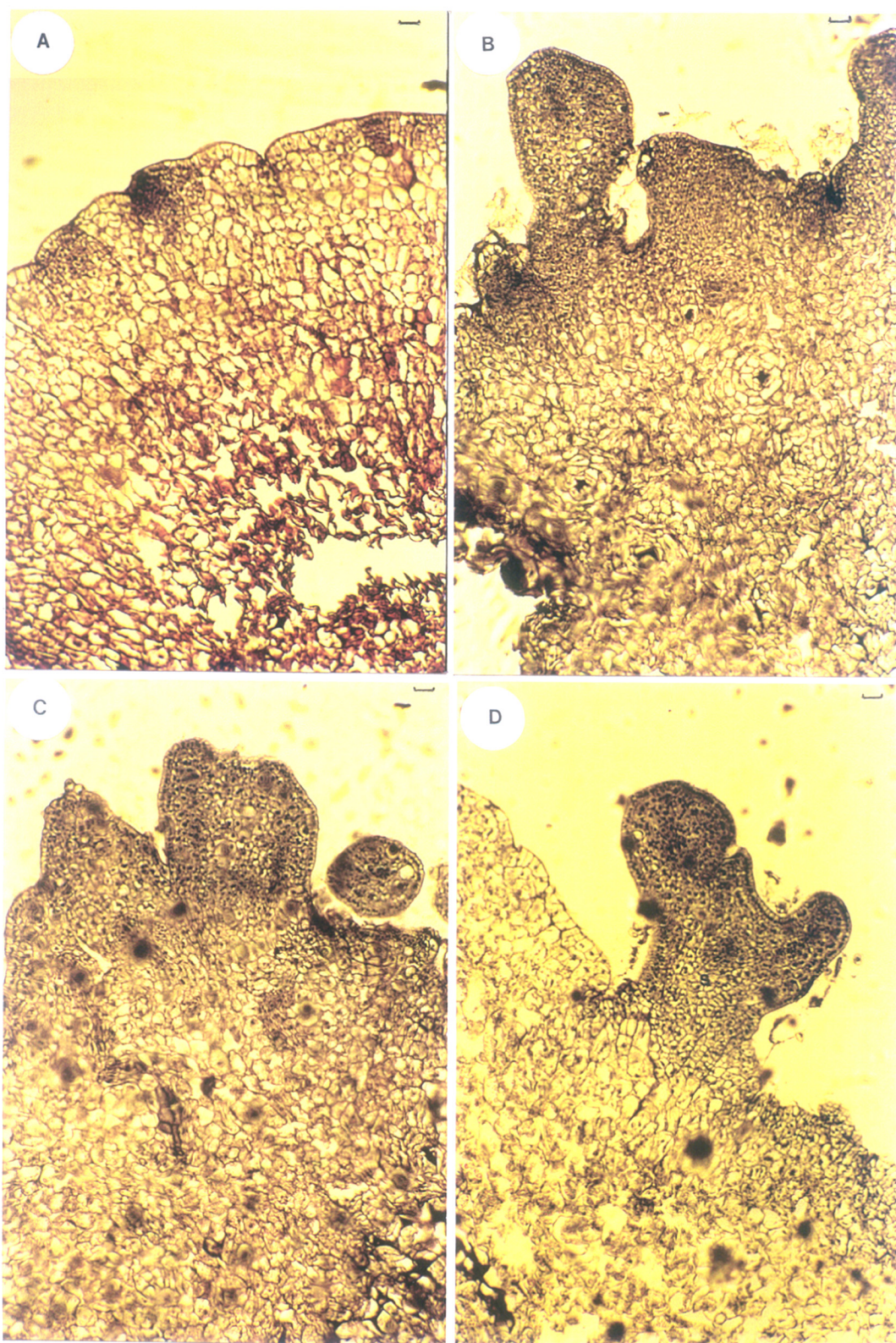


Fig. 4.5-II.

A. Induction of morphogenetic patches in epidermal and subepidermal layers.
Bar = 0.2 mm.

B., C. Formation of meristematic pockets and domes Bar = 0.2 mm.

FIG. 4.5II



4.3.9. *In vitro* multiplication of *Piper colubrinum* using apical and axillary shoot meristems:

In 1946, Ball established the principle of micropropagation by successfully culturing the axillary meristems. Since then several woody species have been successfully multiplied using the meristem explants (Ahuja, 1993).

4.3.9.1 Effect of different basal media on sprouting of apical and axillary shoot meristems: In *Piper colubrinum*, both axillary and apical pre-existing meristems, cultured in different basal media sprouted to form only single shoots (Table 4.8). The sprouting response of both the meristems was not affected by composition of basal media for the apparent differences were not found statistically significant Table (4.8).

Table 4.8. Effect of Basal media on sprouting of apical and axillary meristems in *P. colubrinum* Link.

Basal Medium	Meristems Sprouting (%)	
	Mean $\pm$ s.d.	
	Axillary	Apical
MS	51.0 $\pm$ 1.4 ^a	70.0 $\pm$ 0.0 ^b
Half MS	50.0 $\pm$ 0.0 ^a	65.0 $\pm$ 1.1 ^b
SH	48.0 $\pm$ 5.6 ^a	72.0 $\pm$ 3.5 ^b
WPM	44.0 $\pm$ 2.8 ^a	64.0 $\pm$ 0.9 ^b
B5	46.0 $\pm$ 0.0 ^a	70.0 $\pm$ 0.0 ^b
BD	52.0 $\pm$ 2.8 ^a	60.0 $\pm$ 0.0 ^b

*Values followed by same letter do not differ significantly at 95% confidence level

4.3.9.2. Multiple shoot induction in apical and axillary shoot meristems: To induce multiple shoot formation in the meristem explants, they were cultured in half MS medium supplemented with BA at 0.5, 3.0 and 5.0 mg l⁻¹.

In presence of BA, the two types of meristems responded differently. The nodal explants gave rise to shoot buds directly without any callus formation but shoot tip explants exhibited indirect organogenesis via callus formation.

The nodes cultured in BA at all the 3 concentrations formed shoot buds without any callus formation (Fig. 4.6A, 4.6B, 4.6C) The explants responding for shoot bud formation were subcultured after 4 weeks. The shoot buds on subculture in half MS basal

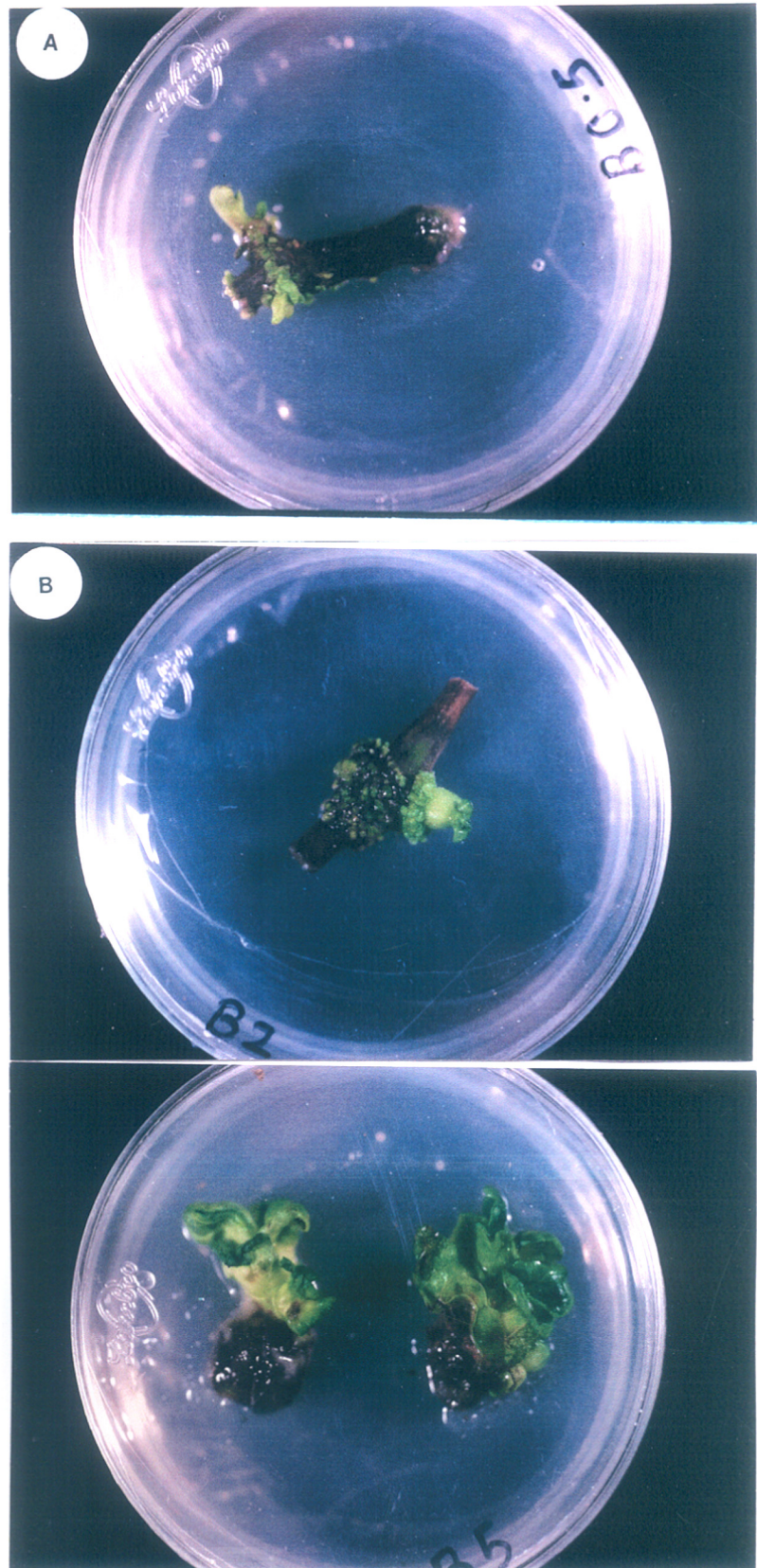
Fig. 4.6

A. Induction of shoot buds in mature nodes of wild pepper in half MS with BA 0.5 mg.l⁻¹

B. Induction of shoot buds in mature nodes of wild pepper in half MS with BA 2.0 mg.l⁻¹

C. Induction and vitrification of shoots in mature nodes of wild pepper in half MS with BA 5.0 mg.l⁻¹

FIG 4.6



medium elongated into shoots. After 2 subcultures of 3 week's duration each, rooting was initiated in the shoots in the same medium.

The percentage of response for multiple shoot induction from nodal explants containing axillary meristems increased with increase in BA concentration. BA at 0.5 mg l^{-1} was sufficient to induce shoot bud formation (Fig. 4.7A). However, BA 3.0 mg l^{-1} (Fig. 4.7B) was the optimum medium for shoot bud induction and supported significantly higher number of shoots per explant (Table 4.9). In BA at 5.0 mg l^{-1} numerous shoot buds were produced but only a few could be converted in shoots. The quality of shoots in BA 5.0 mg l^{-1} was poor and the shoots were stunted and glassy (Fig. 4.7C). Vitrification due to high BA concentration is reported in other plant species too (Boulay, 1984; Aitken-Christie and Jones, 1985; Gaspar, 1987).

The shoot tip explants sprouted and formed single shoots in half MS with BA at 0.5 mg l^{-1} but they induced organogenetic callus in half MS medium with BA 3.0 and 5.0 mg l^{-1} (Fig. 4.7A). The callus induction started 2 weeks after culturing the explants. The callus regenerated shoot buds in the same medium in another 4 weeks. On subculture in half MS basal medium, the shoot buds elongated into shoots (Fig. 4.7B). Each callus formed approximately 6-7 shoots (Table 4.9).

BA at 3.0 and 5.0 mg l^{-1} gave similar results for shoot induction. But in BA 5.0 mg l^{-1} browning of explants resulted in lowered percentage of explants forming shoot buds ultimately reducing the organogenetic efficiency. However culture of the explants in medium containing high BA did not affect the rooting *ex vitro* and acclimatization (Fig. 4.7C).

Fig. 4.7.

A. Organogenetic callus formed in shoot apex explants of wild pepper.

B. Formation of multiple shoots in shoot apex explants of wild pepper.

C. Acclimatization of shoots derived from apical and axillary meristems of wild pepper.

Table 4.9. Effect of BA on *in vitro* multiplication of apical and axillary meristems in *Piper colubrinum* Link.

Explant	BA (mg l ⁻¹)	SBI (%) (Mean ± S.D.)	Shoots/explant (Mean ± S.D.)
Shoot apex	0.5	0	0
	3.0	26.1 ± 3.9 C	5.6 ± 1.0 ^a C
	5.0	12.0 ± 0.0 C	6.5 ± 1.6 ^a C
Node	0.5	31.6 ± 2.6	4.6 ± 1.3 ^A
	3.0	42.0 ± 2.0	6.7 ± 1.8 ^B
	5.0	42.7 ± 2.7	3.3 ± 0.9 ^A

SBI: shoot bud induction, C: callus mediated shoot formation;

Values followed by same superscripts do not differ significantly at 95% confidence level.

4.3.9.3 Histology : Development of *de novo* induced shoot buds in the nodal explant was found to be superficial and asynchronous. In same explant both meristematic patches and well developed shoot buds connected to the original explant by a vascular strand were observed confirming the direct origin of shoot buds from the explant (Fig. 4.8 A).

Indirect origin of meristematic pockets and shoots from shoot tips was confirmed. The shoots as well as the meristematic pockets were located among the loosely arranged callus cells.(Fig. 4.8B)

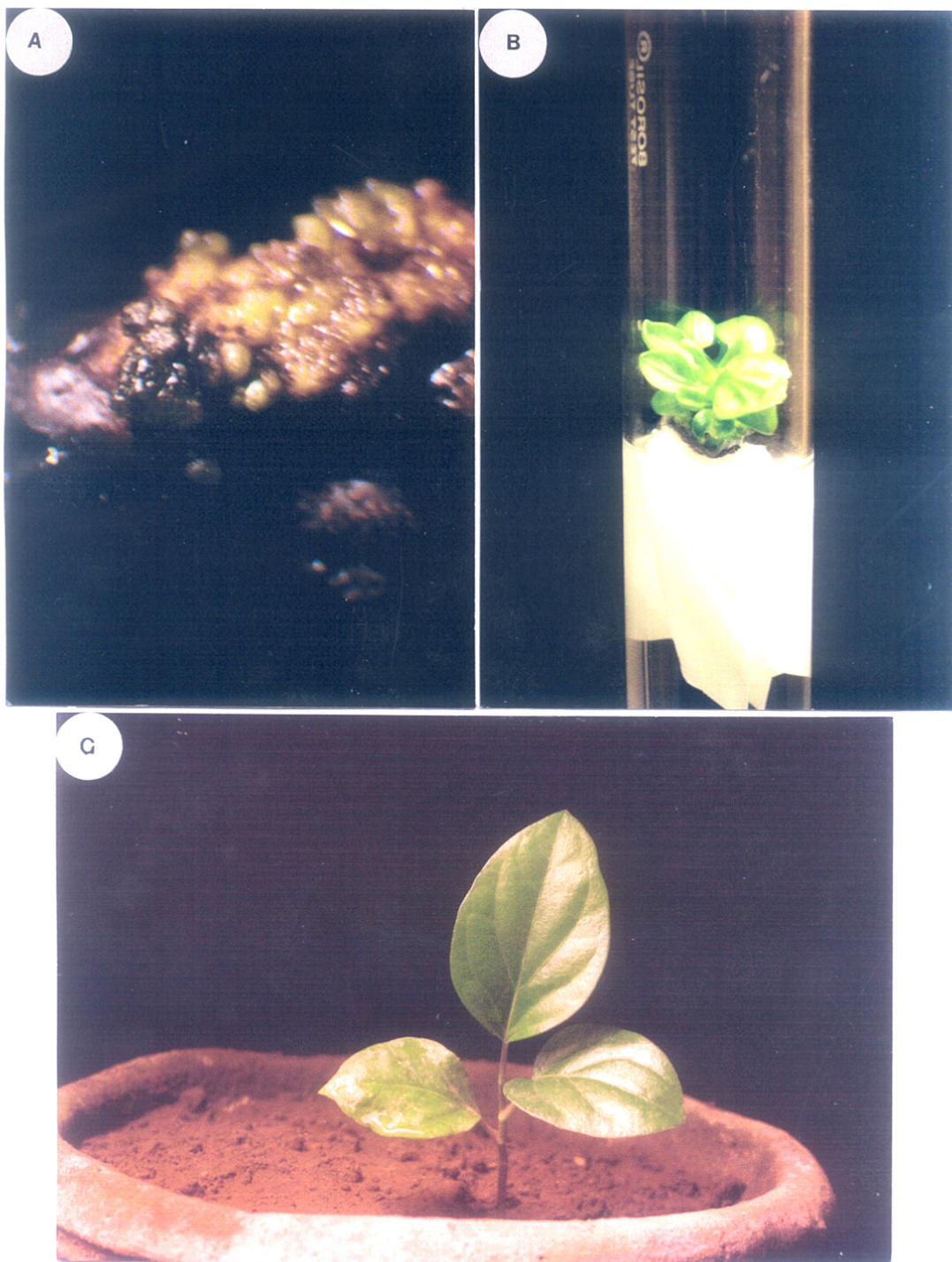
Fig. 4.7.

A. Organogenetic callus formed in shoot apex explants of wild pepper.

B. Formation of multiple shoots in shoot apex explants of wild pepper.

C. Acclimatization of shoots derived from apical and axillary meristems of wild pepper.

FIG. 4.7



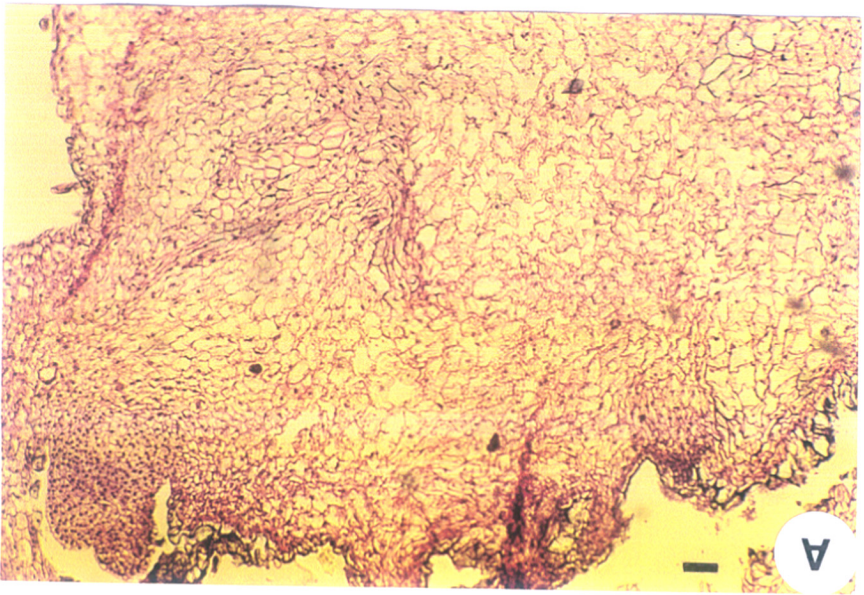
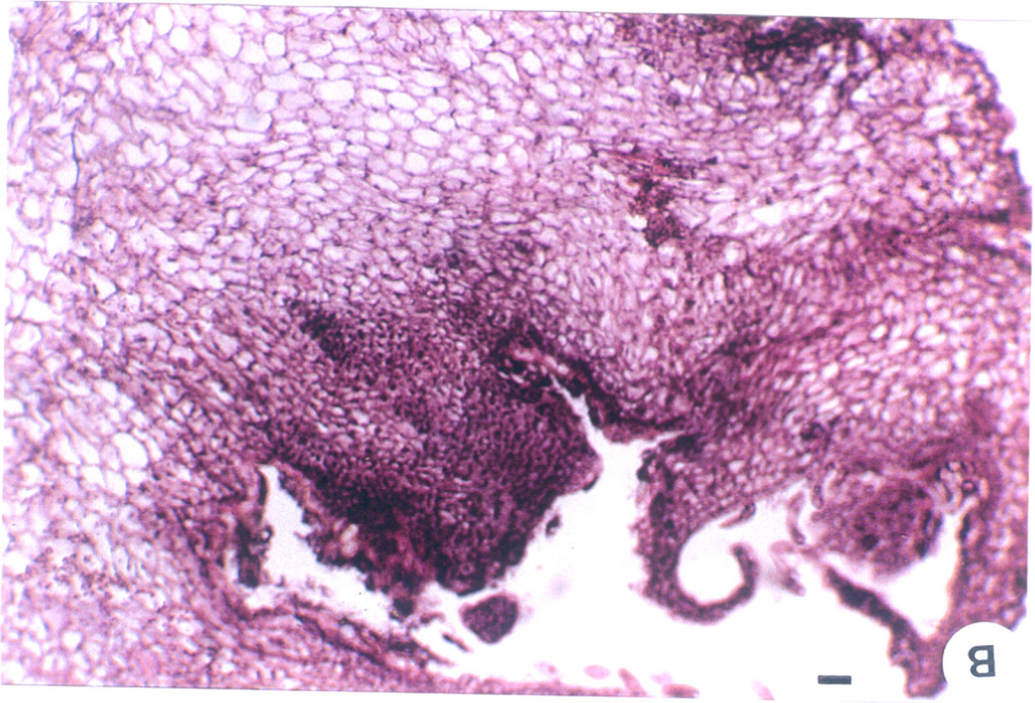


FIG. 4.8

Fig. 4.8

A. Organogenetic structures representing direct shoot bud induction in nodal explants of wild pepper. Bar = 0.2 mm

B. Shoot bud induction in shoot tip explants of wild pepper. Bar = 0.15 mm

4.4 Conclusions:

Optimization of media and culture conditions for culture of mature explants of *P. colubrinum* is presented in this Chapter. The leaf, internode and shoot meristem explants were found to be well suited for induction of organogenesis. MS at half strength was found to be optimum for culture of different explants. BA or TDZ was found to induce organogenesis in the leaf, internode and shoot meristem explants. Three different protocols were standardized for organogenetic plant formation in four different explants. The origin of the shoot buds was confirmed histologically. The shoot buds were elongated in shoots which were rooted and successfully transferred to the soil.

A part of this Chapter has been published as a paper entitled '*In vitro* plant regeneration from leaf callus of *Piper colubrinum* Link.' by Kelkar SM, Deboo GB and Krishnamurthy KV (1996), in *Plant Cell Reports*, 16, 215-218.

CHAPTER 5.

STUDY OF ORGANOGENESIS FROM JUVENILE
EXPLANTS OF *PIPER COLUBRINUM* LINK.

Part A. Induction of direct organogenesis in root, internode, leaf and petiole explants derived from *in vitro* caulogenic plants.

5.1 Introduction

In vitro morphogenesis is the formation (genesis) of organized structures (morphos) in the cultured tissue. It could be defined as *de novo* genesis of structures with known or unknown apparent functions (Tran-Than-Van, 1981). Unlike the animal cells that de-differentiate on culture, the plant cells often retain the capacity to re-differentiate into different organs and even into whole plants. The versatility of response of the plant cells could be attributed to their 'multiprogrammable potentiality' due to which cultured plant cells produce root, shoot, embryos or structures like trichomes and pro-meristemoids (Tran-than-van, 1981; Thorpe, 1993). Based on the end-products, two pathways of morphogenesis are recognized: somatic embryogenesis and organogenesis.

In embryogenesis the end-products are somatic embryos which are bipolar structures that upon germination, produce complete plants. In organogenesis the end-products are unipolar structures like organs such as root, shoots or leaflike structures or sometimes pro-meristemoids i.e. organized masses of meristematic cells halted at certain developmental stages (Thorpe, 1993).

The plants derived *in vitro* from either of these two pathways furnish the requirement of source material for biotechnological manipulations using protoplast or transformation technologies. For such studies the *in vitro* derived plants are preferred because of following reasons:

1. The *in vitro* plants are considered to be germ-free.
2. *In vitro* regeneration provides juvenile material continuously without seasonal constraints.
3. The *in vitro* explants are considered to have better regenerability.

4. The controlled regeneration and growth conditionsv of *in vitro* plants ensure uniformity of experimental material and repeatability of the experiment.

In both organogenesis and embryogenesis two types are recognized: direct and indirect types (Hicks 1980; Dandekar 1992; Fisk and Dandekar, 1993). In the indirect type explant de-differentiates into callus which further re-differentiates into organized structures and in direct type the organized structures appear without any intervening callus stage. Both types of pathways have different applications. Caulogenesis involves rapid cell divisions that could cause somaclonal variations and are useful for assessment, selection and multiplication of useful variants. Whereas direct morphogenesis involves no or few cell divisions and is more useful for production and multiplication of transformants (Hicks, 1980; Dandekar, 1992).

In order to develop highly efficient direct regeneration system in *P. colubrinum*, regeneration potential of shoot meristems, root, internode, leaf and petiole explants of *in vitro* plants derived from leaf-callus (Kelkar *et al.*, 1996) was assessed. This section deals with the studies on regeneration response of different *in vitro* derived explants.

5.2 Materials and Methods:

5.2.1 Plant material : *In vitro* plantlets were raised from leaf calli of greenhouse grown *P. colubrinum* as previously reported (Kelkar *et al.*, 1996). The plantlets, at 4-6 node stage and maintained for 8-10 weeks on half strength MS medium (containing macronutrients at half the concentration) without plant growth regulators, were used as source of explants. Different explants were aseptically cut into pieces of appropriate size, i.e. root (2-3 mm in length), internode (3-5 mm in length), petiole (2-3 mm in length) and leaf (5-8 mm²) and were used for culture. The shoot apices and nodal explants were cut at the base leaving minimum internode tissue.

5.2.2 Media and Culture Conditions: Culture of explants without pre-existing shoot meristems: Aseptically cut root, internode, petiole and leaf explants were cultured on half MS medium supplemented with BA (0.0, 0.2, 0.5, 1.0, 5.0 and 10.0mg l⁻¹) alone, or in combination with 2,4-D (0.5, 1.0mg l⁻¹) or NAA (0.5, 1.0mg l⁻¹) in the pilot experiments. Further, the effect of BA on shoot bud induction and elongation was studied in three parallel experiments:

1. To study the effect of BA concentration on shoot bud induction, all four explant types were cultured on a range of Induction media (IMs) which comprised half strength MS medium with BA (0.2, 0.5, 1.0, 5.0 and 10.0mg l⁻¹) or without BA. The explants which showed shoot bud induction were transferred to different Subculture Media (SMs) after 30 d.
2. To study the effect of continued presence of BA in IM on the elongation of shoot buds and regeneration efficiency, the root, internode, petiole and leaf explants with shoot buds from experiment 1 were subcultured on half strength MS medium with or without BA (0.0, 0.2 and 0.5mg l⁻¹). Further subcultures were done at monthly intervals. The number of shoots more than 1 cm in height were counted after 2 subcultures.

In the pilot experiments, high concentration of BA in IM was found to lower the number of shoots regenerated per explant in all the explant types except root. In order to analyze the interaction between culture period and high BA (5.0, 10.0mg l⁻¹)

concentration in IM on multiple shoot formation, explants from all 4 explant types were cultured on IMs containing BA (5.0, 10.0 mg l⁻¹) for different time intervals (8, 15 and 30 d) and then subcultured on half MS medium without growth regulators. Further subcultures were carried out at monthly intervals. The number of shoots (more than 1 cm in length) regenerated per explant was counted and results were compared against results of experiment 2 where the culture period was of 30 d.

All regenerated shoots from the three experiments were further elongated on half MS without growth regulators for 2 months and then transferred for *ex vitro* rooting and hardening.

Culture of pre-existing shoot meristems: The shoot apices and nodal explants were cultured on half MS medium with or without BA (0.5, 1.0, 2.0, 5.0, 10.0 mg l⁻¹). The sprouted explants as well as explants showing callus formation were subcultured after 3 weeks in half MS medium without any growth regulators. The shoots elongated during subsequent subcultures in the same medium at regular intervals of 3 weeks.

All the media contained 3 % sucrose and 0.6 % agar (Qualigens, India). The pH was adjusted to 5.8 before autoclaving. Each treatment had at least 10 replicates and each experiment was carried out at least thrice. The cultures were incubated at 25 ± 2° C, 16 h photoperiod and light intensity of 30 E.m⁻².s⁻¹ provided by cool white fluorescent tubes.

5.2.3 Acclimatization: Ten to Twenty shoots at 3-5 node stage from each explant type were rooted *ex vitro* and acclimatized as described in Chapter 2.

5.3 Results and Discussion:

5.3.1 Culture of explants without pre-existing shoot meristems: *In vitro* organogenesis is a complex process which involves a cascade of developmental events ultimately expressed as formation of organs or meristemoids etc. Broadly these events could be classified into 3 different phases as follows:

1. Pre-induction phase or acquisition of competence: Certain cells of explants become competent for morphogenesis. This may or may not require a signal such as plant growth regulator. In case of callus-mediated morphogenesis, this phase is equivalent to de-differentiation where the competent cells undergo profuse cell divisions or probably some of the callus cells acquire competence (De Klerk *et al.*, 1997). In case of direct morphogenesis the neo-competent cells do not undergo cell divisions or undergo just a few cell divisions.

2. Determination or Induction phase: In this phase, the competent cells become determined to take either organogenetic or embryogenetic pathway. This is most of the times influenced by signals most likely being plant growth regulators or sometimes media components especially nitrates.

3. Realization or Expression phase: In this phase, the chosen pathway is expressed and becomes visible in the form of shoot buds or pro-embryos etc.

The occurrence of these phases was postulated by Christianson and Warnick (1983, 1985) while working on organogenetic responses in leaf explants of *Convolvulus*. Though originally worked out and confirmed for shoot formation in several species, (Flinn *et al.* 1988; Attfield and Evans, 1991 a, b; De Klerk *et al.*, 1997) the model has been successfully extrapolated for root and embryo formation with slight modification of terminology (De Klerk *et al.*, 1997). Christianson and Warnick (1983, 1985) proposed that the three phases though interlinked may have distinct specific requirements and could be irreversibly inhibited or slowed down in the absence of such requirements.

In the present study, an analogous pattern to the above model was observed for

shoot formation from different explants. We found that in all the three stages, presence of BA was either essential or promotive for all the explant types used. Tissue culture studies carried out so far in *Piper* spp. involve the use of BA for shoot bud induction (Mathews and Rao 1984; Fitchet 1990; Philip *et al.* 1992; Bhat *et al.* 1992, 1995; Aminuddin *et al.* 1993; Meyer *et al.* 1992; Babu *et al.* 1995; Kelkar *et al.* 1996). Based on these reports, BA was used for shoot bud induction in the current experiments. The use of half strength MS basal medium was based on our previous experiments (Kelkar *et al.* 1996).

The combination of BA with auxins 2,4-D and NAA (0.5 and 1.0mg l⁻¹) was found to induce organogenesis in mature leaf explants of *P. colubrinum* (Kelkar *et al.*, 1996). So in the pilot experiments, auxins were used in addition to BA (0.2, 0.5, 1.0, 5.0, 10.0mg l⁻¹). However, these resulted in callus formation as well as other forms of organogenesis described by Tran-Thanh-Van (1981) such as rhizogenesis and formation of leaflike structures. Therefore only BA was used in further experiments.

When used alone in the medium, BA influenced organogenetic induction (Table 5.1) as well as continuation of the organogenetic process (Table 5.2). The inhibition of organogenetic induction caused by high concentration of BA in IM could be traced to an early stage when the explants were exposed to different culture periods (Table 5.3). The effect of BA on different phases of organogenesis can be explained as follows:

5.3.1.1 Effect of BA concentration in Induction Medium (IM) on shootbud induction : The use of half strength MS basal medium alone as IM did not evoke any response other than occasional rhizogenesis (Table 5.1). However the presence of BA in the IM (Induction Medium), supported shoot bud formation in different explants at various frequencies depending on the concentration (Table 5.1). The threshold concentration of BA required for induction of organogenesis and optimal response differed for the different explant types.

Root: After 2 weeks of culture, the roots became intense green and swollen in all the IMs tried, except for the medium containing 0.2mg l⁻¹ BA. The incubation period for initiation of shoot bud induction was relatively long, i.e. 4 weeks for roots as against 2-3 weeks

required by other explant types. By the end of 4th week, small nodule-like structures appeared predominantly on proximal end (Fig. 5.1A) and rarely on the distal end. Such polarization of morphogenetic response in root explants is common in other plants (Rybczynski *et al.*, 1994). The percentage response of shoot bud induction increased with increasing concentration of BA, the lowest being 15.8 at BA 0.5mg l⁻¹ and highest being 86.7 at BA 10.0mg l⁻¹.

Internode: The percentage response of internode explants to form shoot buds was more or less similar in all the concentrations of BA tried (Table 5.1). The shoot buds appeared after 2-3 weeks of culture (Fig. 5.1B) first at cut ends and later on the central portion. The medium with BA at 0.5mg l⁻¹ supported highest (97.5) percentage of explants forming shoot buds whereas lowest percentage response (91.1) was observed at BA 5.0mg l⁻¹ (Table 5.1).

Leaf: Leaf explants formed shoot buds at all concentrations of BA except at 0.2mg l⁻¹. The buds were induced at cut ends after 2-3 weeks (Fig. 5.1C). The response increased gradually along the concentration gradient from 26.5 % at BA 0.5mg l⁻¹ to 97.6 % at BA 5.0mg l⁻¹ but decreased to 88.6 % at BA 10.0mg l⁻¹ (Table 5.1). BA induced callusing at higher concentrations (5 and 10mg l⁻¹) and also caused blackening of explants.

Petiole: Petiole explants showed shoot bud induction at all the concentrations of BA. The lowest % (55.6) of explants forming shoot buds was noted at BA 0.2mg l⁻¹. It increased with increase in concentration of BA up to 5.0mg l⁻¹ (Table 5.1). The shoot buds were induced only at cut ends after 2 3 weeks of culture (Fig. 5.1D).

Fig. 5.1

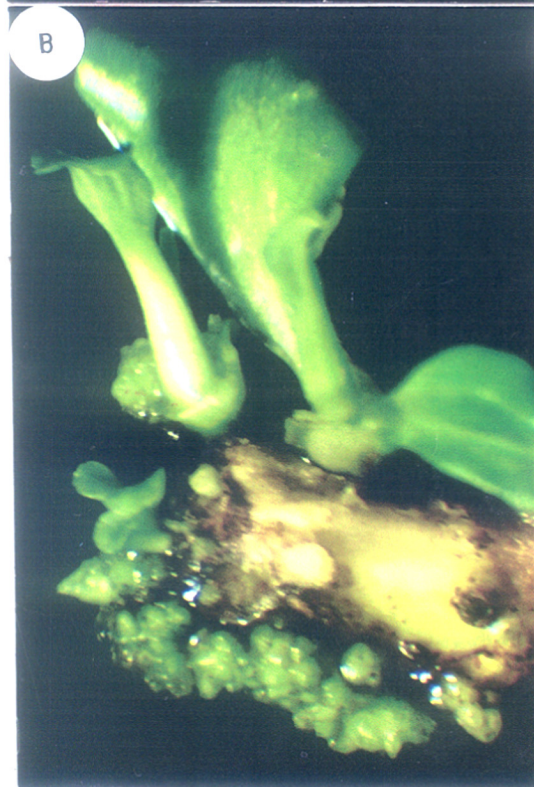
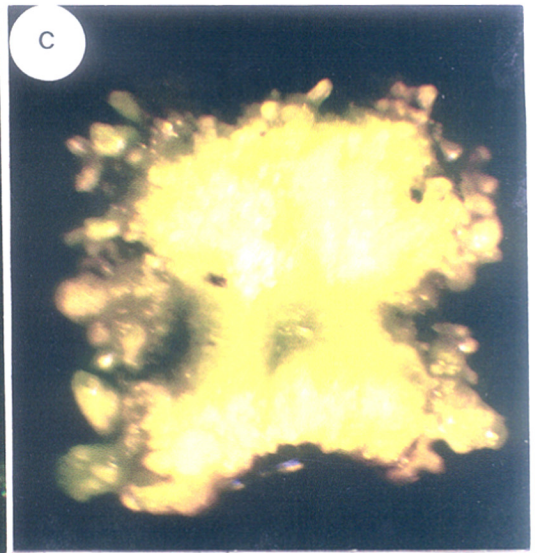
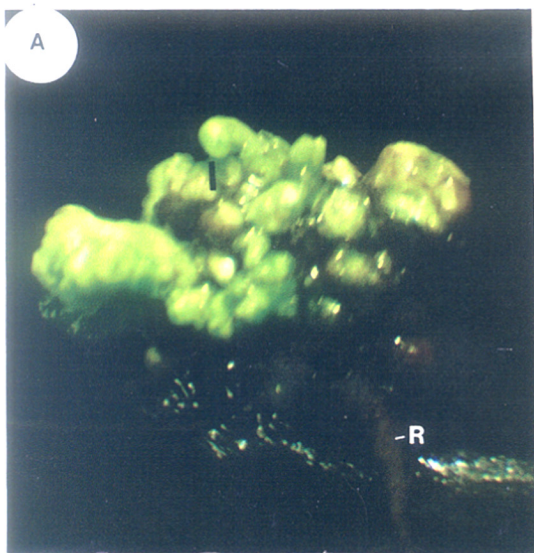
A. Shoot bud formation in root explant of wild pepper cultured in half MS with BA 5.0 mg l⁻¹.

B. Shoot bud formation in internode explant of wild pepper cultured in half MS with BA 0.2 mg l⁻¹.

C. Shoot bud formation in leaf explant of wild pepper cultured in half MS with BA 1.0 mg l⁻¹.

D. Shoot bud formation in internode explant of wild pepper cultured in half MS with BA 1.0 mg l⁻¹.

FIG.5.1



5.3.1.2 Effect of BA concentration in IM on number of shoots regenerated

per explant : The concentration of BA in the IM markedly affected the efficiency of regeneration. Each explant type showed differential preference for maximal response. Absence of BA in IM resulted in lack of any morphogenetic response in all the explant types (Table 5.1).

For root explants, the increase in number of shoots per explant was directly proportional to the increase in BA concentration. This relationship was reversed in petiole and internode explants, where the number of shoots decreased with increase in BA concentration (Table 5.1). The regeneration frequency for leaf explants gradually increased with increase in BA concentration; reaching its maximum at BA 1.0mg/l and then decreased with increase in BA concentration (5.0, 10.0mg/l). In internode and petiole explants the maximal response for number of shoots formed per explant was achieved at BA 0.2mg/l whereas in root explants it was at BA 10.0mg/l (Table 5.1).

The number of shoots formed per root and leaf explant was not significantly different for any treatment (Table 5.1). However, the efficiency of organogenesis (product of percentage of explants responding and the number of shoots formed per explant) was higher in half MS with BA 5.0 and 10.0mg/l for root explant and half MS with BA 5.0 for leaf explant. For petiole explants the regeneration frequency denoted by number of shoots formed per explant was significantly low in half MS with BA 5.0 and 10.0mg/l but the efficiency was similar for all the treatments. Whereas the regeneration frequency in internode explants was comparable for half MS with BA 0.2, 0.5, 1.0 and 5.0mg/l. It was significantly lower in BA 10.0mg/l and so was regeneration efficiency.

Table 5 1. Effect of BA concentration on shoot bud formation in different explant types of *Piper colubrinum*.

BA (mg.l ⁻¹)	% Explants forming shoot buds				Total No. of Shoots per Explant (Mean±S.D.)			
	Root	Pet	Int	Leaf	Root	Pet	Int	Leaf
IM								
B0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B0.2	0.0	55.6	95.6	0.0	0.0	7.2±2.6	11.0±3	0.0
B0.5	15.8	78.3	97.5	26.4	2.0±0.8	6.6±2.7	9.5±2.3	5.3±1.5
B1.0	21.6	93.1	94.8	79.6	2.5±0.7	5.9±2.2	8.8±2.5	6.1±1.9
B5.0	83.5	93.0	91.1	97.6	3.3±1.0	2.6±1.0	5.9±1.2	4.6±1.4
B10.0	86.7	93.0	93.1	88.6	3.5±1.1	2.3±1.0	2.7±0.8	3.6±1.6

IM: Induction Medium. Int :Internode Pet : Petiole

Effect of BA concentration in the Subculture Medium on number of shoots regenerated per explant: Maintenance of BA in SM (Subculture Medium) was not necessary for further growth and elongation of shoot buds (Table 5.2). However, the continuation of BA in SM had a positive effect on the number of shoots formed per explant depending on the explant type and BA concentration used in the IM. In the preliminary experiments, all the concentrations of BA used in IM were used in SM. But presence of BA at 1.0, 5.0 and 10.0mg l⁻¹ in SM caused darkening of leaf explants and callusing in other explant types. Hence only BA 0.2 and 0.5mg l⁻¹ were maintained as SMs. However presence of BA in SM when half MS medium was used as IM did not show shoot bud formation in any of the explants (Table 5.2). This suggests that presence of BA is required for the pre-induction stage i.e. in acquisition of competence.

In internode, petiole and leaf explants the interaction of BA concentration in SM and IM followed a definite pattern. The presence of BA in SM had a significant advantage over its absence when the IM contained lower BA (0.2, 0.5, 1.0, 5.0 mg l⁻¹). However, the differences between SM with or without BA were rendered insignificant when IM contained BA 10.0mg l⁻¹ (Table 5.2).

The combined action of low BA (0.2mg l⁻¹ for internode and petiole; 1.0mg l⁻¹ for leaf) in IM and in SM (0.2 and 0.5mg l⁻¹) was optimal for eliciting high number of shoots per explant (Table 5.2). But the differences between the regeneration response at these two levels of BA, i.e. 0.2 and 0.5mg l⁻¹, were not found to be statistically significant except for internode explant (Table 5.2). High BA (5.0, 10.0mg l⁻¹) in IM gave a low response irrespective of the concentration of BA used in SM (Table 5.2).

In root explants, the interaction between SM and IM seemed to differ. The absence of BA in SM had promoting effect when IM contained high BA (5.0, 10.0mg l⁻¹) but it was not found to be statistically significant.

From the results presented in Tables 5.1 and 5.2, it was found that the regeneration efficiency of the internode explants is the best among the four explant types tested, followed by petiole, leaf and root in consecutive order.

In general, the level of organogenetic competence of the explants was defined by the BA concentration of IM stage itself whereas the SM had a secondary effect on elongation of the shoots formed on the IM.

Table 5 2. Effect of BA concentration of SM on the number of shoots formed per explant in different explant types of *Piper colubrinum*.

BA (mg.l ⁻¹)		No. of shoots per explant (Mean $\pm$ s.d.)			
IM	SM	Root	Petiole	Int	Leaf
0.0	0.5	0.0	0.0	0.0	0.0
	0.2	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0
0.2	0.5	0.0	8.3 $\pm$ 1.6	10.8 $\pm$ 3.2	0.0
	0.2	0.0	8.6 $\pm$ 2.2	13.5 $\pm$ 2.6	0.0
	0.0	0.0	4.0 $\pm$ 1.5*	7.5 $\pm$ 1.7*	0.0
0.5	0.5	2.0 $\pm$ 0.8	7.9 $\pm$ 1.8	10.0 $\pm$ 2.3	5.7 $\pm$ 1.2
	0.2	2.0 $\pm$ 1.0	7.2 $\pm$ 2.4	10.2 $\pm$ 2.0	6.0 $\pm$ 1.3
	0.0	2.0 $\pm$ 1.0	3.1 $\pm$ 1.1*	7.7 $\pm$ 2.1*	3.8 $\pm$ 1.4*
1.0	0.5	2.8 $\pm$ 0.8	7.0 $\pm$ 1.3	9.4 $\pm$ 1.8	6.4 $\pm$ 1.5
	0.2	2.3 $\pm$ 0.8	6.0 $\pm$ 2.2	10.2 $\pm$ 1.9	7.4 $\pm$ 1.6
	0.0	2.3 $\pm$ 0.5	3.9 $\pm$ 2.2*	6.7 $\pm$ 1.9*	4.2 $\pm$ 1.4*
5.0	0.5	2.9 $\pm$ 1.3	3.1 $\pm$ 1.3	5.6 $\pm$ 1.0	4.7 $\pm$ 1.2
	0.2	3.4 $\pm$ 0.9	2.5 $\pm$ 1.0	5.8 $\pm$ 1.4	5.0 $\pm$ 1.6
	0.0	3.6 $\pm$ 0.8	1.9 $\pm$ 0.9	6.3 $\pm$ 1.0*	4.2 $\pm$ 1.4*
10.0	0.5	3.0 $\pm$ 1.0	2.8 $\pm$ 1.1	2.3 $\pm$ 0.6	3.5 $\pm$ 1.2
	0.2	3.7 $\pm$ 1.0	2.4 $\pm$ 0.9	2.8 $\pm$ 0.7	4.0 $\pm$ 1.8
	0.0	4.0 $\pm$ 1.2	1.3 $\pm$ 0.5	3.0 $\pm$ 0.9	3.1 $\pm$ 2.1

*: Differences among the subculture treatments for each induction treatment are significant at 95% confidence level. IM : Induction Medium. SM :Subculture Medium Int: Internode.

5.3.1.3.Effect of culture period on number of shoots formed per explant :

For internode, petiole and leaf explants, high BA in the IM resulted in a high % of shoot

bud induction but lowered the number of shoots per explant irrespective of SM used. (Table 5.1). So to investigate the relationship between high BA (5.0, 10.0 mg l⁻¹) and the culture period on IM, explants were cultured for 8 and 15 d on IM with BA (5.0, 10.0 mg l⁻¹) and then subcultured on half strength MS medium without any plant growth regulators. The results were compared to those in Experiment 2 where culture period was of 30 d.

The reduction in culture period from 30 d to 15 and 8 d caused no statistically significant increase in regeneration frequency in any of the explant types. (Table 5.3). This suggests that inhibitory action of higher dose of BA (5.0, 10.0 mg l⁻¹) was operative even on exposure of the internode, petiole and leaf explants for 8 d, whereas for root explants, culture period of 8d was sufficient to induce optimum response. The gradual increase in number of shoots formed per explant with increase in culture period was not found to be statistically significant.

Table 5 3. The Effect of induction period on number of shoots formed per explant in root, internode, leaf and petiole explants in *P. colubrinum*.

IM BA (mg.l ⁻¹)	Induction period	No. of shoots per explant (Mean ± S.D.)			
		Root	Petiole	Internode	Leaf
5.0	30 D	3.6±0.8	1.9±0.9	6.3±1.0	4.2±1.4
	15 D	3.8±0.9	3.0±0.6	6.8±1.2	4.6±1.8
	8 D	3.1±0.6	2.0±1.0	6.0±0.6	3.0±1.0
10.0	30 D	4.0±1.2	1.3±0.5	3.0±0.9	3.1±2.1
	15 D	3.0±0.7	2.1±0.9	4.0±1.5	4.0±1.5
	8 D	3.0±0.6	1.3±0.5	2.8±0.7	3.1±1.0

IM: Induction Medium

The regenerated shoots (more than 1 cm in height) from all the explants and all the treatments were further elongated on half strength MS medium without growth

Fig. 5.2.

A. Elongated multiple shoots of wild pepper in half MS basal medium.

B. Acclimatization of *in vitro* shoots of wild pepper.

FIG. 5.2



regulators for 7 to 8 weeks (Fig 5.2A).

5.3.1.4. Acclimatization and *ex vitro* Rooting : A total of 10-20 elongated shoots at 4-5 node stage were selected randomly from the regenerants of each explant type and transferred to the sand:soilrite mixture (1:1 ratio) for hardening. Adventitious roots were formed and the plantlets were established in 4-5 weeks (Fig. 5.2B). The degree of hardening success differed for the shoots regenerated from different explants. The shoots derived from roots exhibited 100 percent hardening, whereas shoots regenerated from internode explant showed 88.3% followed by petiole explants (87.2%) and leaf explants (85.0%).

5.3.2. Culture of pre-existing shoot meristems: The shoot apices and nodes cultured in medium containing BA at 0.5, 1.0 and 2mg/l formed multiple shoots. The shoot tip explants, callus was induced in media containing BA 5.0 and 10.0mg/l (Fig. 5.2 A, Kelkar and Krishnamurthy, 1996). This callus produced shoots on subculture onto half MS (Fig. 5.2 B, Table 5.4). Maintenance of the callus in media containing BA 5.0 and 10.0mg/l resulted in proliferation of callus that was not morphogenetic.

In the nodal explants, shoot buds were induced around the sprouting axillary meristems which elongated along with the sprouts (Fig. 5.2C) in media containing low BA (0.5, 1.0, 2mg/l). The elongated shoots derived from nodes as well as shoot apices were rooted *ex vitro* (Fig. 5.3D) and hardened with 100% efficiency.

Table 5.4 Effect of BA in IM on shoot multiplication in pre-existing shoot meristems of *P. colubrinum*

BA (mg.l ⁻¹)	Explant type	Response (%) (mean±s.d.)	No. of Shoots/explant (mean±s.d.)
0.5	Shoot apex	75.0±5.0	3.6±1.5
	Node	80.0±0.0	4.0±1.0
1.0	Shoot apex	100.0±0.0	5.0±1.0
	Node	95.0±5.0	6.2±2.0
2.0	Shoot apex	100.0±0.0	3.9±0.9
	Node	100.0±0.0	5.1±1.0
5.0	Shoot apex	85.0±5.0	4.2±0.8
	Node	100.0±0.0	7.2±2.0
10.0	Shoot apex	100.0±0.0	6.1±1.2
	Node	100.0±0.0	6.3±2.5

Values followed by same superscripts are not significantly different at 95% confidence level for each explant type at different treatments. (Lower case and upper case superscripts denote differences among treatments for nodal explants and shoot apex explants respectively.)

C:callus mediated shoot formation.

Fig. 5.3

A. Morphogenetic callus developed from shoot tip explants in half MS with BA 2.0 mg.l^{-1}

B. Formation of multiple shoots from shoot tip explants of wild pepper.

C. Nodal multiples of wild pepper in half MS with BA 1.0 mg.l^{-1}

D. Elongation of nodal multiples of wild pepper.

FIG. 5.3



Part B.: Isolation of protoplasts using leaf callus and leaves (*ex vitro* and *in vitro*) as source materials in *Piper colubrinum*.

5.4 Introduction

Genetically fixed resistance is the cheapest and most prophylactic procedure of plant protection against several plant diseases. Hence, most desirable and effective strategy has been incorporation of disease resistance genes into commercially acceptable cultivars. When this is not possible by conventional means such as hybridization or grafting, nonconventional techniques such as protoplast-mediated somatic hybridization or genetic transformation with alien genes are used.

A protoplast is a 'naked' plant cell with cell wall removed enzymatically or mechanically. The cell walls provide efficient barriers for restricting entry of foreign bodies into the plant cell. Removal of these barriers facilitates fusion of protoplasts of diverse origin under the influence of agents like electrical charge or polyethylene glycol (PEG). The extent of hybridization could vary from total to partial. In total hybridization nuclear as well as cytoplasmic genomes (chloroplast and mitochondria) of both the partners combine in the fusion product, whereas partial hybridization involves fusion of at least one of the two genomes of one partner with one or more genomes of the other. Accordingly, 2 types of products can result from protoplast fusion : somatic hybrids and cybrids (Stafford, 1991; Warren, 1991). First somatic hybrid plants were recovered by Carlson *et al.*, (1972) in *Nicotiana* spp. using NaNO₃ as a 'fusogen' while upon further refinement in fusion methods, first intergeneric hybrids (Melchers *et al.*, 1978) and first cybrids (Zelcer, 1978) were reported. The somatic hybridization involves the selective influx and combination of nuclear and cytoplasmic genomes.

Somatic hybridization has immense potential and direct application in breeding and improvement of plants by which reproductive isolation barriers could be overcome to generate new and useful combinations of nuclear as well as cytoplasmic genes. But before somatic hybridization can be exploited, it is imperative to establish the following:

1. An efficient *in vitro* regeneration system.
2. Protocols for isolation and culture of protoplasts.
3. Selection systems or markers required for selective recovery of fusion products.

An efficient *in vitro* plant regeneration protocol is important for providing source material for isolation of protoplasts. It can be further manipulated to achieve plant regeneration from protoplasts. Although use of *ex vitro* material for protoplast isolation has been reported, *in vitro* plants are advantageous for their germ-free nature, juvenility and controlled growth conditions. The absence of waxy cuticle in these plants is an added advantage for isolation of protoplasts in large numbers.

Successful isolation and high yield of viable protoplasts determine the efficiency of recovery of somatic hybrids. Several factors control isolation, yield and viability of protoplasts. These include the isolation procedure, the source tissue and its pre-treatment, osmotica and the conditions for release of protoplasts as well as the culture of protoplasts.

In Part B and C of this chapter, preliminary experimentation for standardization of parameters for some of the above mentioned factors for the multiple resistant wild pepper *Piper colubrinum* is described. Standardization of these conditions which are the basic pre-requisites for somatic hybridization would be a useful starting point for transfer of the resistance traits of this species with the economically important but susceptible species i.e. *Piper nigrum* (Black pepper).

5.5 Materials and Methods:

5.5.1 Source material: Three different source materials in form of young leaves of 4-6 year old green-house grown plants, young leaves of *in vitro* shoots and green nodular calli induced in mature leaf explants were used for protoplast isolation.

The first leaves were excised from 4-6 year old greenhouse grown plants. These leaves were washed thoroughly with tapwater and surface sterilized using 0.1% mercuric

chloride. After washing 5-6 times with sterile distilled water the lower epidermis was peeled off mechanically and then the leaves were cut into small pieces (0.5 cm²).

The *in vitro* shoots were derived from root, internode, leaf and petiole explants (Kelkar and Krishnamurthy, 1998) and maintained *in vitro* for 60 days. First 2-3 leaves were cut aseptically and the abaxial surface was gently scratched using a sterile blade. These leaves were then cut into small pieces (approx. 0.5 cm²).

Intense green, nodular organogenetic calli obtained from leaf explants cultured in half MS medium containing BA 2.0mg/l and NAA 0.5mg/l for one month, (Kelkar *et al.*, 1996) were washed to remove agar medium, using sterile distilled water in the laminar air flow cabinet and then blotted on pad of sterile filter paper and used for isolation.

At least 500 mg tissue was used per treatment with 3 replicates and the all the experiments were repeated thrice. 5.5.3 Pre-treatment of the source material: All source materials were preplasmolysed in CPW13M (13% mannitol in CPW salts) (Table 5.5) for 30 minutes. The solution was then removed with a Pasteur pipette before subjecting the tissue for enzymatic digestion.

Table 5.5 Composition of CPW salts

Salt	mg/l
CaCl ₂ 2H ₂ O	1480
KH ₂ PO ₄	27.2
KNO ₃	101
MgSO ₄ 7 H ₂ O	246
CuSO ₄ 5 H ₂ O	0.025
KI	0.16

5.5.2 Enzyme treatment of the source material: Preplasmolysed tissues were treated with enzyme solution (1g per 10 ml.). The enzymes Cellulase R-10 i.e. CR-10 and

Macrozyme R-10 i.e. MR-10 (Yakult Honsha Co., Tokyo, Japan) were dissolved in V47 basal medium (Chapter 2, Table 2.1) supplemented with 2% glucose, 3% sucrose and mannitol (6% or 9% or 13%). The combinations of enzymes used for protoplast isolation are shown in Table 5.6. The pH of the enzyme was adjusted to 5.6 and it was filter sterilized through 0.22 μ m pore size Millipore membrane. The enzyme mixture was dispensed in 5 ml aliquots in sterile test tubes and stored at -200C until required. Tissue digestion was carried out in test-tubes for 4, 8, 12 and 16 h in diffuse light ($4.6 \text{ mol m}^{-2} \text{ s}^{-1}$) on a roller drum apparatus at a speed of 15 revolutions per hour (Model Syn- 152, Emenvée, India).

Table 5.6 Enzyme combinations used for protoplast isolation using leaves of *Piper colubrinum*

Enzyme Conc.	CR10 0.5%	CR10 1.0%	CR10 2.0%
MR10 0.5%	1	2	3
MR10 1.0%	4	5	6

CR10: cellulase R-10, MR-10: macrozyme R-10

5.5.3 Purification of protoplasts: After incubation the enzyme solution was filtered through stainless steel sieve (pore size 74 μ m) and transferred to screw capped centrifuge tubes (15 ml capacity) and centrifuged at 80 x g. The supernatant was discarded and the pellet resuspended in V-47 basal medium and centrifuged for 5 min at 80 x g. Again the supernatant was discarded and the pellet was resuspended in small amount (2ml) of V-47 basal medium. Using a narrow bore pipette the resuspended pellet was slowly suspended on top of 21% sucrose solution in CPW salts (Table contained in a screw-capped centrifuge tube. A small amount (0.5-1.0 ml) V47 basal medium was overlaid and centrifuged at 80 x g. The protoplast band obtained as a ring at the interface was carefully collected using a narrow bore pipette and resuspended in V-47 basal medium and centrifuged 5 min at 80 x g. Then the supernatant was discarded and the washing with V-47 basal medium was repeated twice. The purified protoplasts were suspended in V-47 medium supplemented with growth regulators.

5.5.4 Estimation of protoplast yield and viability: After purification the yield of protoplasts was estimated using a haemocytometer (Pollard and Walker, 1990). The yield of protoplasts is expressed as the number of protoplasts isolated per gram fresh weight of source tissue digested (Fowke and Constabel, 1985; Sagare, 1996). The viability was determined by phenosafranin staining. (1% Phenosafranin was prepared in V-47 basal medium supplemented with 9% mannitol, Widholm, 1976) The stain selectively stains only dead and broken protoplasts. Viability percentage was calculated from the number of intact unstained protoplasts out of total number of protoplasts in at least three microscopic fields.

5.5.5 Incubation and Culture of protoplasts: After this, the pellet was resuspended in a known volume of V-47 plating medium (V-47 basal medium supplemented with BA 1.0mg l⁻¹, NAA 1.0mg l⁻¹ and 2,4-D 1.0mg l⁻¹) contained in disposable petridishes (35 mm diameter). The density was adjusted to 1 x 10⁵ protoplasts per ml with the haemocytometer. The petridishes were incubated in diffused light (4.6 mol m⁻²s⁻¹) at 25 °C.

Protoplast studies in *Piper* spp. as of now are restricted to a brief report in NRCS Annual Report (1990). No details of the work are available till date.

5.6 Results and Discussion:

5.6.1 Effect of source material on protoplast isolation: The source tissue affects the efficiency of protoplast isolation considerably (Ochatt *et al.* 1989, Power *et al.* 1989). Among the three source tissues used in this study, the leaf of green-house grown plants and the organogenetic calli of the mature leaf explants were found to be unsuitable for protoplast isolation. Both these source tissues upon digestion with enzymes produced dark brown solution that on further processing yielded only broken cells and the debris. The browning could have resulted from phenolic exudation. The use of mature leaves also resulted in high rate of contamination. Hence all the further work was carried out with the juvenile leaves of *in vitro* derived plantlets. These *in vitro* leaves after digestion produced yielded protoplasts.

5.6.2 Effect of enzymes on protoplast isolation: The enzymatic method pioneered by Cocking (1960) is the most efficient and popular method of protoplast isolation. Apart from the source material, the cell wall digesting enzymes is the most influential factor affecting protoplast isolation. The concentration and combination of the enzymes as well as the duration of enzyme treatment affected the efficiency of protoplast isolation and the viability of the protoplasts. Over-exposure of the source tissue to the cell wall digesting enzymes leads to degradation of protoplasts whereas less exposure causes reduction in the isolation efficiency.

In the present study, we found that out of the 6 enzyme combinations used (Table 5.6), only 2 were suitable for protoplast isolation from juvenile leaves of *P. colubrinum* (Table 5.7). Out of these two, combination 3 was found to be optimum for protoplast isolation (Fig. 5.4A, B). The isolation efficiency as well as viability count in this combination was better than combination number 6.

The duration of treatment also affected the protoplast isolation. The incubation for 4 h was not sufficient for isolation of protoplasts in any of the enzyme combinations studied. In combination 3, incubation period of 8, 12 and 16 h supported protoplast isolation. The duration of 12h resulted in highest yield of protoplasts as well as highest viability. In combination 6 however the yield and viability were very low and isolation time was higher.

Fig. 5.4

A. Bursting of protoplasts in V-47 medium containing 6% Mannitol

B. Freshly isolated protoplasts from juvenile leaf explants of wild pepper.

FIG. 5.5

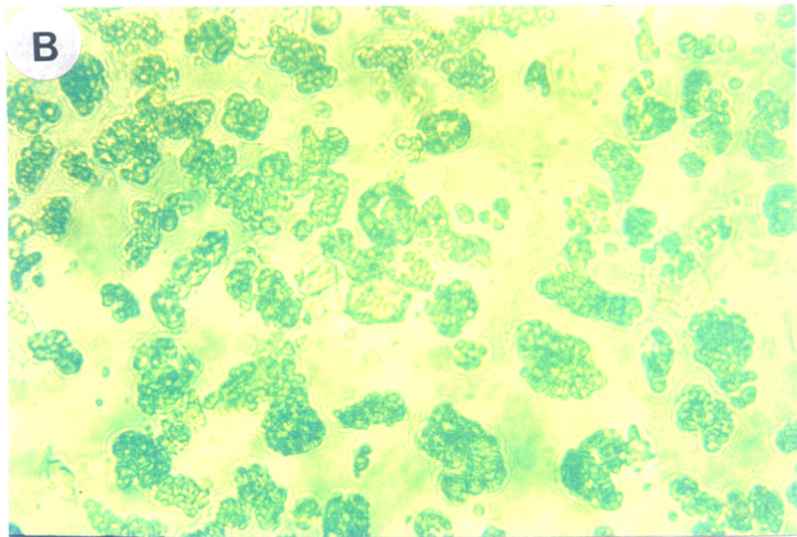
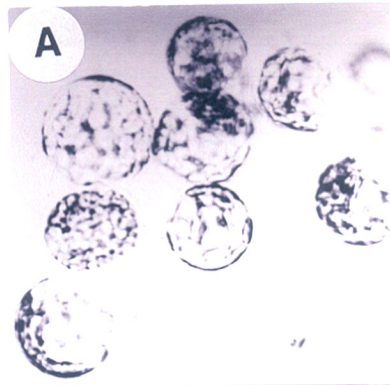


Table 5.7 Effect of enzyme concentration, combination and duration on yield and viability of leaf protoplasts of *Piper colubrinum* Link.

Enzyme Treatment	Duration of treatment (h)	Yield of protoplasts	Viability count
1	4	0	-
	8	0	-
	12	0	-
	16	0	-
2	4	0	-
	8	0	-
	12	0	-
	16	0	-
3	4	0	-
	8	8.2×10^4	60.0%
	12	28.4×10^4	73.7%
	16	16.5×10^4	66.6%
4	4	0	-
	8	0	-
	12	0	-
	16	0	-
5	4	0	-
	8	0	-
	12	0	-
	16	0	-
6	4	0	-
	8	0	-
	12	4.9×10^4	71%
	16	6.8×10^4	51%

5.6.3: Effect of Mannitol concentration on protoplast isolation: The use of mannitol as an osmoticum is common in several woody as well as herbaceous species (Ochatt *et al.*, 1989, Power *et al.*, 1989). In the present study, 3 concentrations of mannitol were used namely 6%, 9% and 13%. The enzyme combination 3 used as well as V-47 medium used for processing and culture of protoplasts contained mannitol at these three concentrations. The yield of protoplasts at these three concentrations of mannitol are presented in Table 5.8. It was found that use of 9% mannitol was optimum for both yield and viability of *P. colubrinum* protoplasts. In 6% mannitol the bursting of protoplasts (Fig.5.3C) occurred and 13% mannitol the protoplasts were somewhat shrunken due to plasmolysis. Low yield and viability in these two concentrations of

mannitol was thus probably due to osmotic imbalance.

Table 5.8 Effect of mannitol concentration on protoplast isolation in the *in vitro* derived leaves of *Piper colubrinum*

Enzyme combination	Mannitol concentration (%)	Yield of protoplasts	Viability count
3	6	8.9×10^4	23.0%
	9	28.4×10^4	73.7%
	13	20.0×10^4	55.5%

5.6.4: Culture of protoplasts: The isolated protoplasts were cultured in plating medium composed of liquid V-47 medium supplemented with BA (1.0mg/l-1), NAA (1.0mg/l-1), 2,4-D (1.0mg/l-1) and 9% mannitol. After 3-5 days of culture no division of the protoplasts was observed and after 7-8 days the protoplasts degenerated, suggesting necessity of manipulation of plant growth regulators and/or the medium for better results in future.

Part C: Kanamycin sensitivity test and Co-cultivation of leaf explants with *Agrobacterium tumefaciens*:

5.7. Introduction:

Plant genetic transformation involves the transfer and incorporation of alien DNA into the plant genome. On the backdrop of initial unsuccessful attempts of transferring naked DNA to plant tissues, the transformation technology got a boost with the discovery of mode of action of the soil bacterium *Agrobacterium*, the causal organism of crown gall disease.. This bacterium was found to contain a plasmid that transferred the tumor-causing principle i.e. the T-DNA to the host plant. Thus the bacterium contained complete machinery for integrative transformation. Further progress was made by 'disarming' the plasmid by removing the crown gall genes while retaining the DNA transfer mechanism intact. The engineering of the plasmids by replacing the crown gall genes with antibiotic resistance genes like Kanamycin resistance genes obtained from bacteria was a step ahead in making the selection of putative transformants easier. This was followed by insertion of the reporter genes such as GUS, GFP and Lux genes.

Though other gene transfer systems such as protoplast mediated naked DNA transfer, microinjection, microprojectile bombardment, pollen transfer and electrophoretic transfer are available, the *Agrobacterium* method still remains the most efficient method of transformation especially for the dicots.

The genetic transformation of plants has immense potential in developing 'plants of future'. It has a lot of scope in overcoming the production constraints by producing high yielding disease and insect resistant plants; in reducing the production cost by reducing the use of insecticides and fertilizers and thereby inducing pollution, in increasing the nutritive value by producing foreign proteins or even vaccines, antibiotics and other pharmaceuticals. In addition to these commercial applications transformation could also be used as a device for in depth basic studies such as the host-pathogen relationships or plant growth regulator interactions at subcellular level.

Presence of distinct, mutually exclusive characters in the protoplasts of the

participant species enables easy selection of fusion product. Intelligent use of existing characters such as morphological or physiological differences has been reported (Stafford, 1992). However in the absence of such characters selectable markers could be introduced in one or both the species by genetic transformation process. The protocols for transformation with marker genes could be further exploited for introducing alien genes useful in conferring resistance or achieving other breeding goals. So far, *Agrobacterium* is the most trusted method of transformation for intact plant cells though other methods like microprojectile bombardment are also being used.

In the present study though our limited objective was to standardize parameters for introduction of marker genes so that the marker-aided selection could be gainfully utilized in future studies on asymmetric hybridization involving wild pepper and black pepper. In this part of the chapter preliminary studies on initiation of *Agrobacterium* mediated transformation of different explants of wild pepper *P. colubrinum* are reported.

So far in *Piper* spp. genetic transformation has been attempted only in cotyledon explants of *Piper nigrum*. Where only callus formation is reported. The results are yet to be confirmed by Southern blot analysis.

5.8 Materials and Methods:

5.8.1 Culture of *Agrobacterium*: *Agrobacterium tumefaciens* strain pGV2260 containing p35SGUSINT plasmid was used for cocultivation of leaf and nodal explants. It carries a chimeric NPTII gene and a GUS gene construct with ST-LS-1 derived intron (Vancanneyt *et al.* 1990). The bacteria were stored in form of glycerol stocks at -700C. About 500 l of the *Agrobacterium* culture was resuspended in YEB medium with antibiotics (Table 5 9). The filter sterilized antibiotics were added to the autoclaved medium under sterile conditions. The bacterium was grown at 200 rpm at 280C for 16 h as optimized earlier (Suhasini, 1997). The 16 h grown culture was used for co-cultivation.

5.8.2 Determination of Kanamycin sensitivity for different explants: Leaf, internode and meristem explants were assessed for their sensitivity to Kanamycin. The explants were cultured in half MS medium with BA which supports shoot formation in all these explants (Kelkar and Krishnamurthy, 1998). The autoclaved medium was supplemented with filter sterilized Kanamycin (50, 100, 200, 400 and 600mg/l) and dispensed in disposable petridishes. At least 20 explants were cultured per treatment and each experiment was repeated twice. The cultured explants were incubated at 250C and under a light intensity of 30 E.m-2.s-1 for 16 h photoperiod. Bleaching of explants was considered as an indicator of lethal reaction of the antibiotic agent.

5.8.3 Co-cultivation of the explants: The explants were cut aseptically and transferred to the *Agrobacterium* culture grown for 16 h for 5, 10 and 15 min and agitated gently. The explants were then blotted on sterile filter paper and cultured on the regeneration medium (Half MS with BA 0.5mg/l). After 1 or 2 or 3 days the explants were washed with sterile distilled water and cultured on same regeneration medium supplemented with 400mg/l Kanamycin and 500mg/l overgrowth of *Agrobacterium*(Claforan Roussel, India). At least 15 explants were cultured per explant type and the experiment was repeated thrice.

Table 5.9 Growth conditions for *Agrobacterium tumefaciens* strain pGV2260-p35SGUSINT: YEB medium and antibiotics.

YEB Medium	
Component	Concentration (mg/l)
Beef extract	5000
Yeast extract	1000
Peptone	5000
Sucrose	5000
MgSO ₄ 7H ₂ O	49.3
Supplementation	
Antibiotic agent	Concentration (µg/ml)
Kanamycin	100
Streptomycin	50
Rifampicin	100

5.9 Results and Discussion:

5.9.1 Effect of Kanamycin concentrations on node, internode and leaf explants of *P. colubrinum*: Phytotoxicity of Kanamycin is well known though in a few plants it is reported to enhance regeneration (Tosca *et al.*, 1992,1996; Mathias and Boyd, 1986) . All the explant types cultured in regeneration medium (Half MS with BA 0.5mg/l-1) containing Kanamycin at 50-600mg/l-1 were adversely affected as indicated by bleaching or browning of tissues due to chlorosis or necrosis. The internode explants were most sensitive to the antibiotic agent (Table 5.10). At all the concentrations of Kanamycin, internode explants became brown or white. None of the internode explants undergo morphogenesis as against the internode explants in the control medium without Kanamycin, 100% of the explants remained green and 76% of which produced shoot buds.

The leaf explants were found to be least sensitive to Kanamycin among the 3 explants used and showed 100% bleaching only at 600mg/l-1, though there was a steady

increase in bleaching percentage proportional to increase in concentration of Kanamycin. Similarly the percentage of explants responding for organogenesis decreased with increase in concentration of Kanamycin. In the control medium 25% explants formed shoot buds. In medium containing Kanamycin at 50 and 100mg/l the percentage of leaf explants forming shoot buds reduced to 10% and 6% respectively. At concentrations 200mg/l and more Kanamycin inhibited organogenesis in leaf explants (Table 5.10, Fig. 5.5A).

The nodal explants could tolerate Kanamycin upto 200mg/l. The sprouting of axillary buds on nodal explants was not affected by Kanamycin upto 400mg/l (Table 5.10). However at the higher concentrations, bleaching of nodal explants and subsequently bleaching of sprouts occurred.

5.9.2 Effect of co-cultivation with *Agrobacterium* on node, internode and leaf explants of *P. Colubrinum*: The explants treated with *Agrobacterium* culture for 15 minutes and cocultivated for 3 days remained green for 3-4 weeks (Table 5.11). But none showed morphogenetic response. These explants eventually turned brown and died. The axillary buds on nodes sprouted to form single albino shoots which did not survive on selection medium. All the treated explants were covered with overgrowth of *Agrobacterium* which eventually resulted in fatal result. The untreated explants showed 100% mortality in selection medium. A reduction in cocultivation time from 15 to 10 and 5 min resulted in marginal increase in percentage of green explants. But could not overcome the growth of bacteria. Reduction in co-cultivation time from 3 days to 2 and 1 day also was unsuccessful in controlling the bacterial overgrowth that led to death of the explants.

The bacterial overgrowth could only be checked by frequent washing of treated explants with 2000mg/l Cefotaxime. But it was found to be detrimental to the explants which turned brown and died.

Similar problem has been confronted in Apple transformation studies (Hammerschlag et al, 1997) solved by use of lower pH and antibiotics. From our preliminary investigation it appears that some such alternative approach such as

optimizing density of *Agrobacterium* growth must be followed to achieve successful transformation in *Piper* spp.

Table 5.10 Effect of different concentrations of Kanamycin on different explants of *P. colubrinum* Link.

Kanamycin (mg.l ⁻¹)	Explant	% Green explants Mean $\pm$ s. d.	% white/brown explants Mean $\pm$ s. d.
50	Node	90.0 $\pm$ 0.0 (81.9)	10.0 $\pm$ 0
	Leaf	91.6 $\pm$ 8.3 (10.0)	8.4 $\pm$ 1.5
	Internode	0	100.0 $\pm$ 0.0
100	Node	41.6 $\pm$ 8.3 (80.0)	58.5 $\pm$ 8.5
	Leaf	36.5 $\pm$ 6.5 (5.5)	63.5 $\pm$ 6.5
	Internode	0	100.0 $\pm$ 0.0
200	Node	0	100.0 $\pm$ 0.0
	Leaf	40 $\pm$ 10.0 (0.0)	50 $\pm$ 10.0
	Internode	0	100.0 $\pm$ 0.0
400	Node	0	100.0 $\pm$ 0.0
	Leaf	7.5 $\pm$ 2.5 (0.0)	92.5 $\pm$ 2.5
	Internode	0	100.0 $\pm$ 0.0
600	Node	0	100.0 $\pm$ 0.0
	Leaf	0	100.0 $\pm$ 0.0
	Internode	0	100.0 $\pm$ 0.0
0	Node	100.0 $\pm$ 0.0 (100.0)	0
	Leaf	100.0 $\pm$ 0.0 (25.0)	0
	Internode	100.0 $\pm$ 0.0 (76.0)	0

Figures in parentheses represent percentage of sprouting among nodal explants and percentage of explants forming shoot buds for leaf and internode explants.

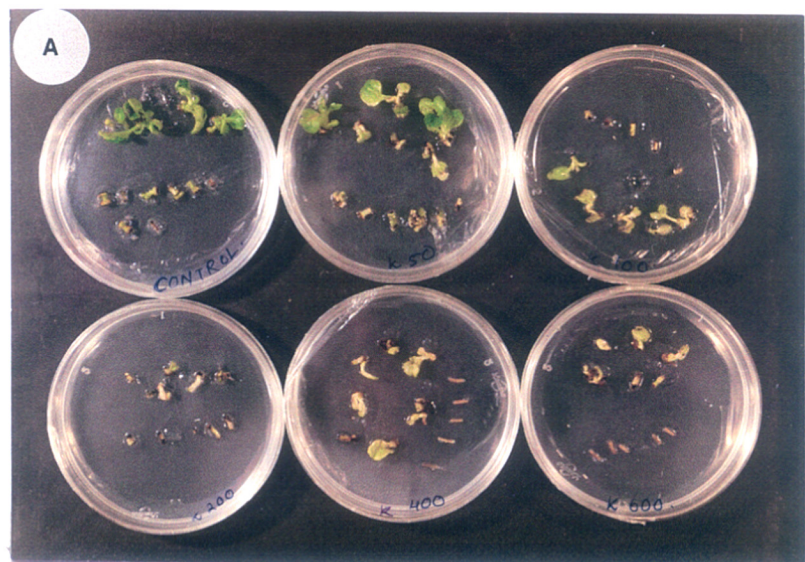
Table 5.11 Percentage response for survival after 3 days of different explants on selection medium after treatment with *Agrobacterium* for three time intervals.

Explant	Co-cultivation time (Min)	% Green explants (Mean $\pm$ s.d.)
Node	15	71.1 $\pm$ 5.9
	10	75.0 $\pm$ 0.0
	5	71.5 $\pm$ 5.6
	0	0
Leaf	15	79.3 $\pm$ 3.3
	10	80.0 $\pm$ 0.0
	5	83.0 $\pm$ 3.0
	0	0
Internode	15	79.5 $\pm$ 7.5
	10	72.0 $\pm$ 0.0
	5	83.5 $\pm$
	0	0

Fig. 5.5

A. Kanamycin sensitivity test carried out for leaf, internode and nodal explants of wild pepper.

FIG. 5.5



5.10. Conclusions:

In the present chapter, an efficient protocol was established for direct shoot regeneration from root, internode, leaf and petiole explants of *in vitro* raised plantlets of the Phytophthora-resistant wild pepper. Presence and concentration of BA in induction as well as maintenance phases of culture influenced efficiency of organogenesis. The effect of culture duration was found to be significant. The origin of shoot buds was confirmed histologically.

The shoot apices and nodes when cultured in half MS medium supplemented with low BA formed multiple shoots. But at higher concentrations of BA, the shoot apices formed organogenetic calli that on further subcultures formed shoots.

A very humble beginning was made in protoplast and transformation studies. Parameters like enzyme concentrations, duration of treatment with enzymes and osmoticum for isolation of protoplasts were studied. However the culture and maintenance of protoplasts was not successful. Kanamycin sensitivity test was carried out for leaf, internode and meristem explants. But the co-cultivation experiments were not successful.

A part of this Chapter was published in 'Biological Applications of Plant Cell and Tissue Culture' (ed. G.A. Ravi Shankar, Oxford, IBH) as a chapter entitled Direct differentiation in wild pepper *Piper colubrinum* Link. by Kelkar SM and Krishnamurthy KV (1997), pp. 146-150.

A part of this Chapter has been published as a paper entitled 'Adventitious shoot regeneration from root, internode, leaf and petiole explants in *Piper colubrinum* Link.' by Kelkar SM and Krishnamurthy KV (1998), in Plant Cell Reports, 17, 721-725.

CHAPTER 6.

STUDIES ON REGENERATION POTENTIAL OF

MATURE EXPLANTS OF THREE CULTIVARS

OF *PIPER NIGRUM* L.

6.1. Introduction :

Vegetative propagation in nature is the most important means of conserving elite genotype as adopted by several horticultural crops, especially woody perennials and further exploited in the conventional methods of crop improvement. It could be defined as production of progeny that is genetically identical or near identical to the parent plant from vegetative or somatic parts of plant. The progeny is called 'Clone' from which the method derives its synonym 'clonal propagation'.

Micropropagation *in vitro* is equivalent to clonal propagation *ex vitro*. It involves production of shoots *in vitro* from different somatic cells, tissues or organs such as buds, shoot apices, leaf, stem, root etc. The process of micropropagation usually consists of 3 steps namely, induction of shoot buds, elongation of shoot buds into shoots and rooting of shoots to form plantlets.

6.1.1. Importance of mature explants: The explants used for micropropagation could be derived from seedlings or from mature plants. However, use of mature explants is preferred owing to the basic principle of clonal propagation that emphasizes 'clonal fidelity' to the elite genotype of proven parent. The use of mature explants enables us to capture the unique and mostly non-additive gene combinations that impart superiority to the parent plant and are very likely to be lost in sexual recombination (Bonga and Durzan, 1992). In addition, use of mature explants could be essential for reasons specific to certain plants or groups of plants such as low seed set, difficulties in seed germination, dioecy or endangered state of a species.

6.1.2. Methods of micropropagation: The use of *in vitro* technique of clonal mass propagation is the most advanced application of plant tissue culture (Thorpe, 1984) and it could be achieved by either of the following methods:

1. Shoot apex culture: The first successful culture of shoot apical meristem was achieved by Robbins (1922) followed by Loo (1945), Ball (1946), Wetmoere and Morel (1946). But its application became widespread among other plants after Morel and Martin (1952) used it for regenerating virus-free Dahlias.

2. Single node culture: This method was first used for *Asparagus* (Galston 1947, 1948). In this method, a single axillary bud is isolated and cultured on basal medium which sprouts forming a solitary shoot that is then transferred to rooting medium.

3. Enhanced axillary bud sprouting: The principle of removal or suppression of apical dominance is used in this method. Though this method was used first in 1967 for multiple shoot formation in carnation (Hacket and Anderson, 1967), the principle was established as early as in 1925 (see, Pierik, 1987).

4. Adventitious shoot formation: In this method explants derived from mature mother plant are induced for direct regeneration of shoot buds usually mediated by cytokinins. These shoot buds are further made to elongate and root.

5. Somatic embryogenesis: This is an alternate pathway where cells of explant are made to differentiate into distinctly bipolar structures i.e. somatic embryos, either directly or via callus formation. These somatic embryos after maturation and germination form somatic plantlets.

6.1.3. Micropropagation of *Piper nigrum* L. from mature explants:

The potential of *Piper nigrum* L. for vegetative propagation *ex vitro* has been exploited for developing micropropagation systems *in vitro*. Multiplication of pepper through shoot tip cultures (Philip *et al.*, 1992), single node culture (Chua, 1981; Chua and Talib, 1985; Fitchet, 1988, 1990 a, b and Nazeem *et al.*, 1992) and adventitious bud formation (Bhat, *et al.* 1995) has been reported. Most of these reports have attempted regeneration in the cultivar Kuching (Chua 1981; Chua and Talib 1985; Fitchet 1988, 1990 a,b).

Philip *et al.* (1992) used shoot tips of cv. Karimunda and Panniyur-1 whereas the remaining reports do not provide any details regarding the varietal status of source plants.

Present study was carried out to deduce response of three high yielding varieties cv. Karimunda, Panniyur-1 and Subhakara *in vitro* of black pepper (*Piper nigrum* L.). The effect of different plant growth regulators on *in vitro* responses of internode and leaf explants as well as sprouting and multiple shoot induction in the apical and axillary meristem explants derived from mature plants of black pepper (*Piper nigrum* L.) are discussed in this Chapter.

6.2. Materials and methods:

6.2.1 Preparation of Explants: Shoot apices (2 cm long), nodal explants (2-3 cm long), green leaves (from first 2-3 nodes starting from shoot apex) and internodes were excised from 6-8 year old green-house grown plants of three varieties of black pepper, i.e. Panniyur-1, Karimunda and Subhakara. All explants were washed under running tap-water for 5 minutes and then pretreated with 1.0 % aqueous solution of systemic fungicide Bavistin (BASF, India) for 120 minutes. The pretreated explants were rinsed with water and then surface-disinfected with 70% ethanol for 1 min. Then 15 explants were transferred to 250 ml sterile flasks in Laminar air flow cabinet and surface sterilized with 0.1% mercuric chloride (10 min for node and internode explants; 5 min for leaf and shoot tip explants). The surface sterilized explants were rinsed with sterile water 5-6 times. Leaf and internode explants were cut aseptically into pieces (0.5-1.0 cm²) and then cultured on respective media. The basal portion of meristem explants was cut before transfer to medium.

6.2.2 Media and culture conditions:

Leaf and internode explants: Leaf and internode explants were cultured in disposable plastic dishes (55 mm diameter, Laxbro, India) containing agar solidified MS medium supplemented with different growth regulators at various concentrations and combinations (Table 6.1).

Shoot tip explants: For the initiation of sprouting, shoot tip explants were cultured in tubes containing different solid or liquid basal media i.e. MS, half MS, WPM, SH, B5 and Blaydes medium in inverted and upright orientations.

Shoot tips were cultured in WPM supplemented with BA at 0.5, 1.5, 3.0 and 5.0mg l⁻¹ for multiple shoot induction. The explants responding for shoot formation were subcultured after 6-8 weeks in WPM basal medium and subsequent subcultures were carried out at monthly intervals. The clumps of shoots originated in BA containing media were transferred to solid WPM first in tubes and then in 250 ml flasks for elongation.

Nodal explants: Effect of different basal media and cytokinins on axillary bud sprouting was studied by culturing the nodal explants in upright position in different basal media (MS, half MS, WPM, SH, B5 and Blaydes medium) in solid or liquid state.

Axillary buds from nodal explants cultured in liquid or solid half MS medium

sprouted to form solitary shoots. These solitary sprouts were subcultured in the same medium containing cytokinins BA, Kinetin and TDZ individually at 0.5, 2.0, 3.0 and 5.0mg l⁻¹ for multiple shoot induction.

The nodal explants were cultured in liquid as well as solid half MS supplemented with cytokinins BA, Kinetin and TDZ at 0.5, 2.0, 3.0 and 5.0mg l⁻¹, individually contained in tubes. Half MS without plant growth regulators served as control for this experiment. To check the effect of ethylene, explants were cultured in half MS medium containing AgNO₃ at 10.0, 15.0 and 25.0mg l⁻¹ with or without BA (5.0mg l⁻¹).

After 6 weeks, the explants with shoot buds at the nodal region as well as axillary bud sprouts showing multiple shoot formation were subcultured on half MS without plant growth regulators for shoot elongation.

Rooting of shoots derived from shoot tip and nodal explants: After 2-3 passages (4-6 weeks), clumps of elongated shoots (2-3 cm in height) developed from shoot tips as well as nodes were separated into single shoots. These single shoots were transferred to fresh half MS basal medium. The shoots rooted within 2 weeks.

All the media contained 2% sucrose (Qualigen's India) and the pH was adjusted to 5.8 before autoclaving. The explants were cultured in culture tubes (Borosil 22 mm diameter) in liquid media on filter paper supports. The solid medium was solidified with 0.7% agar (Qualigen's India). All the experiments contained at least 10 replicates and were repeated twice. The growth regulators except IAA were autoclaved with medium. Filter sterilized IAA was separately added to the autoclaved medium under aseptic conditions. Cultures were incubated at a light intensity of 30 $\mu\text{E.m}^{-2} \text{ s}^{-1}$ at 16 h photoperiod provided by cool fluorescent tubelights and $25 \pm 2^{\circ} \text{C}$.

6.2.3 Acclimatization: Rooted plantlets were transferred to pots containing soil: sand mixture at 1:1 ratio and were hardened in the green-house as described in Chapter2.

6.3. Results and Discussion:

Leaf and internode explants derived from the green house grown vines of three cultivars of black pepper were cultured in MS medium based on previous reports (Chua, 1981, 1983; Mathews and Rao, 1984). The explants showed differential response to the different growth regulators.

The apical and axillary meristems in form of shoot tips and nodal explants were cultured in different basal media (MS, half MS, WPM, SH, B5 and Blaydes medium) for sprouting. They sprouted to form single shoots in all these basal media. Shoot tips cultured in WPM supplemented with BA ($0.5 - 5.0\text{mg l}^{-1}$) and nodal explants cultured in half MS with BA and TDZ ($0.5-5.0\text{mg l}^{-1}$) resulted in multiple shoot induction.

6.3.1 Effect of different plant growth regulators on internode and leaf explants:

Mature internode and leaf explants cultured in half MS with different plant growth regulators (Table 6.1) failed to show any morphogenetic response except callus or root formation. Different growth regulators (BA, Kinetin, 2,4-D, NAA, 2,4,5-T, Dicamba, Picloram, TDZ) used individually or in combination and at various concentrations ($0.1-10.0\text{mg l}^{-1}$) resulted into either callus or root formation. The calli formed in leaf and internode explants different media combinations though of different colour (White, Greenish white and Green), were of same loose, friable consistency (Figs. 6.1A, B, C). BA and Kinetin at 2.0 , and 5.0mg l^{-1} caused darkening of the explants or calli (Table 6.1). Among the auxins, 2,4,5-T was least effective in evoking morphogenetic response in either explant type (Table 6.1). Whereas NAA, 2,4-D resulted in root formation. TDZ also induced rhizogenesis at 5.0mg l^{-1} (Table 6.1). None of the calli induced by different growth regulators regenerated into shoot buds even after 5-6 subcultures in the same or growth regulator free medium.

Table 6.1 Effect of different plant growth regulators added to MS basal medium on leaf and internode explants of three cultivars of *P. nigrum* L.

No.	PGR/s	Concentration mg l ⁻¹	Panniyur-1		Karimunda		Subhakara	
			L	I	L	I	L	I
1	BA	0.1	-	-	-	-	-	-
2		0.5	-	-	-	-	-	-
3		1.0	+G	+G	+G	+G	+G	+G
4		2.0	+GW	+GW	+GW	+GW	+GW	+GW
5		5.0	+GW/D	+W	-D	+W	+GW	+W
6	Kin	0.1	-	-	-	-	-	-
7		0.5	-	-	+W	+W	-	+W
8		1.0	+WD	-	-D	-	+W	+W
9		2.0	+W;D	-	-D	-D	-D	+W;D
10		5.0	+G;D	+W/D	-D	-D	-D	-D
11	2,4-D	0.1	-	-	-	-	-	-
12		0.5	-	-	+W	-	+W	-
13		1.0	+W	-	+W	+W	+W	+W
14		3.0	+W	-R	+W;R	+W	+W;R	+W
15		5.0	+W;R	-R	+W;R	+W	+W;R	+W;R
16		10.0	+W	+W	ND	ND	ND	ND
17	NAA	0.1	-	-	-	-	-	-
18		0.5	-	+W;R	-	-R	-	-
19		1.0	+W	+W	+W	+W	+W	+W
20		3.0	+W	+W;R	+W;R	+W	+W;R	+W;R
21		5.0	+R	+W;R	+W;R	+R	+W;R	+W;R
22		10.0	-R	ND	ND	ND	ND	ND
23	2,4,5-T	0.1	-	-	-	-	-	-
24		0.5	-	-	-	-	-	-
25		1.0	-	-	-	-	-	-
26		3.0	-	-	-	-	-	-

Table 6.1 contd.

No.	PGR/s	Concentratio mg l ⁻¹	Panniyur-1		Karimunda		Subhakara	
			L	I	L	I	L	I
27	2,4,5-T	5.0	+W	-	+W	-	+W	-
28		10.0	+W	+W	+W	+W	+W	+W
29	Dicamba	0.1	+W	-	+W	-	+W	-
31		1.0	+W	-	+W	+W	+W	+W
32		3.0	+W	+W	+W	+W	+W	+W
33		5.0	+W	+W	+W	+W	+W	+W
34		10.0	-	+W	-	+W	-	+W
35	Picloram	0.05	+W	-	-	+W	-	+W
36		0.1	+W	-		+W	-	+W
37		0.25	+W	-	+W	+W	+W	+W
38		0.5	+W	-	+W	+W	+W	+W
39		1.0	+W	+W	+W	+W	+W	+W
40	TDZ	0.5	+G	-	+G	+W	+G	+W
41		1.0	+G	-	+G	+W	+G	+W
42		3.0	+G	+G	+G	-	+G	+W
43		5.0	+W;R	+G	+G	-	-R	+W
44		10.0	ND	ND	-R	-	ND	ND
45	BA + 2,4-D	0.1 + 0.5	-	-	-	-	-	-
46		0.1 + 1.0	+W	-	-	-	+W	-
47		0.1 + 2.0	+W	-	-	-	-	-R
48		1.0 + 0.5	+G;R	+W	+G	+W	+G	+W
49		1.0 + 1.0	+G;R	+W	+G	+W	+W	-
50		1.0 + 2.0	+W;R	+W	+W	+W	+W	+W
51		2.0 + 0.5	+G;R	+W	+G	+GW	+G	+GW
52		2.0 + 1.0	+G;R	+W	+G	+GW	+G	+GW
53		2.0 + 2.0	+W;R	-	+W	-	-	-
54		5.0 + 0.5	-D	-	+G	+W	+G	-
55		5.0 + 1.0	-	+W	+G	+W	+W	+W
56		5.0 + 2.0	+W	+W	+W	+W	+W	+W

Table 6.1 contd.

No.	PGR/s	Concentration mg l ⁻¹	Panniyur-1		Karimunda		Subhakara	
			L	I	L	I	L	I
57	BA+ NAA	0.1 + 0.5	-	-	-	-	-	-
58		0.1 + 1.0	-	-	-	-	-	-
59		0.1 + 2.0	+W	-	+W	-	-R	-
60		1.0 + 0.5	+W		+W		+W	
61		1.0 + 1.0	-	-	+W	-	-	-
62		1.0 + 2.0	+W	+W	-	-	-	-
63		2.0 + 0.5	+G	+G	+G	+G	+G	+G
64		2.0 + 1.0	+G	+G	+G	+G	+G	+G
65		2.0 + 2.0	+W	+W	+W	+W	+W	+W
66		5.0 + 0.5	-D	+W	-	+W	+W	+W
67		5.0 + 1.0	+WN;D	+WN	+WN;D	+WN	+WN	+WN
68		5.0 + 2.0	-	+W	+W	+W	+W	+W
69	Kin + 2,4-D	0.1 + 0.5	-	-	-	-	-	-
70		0.1 + 1.0	-	-	+W	-	+W	-
71		0.1 + 2.0	+W	+W	+W	+W	+W	+W
72		1.0 + 0.5	+W	+W	+W	+W	+W	+W
73		1.0 + 1.0	+W;R	+W	+W	-	-	-
74		1.0 + 2.0	-R	+W	-R	+W	-	+W
75		2.0 + 0.5	+G	+WN	+G	+WN	+G	+WN
76		2.0 + 1.0	+W	+WN	+G	+WN	+G	+WN
77		2.0 + 2.0	-	+W	+W	+W	+W	+WR
78		5.0 + 0.5	-	+W	-	+W	-	+W
79		5.0 + 1.0	+G/D	+WN		+WN	+G/D	+WN
80		5.0 + 2.0	+G/D	+WN	+G/D	+WN	+GD	+WN
81	Kin + NAA	0.1 + 0.5	-	-	-	-	-	-
82		0.1 + 1.0	-	+W	+W	-	+W	+W
83		0.1 + 2.0	+W;R	+W	-	-	-	-
84		1.0 + 0.5	-	+W	+W	+W	+W	+W
85		1.0 + 1.0	-	-	+W	+W	+W	+W
86		1.0 + 2.0	-R	-	-	-	+W	-
87		2.0 + 0.5	+GWD	+W;D	+GW; D	+W;D	+GW D	+W ;D
88		2.0 + 1.0	+GW	+W	+W	+W	+W	+W
89		2.0 + 2.0	-	-	-	-	-	-
90		5.0 + 0.5	-D	-D	-D	-D	-D	-D
91		5.0 + 1.0	-	-	-	-	-	-
92		5.0 + 2.0	+GW	-	+GW	-	-	+W

Table 6.1 contd.

93	BA + IAA	1.0 + 1.0	+G	+G	+G	+G	+G	+G
94		1.0 + 2.0	+G;R	+G;R	+G;R	+G;R	+G;R	+G;R
95		1.0 + 5.0	+W;R	+W;R	+W;R	+W;R	+W;R	+W;R
96		2.0 + 1.0	+G	+W	+G	+W	+G	+W
97		2.0 + 2.0	+W;R	+W;R	+W;R	+W;R	+W;R	+W;R
98		2.0 + 5.0	+W;R	+W;R	+W;R	+W;R	+W;R	+W;R
99	BA + NAA + 2,4-D	0.5 + 1.0 + 1.0	+W	+W	+W	+W	+W	+W
100		2.0 + 1.0 + 1.0	+W	+W	+W	+W	+W	+W
101	Kin + NAA + 2,4-D	0.5 + 1.0 + 1.0	-	+W	+W	+W	+W	+W
102		2.0 + 1.0 + 1.0	+W	+W	+W	+W	+W	+W

PGR: Plant growth regulator; Conc.: Concentration; W: White callus; G: Green callus; GT: Green translucent callus; GW: Whitish green callus; GN Green nodular callus; - No callus formation; + callus formation; R: Root induction; D: Darkening of explant / callus; ND: Not done Kin : Kinetin.

6.3.2 Effect of different basal media on sprouting of apical meristems: Shoot tip explants of the three cultivars cultured in different basal media solidified with agar showed high percentage of necrosis (Table 6.2, Fig. 6.1D) and hence low percentage of sprouting. WPM supported highest percentage of sprouting. This could have resulted from higher calcium content of the medium that prevents shoot tip necrosis (Herman, 1994). Use of WPM has been reported to control shoot apex necrosis in various plant systems (Lingington 1993; Brand and Lineberger, 1986; Lakshmi Sita, 1993). Hence WPM was used in all further experimentation with shoot apices.

The use of liquid cultures is recommended for controlling shoot tip necrosis (Abousalim and Mantell, 1994). But use of liquid medium for shoot tip cultures of *Piper nigrum* resulted in delayed necrosis. The number of necrotic shoots in liquid medium though less, was found to be comparable to that in solid medium. Culturing of shoot apices in an inverted position in solid half MS medium however, decreased percentage of necrotic explants in all three cultivars (from 70-80% to 50-60%). This supports the hypothesis put forth by Abousalim and Mantell (1994) that shoot tip necrosis is caused by insufficient distribution of calcium rather than by poor uptake of calcium. Thus in

effect both solid and liquid media gave similar results for shoot tip cultures.

Table 6.2 Effect of Basal media (Solid) on sprouting of apical and axillary meristems in 3 cultivars of *P. nigrum* L.

Cultivar	Meristem	Percentage of explants sprouting (Mean $\pm$ s.d.)					
		MS	Half MS	SH	WPM	B5	BD
Panniyur-1	Axillary	55.0 $\pm$ 5.0	50.0 $\pm$ 0.0	49.0 $\pm$ 1.0	50.0 $\pm$ 0.0	61.0 $\pm$ 1.0	55.0 $\pm$ 3.0
	Apical	20.0 $\pm$ 0.0	18.0 $\pm$ 2.0	30.0 $\pm$ 2.0	40.0 $\pm$ 5.0	34.0 $\pm$ 0.0	22.0 $\pm$ 2.0
Karimunda	Axillary	38.0 $\pm$ 2.0	47.5 $\pm$ 2.0	59.0 $\pm$ 2.0	50.0 $\pm$ 0.0	50.0 $\pm$ 2.0	43.0 $\pm$ 3.0
	Apical	12.0 $\pm$ 4.0	23.0 $\pm$ 3.0	20.0 $\pm$ 0.0	34.0 $\pm$ 4.0	20.0 $\pm$ 6.0	15.0 $\pm$ 5.0
Subhakara	Axillary	40.0 $\pm$ 0.0	55.0 $\pm$ 5.0	33.0 $\pm$ 3.0	40.0 $\pm$ 0.0	33.0 $\pm$ 7.0	34.0 $\pm$ 0.0
	Apical	17.0 $\pm$ 2.0	19.0 $\pm$ 0.0	12.0 $\pm$ 6.0	29.0 $\pm$ 2.0	15.0 $\pm$ 5.0	12.0 $\pm$ 0.0

6.3.3 Effect of different basal media on sprouting of axillary meristems: The axillary buds present on nodal explants sprouted in all the basal media tested. In the pilot experiments, both solid and liquid media gave similar results for sprouting. Among the cultivars, cv. Panniyur-1 showed the highest percentage of sprouting (61%) in B5 medium followed by Karimunda (59%) in Sh medium. The cv. Subhakara showed lower percentage of sprouting as compared to the other two cultivars. The differences between media treatments were not found to be statistically significant. For any cultivar (Table 6.2). Thus all the basal media supported sprouting of axillary buds with same efficiency. Half MS medium was randomly chosen for cultures of nodal explants.

The percentage of nodal explants sprouted was significantly higher than the shoot tips. Besides, the number of nodes available for culture per plant is also much higher than the shoot tips; hence, nodal explants were found to be more suitable as experimental material.

6.3.3 Effect of cytokinins on sprouting of axillary buds: The axillary buds on the nodal explants sprouted to produce single shoot per explant in half MS medium with or without cytokinins though the percentage response varied (Table 6.3). The sprouting of axillary buds and resultant formation of single shoots is known to be affected by cytokinins (Wertheim and Easterbrook, 1994; Kamineck, 1992). In the present study, with increase in concentration of BA and TDZ, the percentage of sprouting increased in

Legends.

Fig. 6.1

A. White callus induced in stem explants of cv. Panniyur-1 in Full MS medium supplemented with 2,4-D (0.5 mg l^{-1})

B. Green callus induced in stem explants of cv. Panniyur-1 in Full MS medium supplemented with BA(2.0 mg l^{-1}) and NAA (0.5 mg l^{-1})

C. Whitish green callus turning brown in leaf explants of cv. Karimunda cultured in Full MS medium with BA (5 mg l^{-1})

D. Necrotic shoot tip of cv. Subhakara in Full MS basal medium.

E. Axillary bud sprouting and solitary shoot formation along with callus formation at the base of nodal explant in cv. Subhakara in half MS medium with kinetin 3.0 mg l^{-1}

FIG 6.1

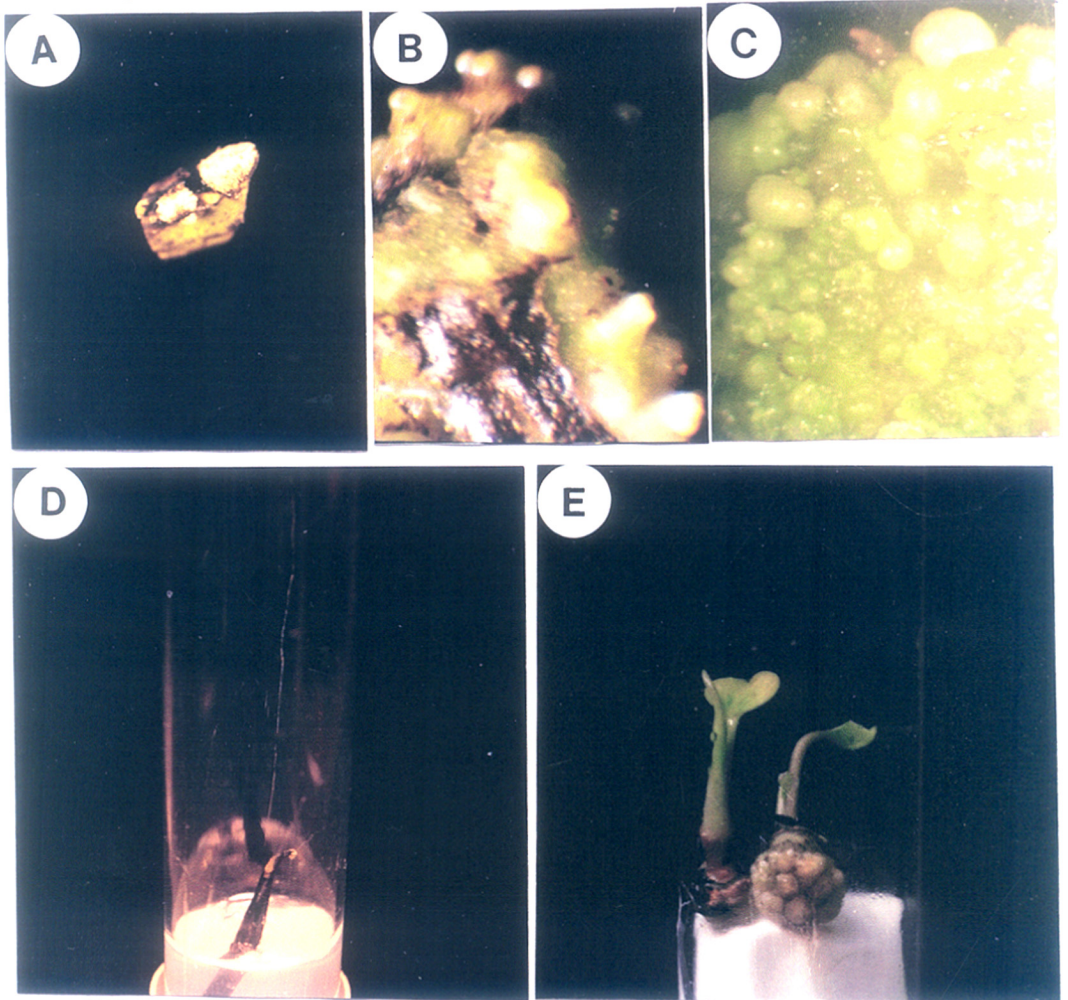


Fig. 6.2

A. Necrosis of shoot tip of cv. Karimunda cultured in WPM leading to sprouting of axillary bud as indicated by bulging of the bud (indicated by an arrow)

B. Enhanced axillary bud sprouting due to necrosis of shoot tip in cv. Subhakara cultured in basal WPM

C. Enhanced axillary bud sprouting in shoot tip explant of cv. Panniyur-1 cultured in WPM supplemented with BA 1.5 mg l^{-1}

D. Enhanced axillary bud sprouting in shoot tip explant of cv. Subhakara cultured in WPM supplemented with BA 3.0 mg l^{-1}

E. Enhanced axillary bud sprouting in shoot tip explant of cv. Karimunda cultured in WPM supplemented with BA 3.0 mg l^{-1}

FIG. 6.2



cv. Panniyur-1 and Karimunda, while no significant difference was observed in cv. Subhakara (Table 6.3).

Table 6.3 Effect of different cytokinins on axillary bud sprouting in different cultivars of *Piper nigrum* L.

Cytokinin	Conc. mg.l ⁻¹	Percentage response for axillary bud sprouting (Mean $\pm$ s.d.)		
		Panniyur-1	Karimunda	Subhakara
BA	0.5	50.0 $\pm$ 0.0 ^a	52.5 $\pm$ 6.0 ^a	52.5 $\pm$ 2.0 ^a
	3.0	61.0 $\pm$ 3.0 ^a	57.0 $\pm$ 0.7 ^{ab}	51.0 $\pm$ 1.0 ^a
	5.0	77.0 $\pm$ 4.0 ^b	67.0 $\pm$ 3.0 ^b	48.0 $\pm$ 2.0 ^a
TDZ	0.5	46.3 $\pm$ 0.8 ^a	56.0 $\pm$ 2.0 ^{ab}	60.0 $\pm$ 0.5 ^a
	3.0	59.0 $\pm$ 0.8 ^a	60.0 $\pm$ 2.0 ^b	53.0 $\pm$ 2.0 ^a
	5.0	63.0 $\pm$ 1.0 ^a	67.0 $\pm$ 2.0 ^b	57.0 $\pm$ 1.0 ^a
Kinetin	0.5	66.6 $\pm$ 0.0 ^a	67.5 $\pm$ 2.0 ^b	60.0 $\pm$ 0.0 ^a
	3.0	55.5 $\pm$ 1.0 ^a	52.7 $\pm$ 2.0 ^a	56.3 $\pm$ 0.8 ^a
	5.0	50.0 $\pm$ 0.0 ^a	46.4 $\pm$ 3.0 ^a	50.0 $\pm$ 0.0 ^a
Control	0.0	50.0 $\pm$ 0.0 ^a	47.5 $\pm$ 5.0 ^a	55.0 $\pm$ 5.0 ^a

Values followed by same letter do not show significant differences among the treatments for each genotype at 95% confidence interval.

The presence of Kinetin in medium supported single shoots at all the concentrations tried and the percentage of single shoot formed from sprouting was equivalent to that of the control (Table 6.3). In media containing Kinetin callus formation occurred at the basal region of the single shoots (Fig. 6.1E). The differences in sprouting percentage of different cultivars were not found to be statistically significant for any cytokinin treatment.

6.3.4 Multiple shoot induction in shoot tip explants: Necrosis of shoot tips cultured in all different basal media including WPM resulted in decapitation that enhanced sprouting of axillary buds in acropetal succession (Fig. 6.2A, B). Results similar to decapitation induced enhanced axillary bud sprouting were obtained in WPM containing BA 1.5 and 3.0mg l⁻¹ in all the cultivars. BA at 5.0 mg l⁻¹ inhibited multiple shoot formation (Table 6.4) In WPM containing BA 1.5 and 3.0 mg l⁻¹, within 6-8 weeks, sprouting of axillary buds in addition to shoot apices (Fig. 6.2 C, D, E) resulted in formation of a clump of 3-4 shoots which was then transferred to solid WPM basal

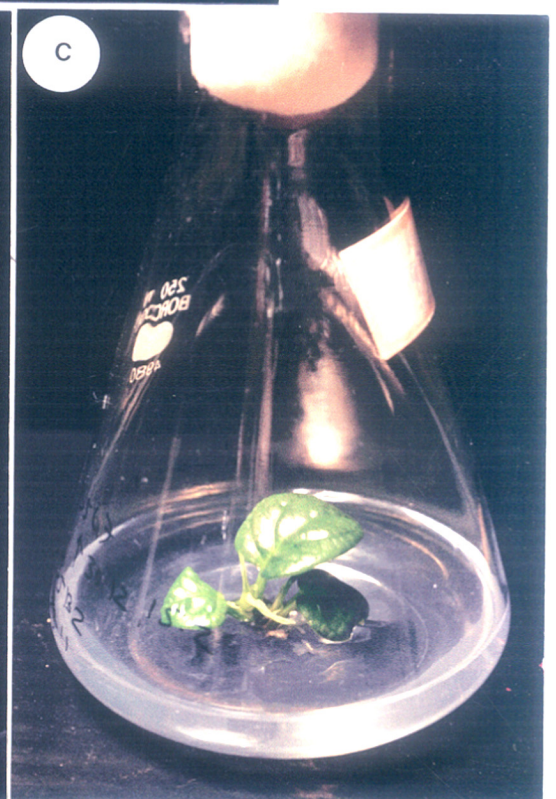
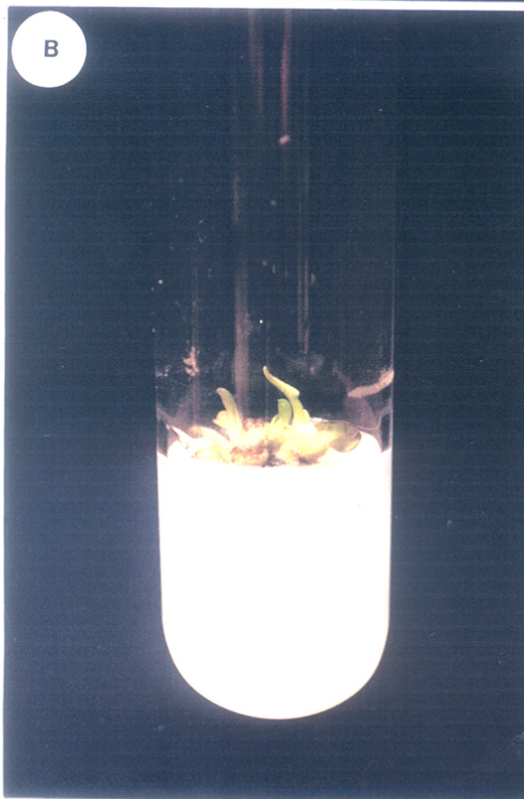
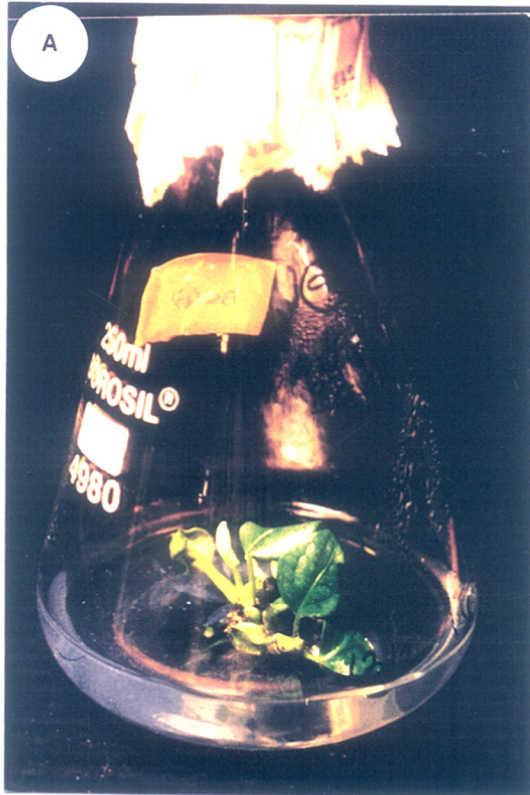
Fig. 6.3.

A. Clump of shoots cultured in WPM developed from enhanced axillary bud sprouting in shoot tip explant of cv. Karimunda.

B. Multiple shoot formation in cv. Panniyur-1 by axillary bud sprouting in WPM with BA 3.0

C. Elongation of single shoot of cv. Panniyur-1 in WPM.

FIG.6.3



medium for elongation (Fig. 6.3A). On elongation, each shoot with one node was separated from the clump and cultured in medium containing BA (1.5 and 3.0mg l⁻¹). These shoots remained stunted but formed multiple shoots after 8-10 weeks in the same medium by axillary sprouting (Fig. 6.3B). These shoots were elongated in WPM basal medium (Fig. 6.3C). Intra- or inter-cultivar significant differences were not observed for enhanced axillary sprouting and all the tree cultivars behaved uniformly for this response.

Table 6.4 Effect of BA on enhanced axillary sprouting in shoot tip explants of three cultivars of *P.nigrum* L.

BA conc. (mg.l ⁻¹)	No. of shoots per explant (Mean $\pm$ S.D.)		
	Panniyur-1	Karimunda	Subhakara
0.5	1.0 $\pm$ 0.0 ^a	1.0 $\pm$ 0.0 ^a	1.0 $\pm$ 0.0 ^a
1.5	2.7 $\pm$ 1.3 ^a	2.5 $\pm$ 1.1 ^a	2.3 $\pm$ 0.9 ^a
3.0	2.6 $\pm$ 0.6 ^a	2.1 $\pm$ 0.8 ^a	2.3 $\pm$ 0.9 ^a
5.0	1.0 $\pm$ 0.0 ^a	0.0 ^a	1.0 $\pm$ 0.0 ^a
0.0	0.8 $\pm$ 0.3 ^a	1.0 $\pm$ 0.0 ^a	1.0 $\pm$ 0.0 ^a

Values followed by same superscripts do not show significant differences at 95% confidence interval.

6.3.5 Multiple shoot induction in nodal explants via enhanced axillary sprouting :

Axillary buds of 3 cultivars of black pepper sprouted on control medium (half MS without growth regulators) to form solitary shoots. The axillary sprouts with 1-2 nodes were excised from original explants and subcultured in half MS medium containing BA, Kinetin and TDZ at 0.5, 2.0, 3.0 and 5.0mg l⁻¹. Kinetin (3.0, 5.0mg l⁻¹) or BA (0.5, 3.0mg l⁻¹) supported the sprouting of axillary buds of these 'Stage 2 cultures' in presence of the growing shoot apex, nullifying the apical dominance exerted by the apical meristem. This led to formation of 2-4 shoots per axillary sprout depending on the number of nodes and axillary buds present (Table 6.5, Fig. 6.4 A, B, C, D). The multiple shoots were elongated in half MS basal medium (Fig. 6.4D) Kinetin at 0.5mg l⁻¹ supported only the elongation of the shoot apex whereas BA at 5.0mg l⁻¹ resulted in swelling and browning of the cultures. TDZ induced callusing and rarely rooting but no shoot formation was observed (Data not tabulated).

Fig. 6.4

A. Secondary axillary bud sprouting in axillary sprouts of cv. Subhakara in half MS with BA 5.0 mg l^{-1}

B. Secondary axillary bud sprouting in axillary sprouts of pannityur-1 in half MS with kinetin 5.0 mg l^{-1}

C. Secondary axillary bud sprouting in axillary sprouts of and Karimunda in half MS with BA 3.0 mg l^{-1}

D. Elongation of multiple shoots in cv. Karimunda in half MS.

FIG. 6.4

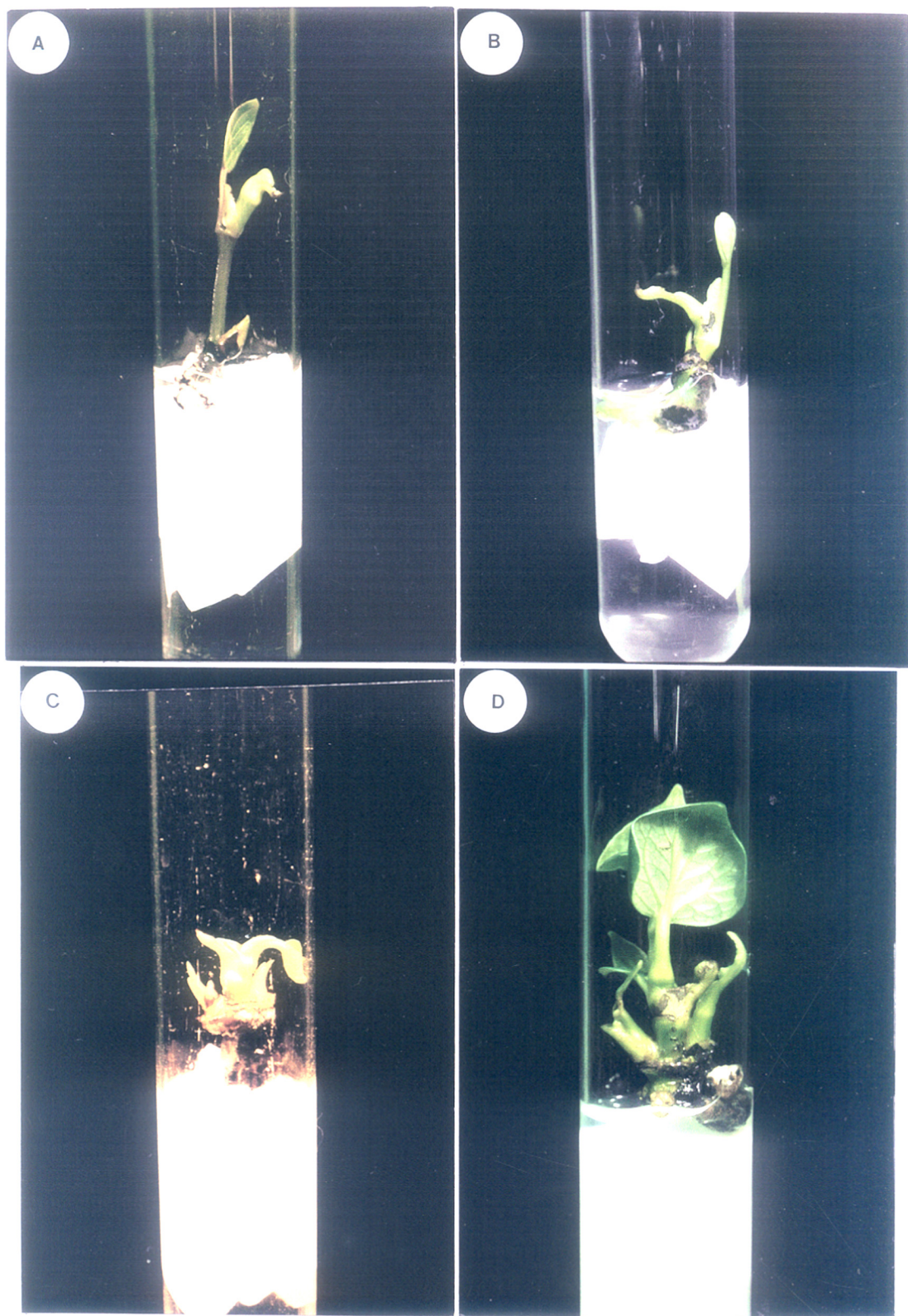


Table 6.5. Effect of different cytokinins on enhanced axillary sprouting in nodal sprouts of different cultivars of *Piper nigrum* L.

Cytokinin	Conc.	Percentage of explants responding mean $\pm$ s.d.			No. of shoots formed per explant Mean $\pm$ s.d.		
		Panniyur-1	Karimunda	Subhakara	Panniyur-1	Karimunda	Subhakara
BA	0.5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	12.5 $\pm$ 1.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	2.5 $\pm$ 0.5
	3.0	20.0 $\pm$ 0.0	10.0 $\pm$ 0.0	25.0 $\pm$ 0.0	2.6 $\pm$ 0.5	3.2 $\pm$ 0.5*	2.2 $\pm$ 0.4
	5.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Kinetin	0.5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	3.0	25.0 $\pm$ 0.0	20.0 $\pm$ 0.0	40.0 $\pm$ 0.0	2.0 $\pm$ 0.0*	2.2 $\pm$ 0.4	2.5 $\pm$ 0.5
	5.0	30.0 $\pm$ 0.0	35.0 $\pm$ 5.0	45.0 $\pm$ 5.0	2.7 $\pm$ 0.5	2.2 $\pm$ 0.4	2.3 $\pm$ 0.5

Values followed by asterik are significantly different at 95% confidence interval at different concentrations of same growth regular for each genotype for MS (Multiple Shoot formation) response.

6.3.6 Adventitious shoot induction in nodal explants: Multiple shoot formation occurred from the nodal explants, by adventitious shoot bud induction at the nodal region only in half MS containing BA (3.0 and 5.0mg l^{-1}) and TDZ (0.5, 3.0 and 5.0mg l^{-1}) (Table 6.6). The nodal explants swelled and formed a ring of small shoot buds in the 4th week of culture (Fig. 6.5A, B, C, D). The percentage of explants forming multiple shoots increased with the increase in the concentration of BA and TDZ (Table 6.6).

The localized formation of shoot buds only at the nodal region could have resulted from the restricted presence of competent cells at this region. The presence of right type of cells is postulated as an important pre-requisite for successful morphogenesis (De Klerk *et al.* 1997). Such cells upon exposure to proper stimulus (which is BA or TDZ in this case) become determined to form specific organs according to the selected morphogenetic pathway (i.e. shoot buds in this case).

At the end of 6th week of culture the explants with shoot buds were transferred to half MS for shoot elongation. After 4-6 weeks, shoot buds elongated to form shoots (Figs. 6.6A, B, C). The shoots measuring 0.5-1.0 cm. in height were counted to determine the number of shoots per explant. For further elongation, the shoots were transferred to conical 250 ml flasks (Figs .6.7A, B, C).

The elongated shoots from clusters of multiple shoots sometimes showed formation of secondary nodal ring of shoot buds (Figs. 6.6C). The buds from such nodes

Fig. 6.5

A. Formation of nodal structures in cv. Panniyur-1 in half MS with BA 3.0 mg l⁻¹

B. Formation of adventitious nodal structures in cv. Karimunda in half MS with BA 5.0 mg l⁻¹

C. Formation of adventitious nodal structures in cv. Subhakara in half MS with TDZ 3.0 mg l⁻¹

D. Histology of nodal explant in cv. Panniyur-1 responding for adventitious shoot formation showing direct origin of shoots.(Bar = 1mm)

FIG. 6.5

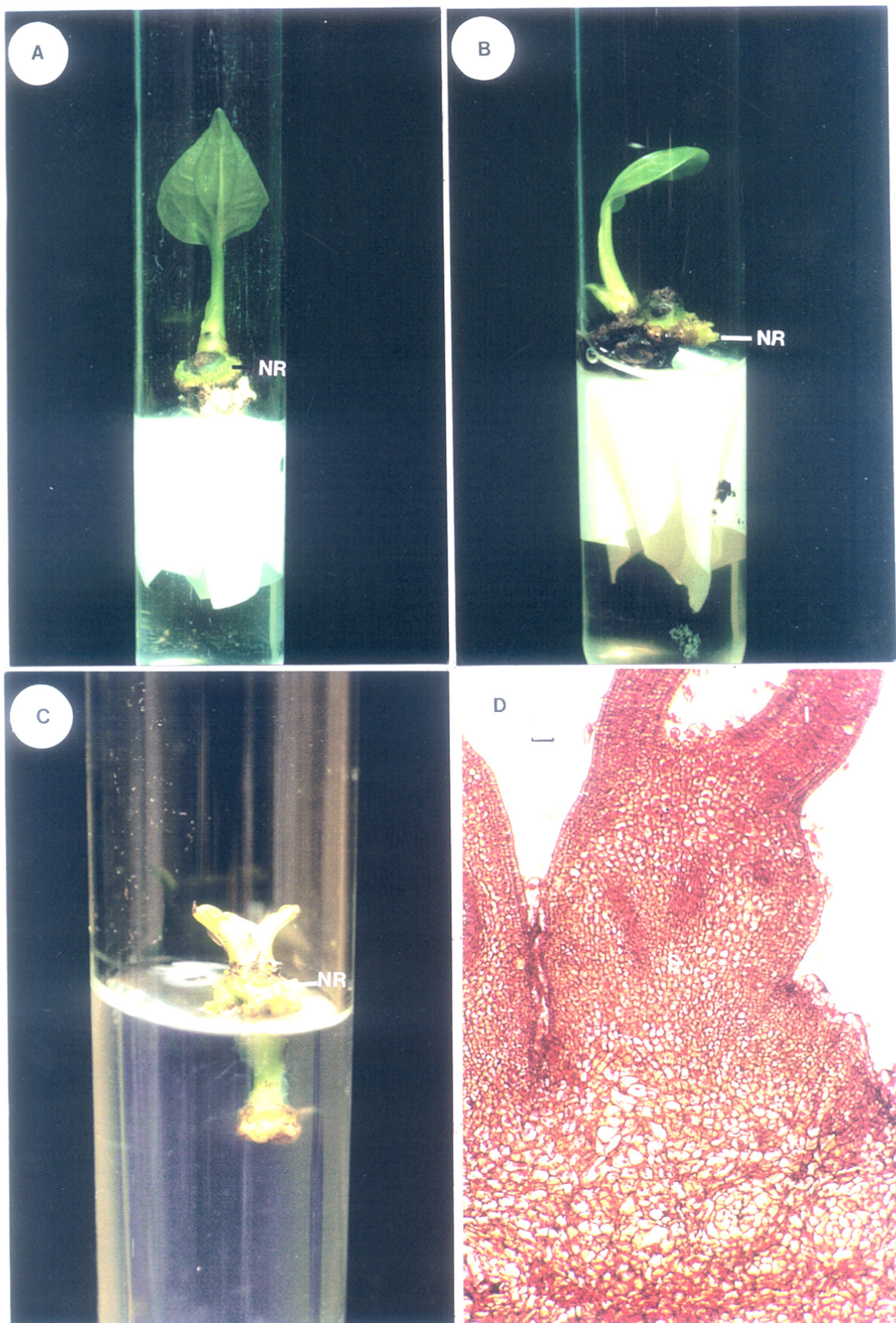


Fig. 6.6

A. A part of nodal explant showing conversion of shoot buds of cv. Subhakara in half MS

B. Conversion of shoot buds of cv. Karimunda in half MS

C. Conversion of shoot buds of cv. Panniyur-1 in half MS

FIG.6.6

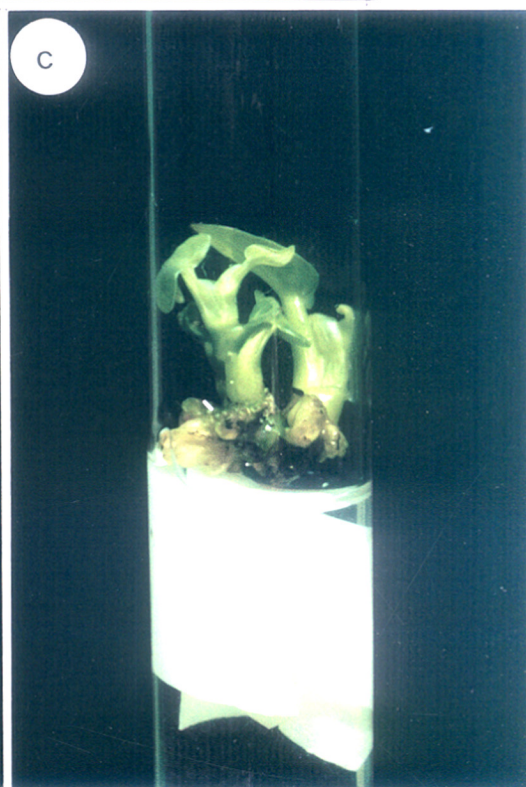
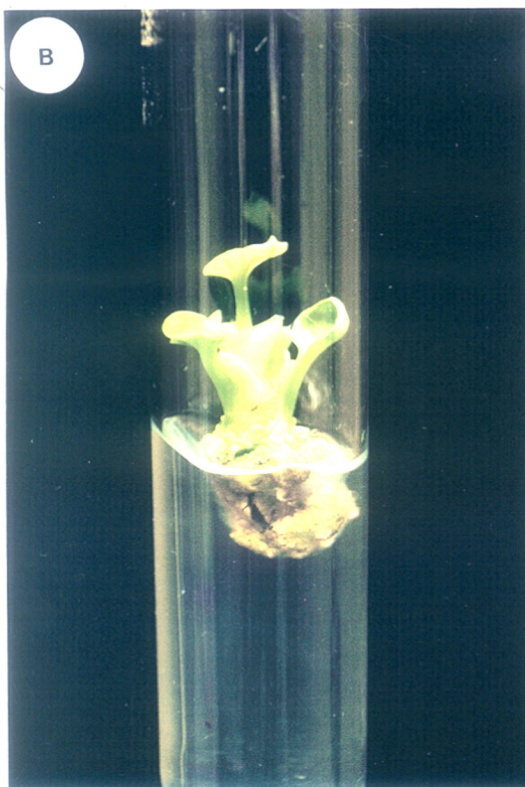
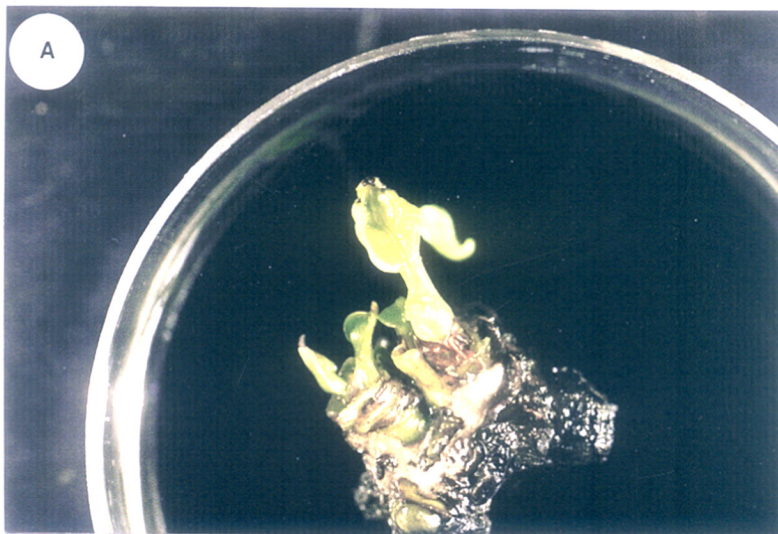
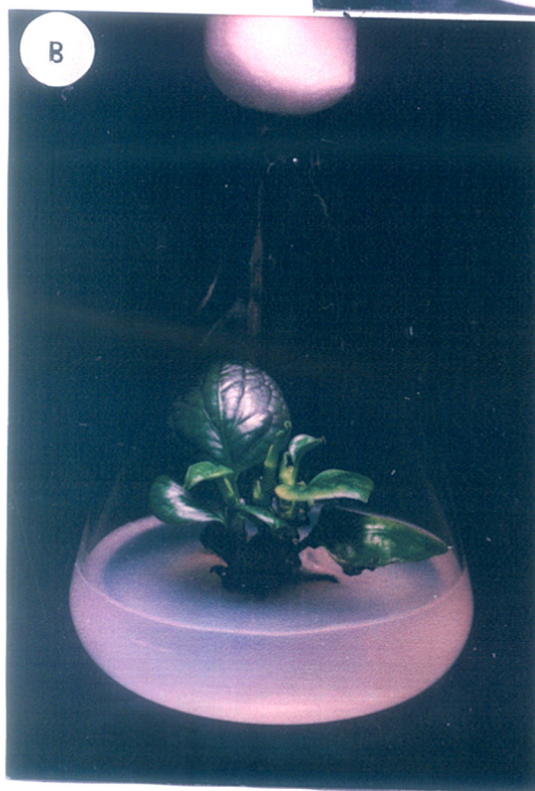


Fig. 6.7

A-C Elongation of *in vitro* derived shoots of cv. Subhakara, Karimunda and Panniyur-1 in half MS

FIG. 6.7



elongated into shoots on half strength MS medium without growth regulators.

BA at lower concentrations (0.5mg l^{-1}) could not induce multiple shoots in any of the cultivars. The number of shoots produced per explant was not significantly different at BA 3.0 and 5.0mg l^{-1} in cv. Panniyur-1. But in cv. Karimunda and Subhakara the number of shoots was significantly higher at BA 5.0 than BA 3.0mg l^{-1} (Table 6.6). BA at 5mg l^{-1} was found to be the optimum for multiple shoot induction in all the cultivars which is higher concentration as compared to the previous report by Bhat *et al.* (1995). The percentage response for shoot bud formation and number of shoot buds formed was found to be higher in our studies as compared to that reported by Bhat *et al.* (1995).

TDZ at 0.5mg l^{-1} had the highest number of shoots which decreased at higher concentrations of 3.0 and 5.0mg l^{-1} , (Table 6.6). The number of shoots produced per explant at TDZ 0.5mg l^{-1} was comparable to BA 3.0mg l^{-1} but lower than at BA 5.0mg l^{-1} (Table 6.6) in all the cultivars. At higher concentrations though the percentage response improved the number of shoots per explant was reduced. The reduction in number of shoots per explant was found to be statistically significant. This was accompanied by callusing at node. No significant differences were observed among the three cultivars for response to different concentrations of TDZ.

(Bhat *et al.*, 1983). The mechanism of action

Table 6.6 Effect of different cytokinins on multiple shoot formation in different cultivars of *Piper nigrum* L.

Cytokinin mg.l^{-1}	Percentage of explants forming *MS Mean $\pm$ s.d.			No. of shoots formed per nodal explant Mean $\pm$ s.d.		
	Panniyur-1	Karimunda	Subhakara	Panniyur-1	Karimunda	Subhakara
BA 0.5	-	-	-	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
BA 3.0	45.0 ± 5.0	37.8 ± 2.0	33.3 ± 0.0	5.4 ± 0.7^a	3.8 ± 0.7^a	2.7 ± 0.6^a
BA 5.0	59.9 ± 6.0	51.6 ± 1.0	47.7 ± 2.0	6.1 ± 0.9^a	5.6 ± 0.7^b	4.8 ± 1.0^b
TDZ 0.5	33.0 ± 4.0	40.0 ± 2.0	50.0 ± 0.0	5.0 ± 0.8^a	4.4 ± 0.8^a	$4.1 \pm 1.0^{b,c}$
TDZ 3.0	48.5 ± 0.0	41.5 ± 2.0	42.2 ± 0.6	2.8 ± 1.0^b	2.8 ± 0.4^c	3.3 ± 1.0^c
TDZ 5.0	52.3 ± 0.8	50.6 ± 3.0	51.6 ± 0.3	2.6 ± 0.5^b	2.6 ± 0.8^c	$2.5 \pm 0.5^{a,c}$
Control	-	-	-	0.0 ± 0.0^c	0.0 ± 0.0^d	0.0 ± 0.0^d

*MS - multiple shoot formation, Values followed by same letter are not significantly different at 95%

confidence interval at different concentrations of same growth regular for each genotype for MS response.

The quality of shoots was better in the medium containing BA than TDZ. In TDZ

containing media, most of the shoot buds became vitrified and only a few elongated to form shoots. Vitrification of shoots due to TDZ has been reported in many other species (Huetteman and Preece, 1993).

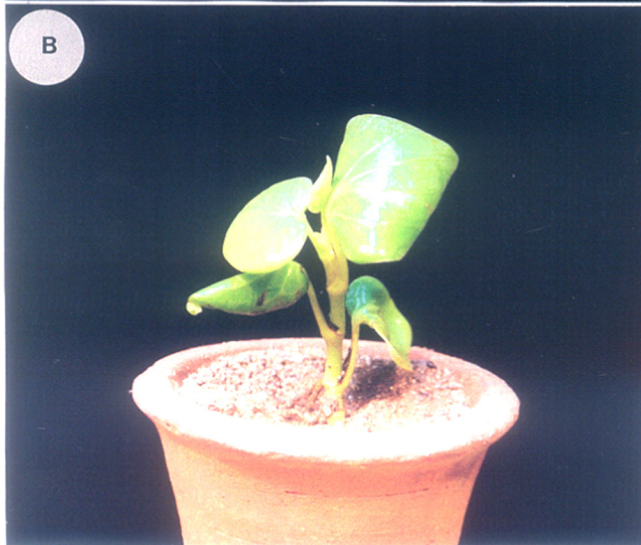
In presence of both TDZ and BA numerous shoot buds were formed but only a few could be elongated into shoots. The reduction in number of shoots could have resulted due to apical dominance exerted by shoots elongated earlier as reported by Busing *et al.* (1993) in soybean. Alternatively, this could have resulted from supra-optimal concentrations of cytokinins which act as a limiting factor and cause suppression of the elongation of shoots. In extreme cases this could lead to irreversible dedifferentiation of the shoot buds and the cells may lose the acquired organogenetic competence as observed in some axillary buds cultured in media containing TDZ at 5.0 mg l⁻¹.

6.3.6.1. Effect of silver nitrate on multiple shoot formation: In this study, BA at 5.0mg l⁻¹ was found to be the optimum for shoot induction in nodal explants of 3 cultivars of black pepper. BA is found to be the most effective growth regulator in *Piper* spp. (Mathews and Rao, 1984; Fitchet, 1990; Philip *et al.*, 1992; Bhat *et al.* 1992, 1995; Kelkar *et al.*, 1996; Kelkar and Krishnamurthy, 1998) and in many other plant species (Flick *et al.*, 1983). The mechanism of action of BA is not yet completely understood. It is proposed that BA causes organogenetic induction indirectly through ethylene production (Lieberman, 1979) which in turn inhibits DNA synthesis and re-programs cell fate as found in shoot apices of soybean (Busing *et al.* 1994). Both positive and negative actions of ethylene on shoot induction are observed. AgNO₃ an inhibitor of ethylene action is reported to enhance shoot induction (Kong and Yeung, 1994; Hyde and Phillips, 1996).

In order to assess the possible role of ethylene in pepper shoot induction, nodal explants were cultured in half strength MS supplemented with BA at 5.0mg l⁻¹ and AgNO₃ at 5.0, 15.0 and 25.0mg l⁻¹. The addition of AgNO₃ caused reduction in number of shoots per explant (Table 6.7). This is suggestive of probable positive role of ethylene in shoot bud induction in nodal explants of all the three black pepper cultivars.

Table 6.7 . Effect of AgNO₃ on multiple shoot induction in different cultivars of *Piper*

Fig. 6.8 A-C Acclimatization of *in vitro* derived shoots of cv. Subhakara, Karimunda and Panniyur-1.

FIG. 6.8

nigrum L.

Cytokinin (mg.l ⁻¹)	AgNO ₃ (mg.l ⁻¹)	No. of shoots per nodal explant (mean ± s.d.)		
		Panniyur-1	Karimunda	Subhakara
BA 5.0	5.0	4.4 ± 1.0	4.0 ± 0.0	4.0 ± 1.0
	15.0	4.3 ± 1.0	4.3 ± 1.0	4.4 ± 0.7
	25.0	4.9 ± 0.8	4.0 ± 1.0	4.0 ± 0.7
	0.0	6.1 ± 0.9*	5.6 ± 0.7*	4.8 ± 1.0

Values followed by asterisk are significantly different from each other at 95% confidence interval among the treatments for each genotype.

6.3.6.2 Acclimatization: The shoots separated from clusters of multiple shoots as well as the solitary sprouts from both types of meristems elongated further and rooted in the elongation medium consisting of half MS. The rooted plants were transferred to soil:soilrite mixture (1:1) and hardened. Out of total 32 shoots (belonging to all 3 varieties) 26 survived in the soil (Fig.6.8A, B, C).

6.2.6.3 Histology : The direct origin of adventitious shoot buds formed in the nodal region was confirmed by histology. The T.S. passing through nodal ring (Fig.6.5D) of cv. Panniyur-1 showed epidermal cells as well as cells of a few subepidermal layers undergoing divisions. Different stages of shoot bud morphogenesis such as morphogenetic patches, dome formation and shoot bud induction were found in one and the same section. A well formed shoot is projected in the figure. At some places the epidermal cells expressed their morphogenetic potential in formation of trichomes. All the morphogenetic stages were located in the vicinity of epidermis. The presence of cell division in epidermis, cytoplasm-rich state of the epidermal cells suggest epidermal origin of the shoot buds. However this observation needs to be confirmed by histological analysis of explants cultured for different durations. The direct epidermal origin of the shoot buds is suggestive of suitability of this regeneration system for genetic transformation studies

6.4 Conclusions

In this chapter different methods of shoot multiplication using mature explants in three cultivars of *P. nigrum* are presented. Forced axillary branching in shoot apices and solitary axillary sprouts as well as adventitious organogenetic shoot induction in nodal explants was used for multiple shoot formation in the meristem explants. It was found that shoot tip necrosis could be effectively controlled by using WPM. Adventitious shoot bud induction in the nodal explants was induced by BA and TDZ. Nodal explants were found to be superior to shoot tip explants, because, more number of nodes are available per vine and as the number of shoots produced per node is higher. The shoots derived *in vitro* were successfully rooted *ex vitro* and hardened.

The work presented in this Chapter is communicated to the Journal 'Plant Cell Reports' in form of a paper entitled 'Effect of different plant growth regulators on plant regeneration from nodal explants of three cultivars of black pepper (*Piper nigrum* L.)

CHAPTER 7

STUDIES ON *IN VITRO* CULTURE OF SEEDS,
EMBRYOS AND JUVENILE EXPLANTS OF
DIFFERENT CULTIVARS OF *PIPER NIGRUM* L.

7.1. Introduction:

The aim of micropropagation is large scale production of true to type progeny and for this reason the mature explants derived from elite plants are preferably used for tissue culture. However, the mature material is associated with heavy microbial contamination and exudation of phenolics that hamper the cultures at the establishment stage itself. These problems are discussed in details in Chapter 3 of this thesis with reference to *Piper* spp. Apart from these problems, mature explants are often difficult to regenerate. Since the cells of mature explants are less plastic and fully differentiated, it is difficult to reprogram these cells and regain the morphogenetic competence. It has been observed that in several tree species the micropropagation is not possible through mature material. For such species use of juvenile or rejuvenated material is necessary (Pierik, 1987; Brar and Khush, 1994; Ahuja, 1994).

The use of juvenile explants is beneficial because, these explants are relatively clean from contaminants, does not exude phenolics and have more regeneration potential. (Pierik, 1987; Brar and Khush, 1994; Ahuja, 1994). In vegetatively propagated plants the culture of seed-derived juvenile explants offer an added advantage of introducing genetic combinations different from the existing ones. Continuous vegetative propagation of any plant results in the loss of natural genetic variation and thereby narrowing the genetic base. This could create problems in crop improvement programs, especially those involving quantitative trait loci (QTLs) such as high yield and disease resistance. New genetic combinations may have some useful traits that could prove valuable in crop improvement as the availability of genetic variation is the basic pre-requisite for any crop improvement program. The culture of juvenile explants involves following techniques:

Direct Seed culture: The culture of whole seeds in a medium containing growth regulators could give rise to multiple shoots. This procedure was first reported by Malik and Saxena, (1992) in *Phaseolus vulgaris*. They obtained prolific shoot formation from cotyledonary node and the region surrounding shoot apex of growing seedling when the seeds were germinated in a medium containing TDZ. Similar results have been obtained in *Lathyrus* spp. (Malik, *et al.*, 1993), peanut (Zhijian Li, *et al.*, 1994), and mungbean (Narciso, *et al.*, 1997). Besides shoot multiplication, *in vitro* seed germination is also an

important method for obtaining juvenile material of plants having seeds that are difficult to germinate (Forest trees); have low or short viability (banana, plantains, black pepper); or require special mediators for seed germination (orchids, sandalwood).

Embryo culture: The zygotic embryo is the most potent explant type for induction of morphogenesis (Tran than van, 1986). It has been used for induction of shoots or somatic embryos in diverse species of angiosperms as well as gymnosperms (Raghavan, 1976).

Culture of seedling explants: The culture of seedling explants is often carried out in woody plants to standardize parameters for morphogenetic induction that could be extrapolated in mature material which is difficult to regenerate and contains contaminants. However it is also used for mutagenesis and for micropropagation (Pierik, 1987).

Relevance of culture of juvenile explants in *Piper nigrum*: Black pepper (*Piper nigrum* L.) is usually propagated by rooted cuttings. Only two hybrid varieties; Panniyur-1 and Panniyur-3 have been released in this crop so far (Pillay, *et al.*, 1994). There is a limited intrinsic heterozygosity derived from out-crossing ancestors. The asexual reproduction has resulted in preservation of this limited heterozygosity, but simultaneously, it has resulted in stagnation of the genetic pool. To widen the genetic base of a crop, inherent genetic variability must be preserved and new variation must be introduced. It is necessary to bring about improvement in pepper for higher yield and disease resistance. These two traits form the basis of black pepper improvement programs carried out using either conventional plant breeding methods or biotechnological approaches. The culture of seed-derived explants is one of the biotechnological methods for exploitation of existent variation and for introducing new variation through manipulation of cultural micro and macro-environment (Philip, *et al.*, 1996). Further the use of seed derived juvenile explants circumvents the problems of contamination, phenolics and recalcitrance associated with mature material in *Piper* spp.

In black pepper, the seed culture is not reported but *ex vitro* seed germination has been recorded (Chaudhury and Chandel, 1994). The process of seed germination could be exploited to preserve intrinsic genetic variability and to induce new combinations by mutagenesis (Ravindran and Nair, 1992). It could also prove useful in obtaining germ free source plants and juvenile explants that have better regenerability to be manipulated

in tissue culture. These aspects are vital for *Piper* spp. where mature explants are known to harbor microbial contaminants (Fitchet, 1990; Philip, *et al.*, 1992; Bhat, *et al.*, 1992; Aminuddin, *et al.*, 1993; Meyer, *et al.*, 1992; Kelkar, *et al.*, 1996).

There is only one report on somatic embryogenesis in black pepper (Joseph, *et al.*, 1996) where zygotic embryos have been used as an explant.

The use of whole seedling explants is reported here for the first time in black pepper. The use of seedling derived explants has been demonstrated by Mathews and Rao, (1984) for organogenetic induction. The use of seedling explants to induce variability is also reported (Philip, *et al.*, 1997).

7.2. Materials and Methods:

7.2.1. Seed germination *in vitro*:

Red, ripe drupes of seven different cultivars of black pepper were collected from Dapoli, Maharashtra and Calicut, Kerala in India. The drupes were washed and seeds were exposed by removing the pulp. The seeds (along with stony endocarp) were washed under running tap water for 30-40 minutes. The seeds of cv. Panniyur-1 were available in sufficient quantities to assess the effect of different factors on the germination process and were used for optimization studies. The seeds of other six cultivars namely, Panchami, Kuthiravally, Kutching, Karimunda, Aimpiriyam and Valiakaniakadan were used along with seeds of Panniyur-1 in defining the effect of genotype on seed germination in one of the experiments.

All seeds were pre-treated with acid for 10 min as described earlier (Chaudhury and Chandel, 1994) and rinsed with water. The pre-treated seeds were surface sterilized with 0.1% mercuric chloride for 10 minutes, (Mathews and Rao, 1984) washed thoroughly with sterile distilled water and cultured in different media (approximately 12 ml) contained in disposable petridishes (55 mm diameter, Laxbro India). All the media contained 2% sucrose and the pH was adjusted to 5.8 before autoclaving at 15 kPa for 20 minutes. The seed cultures were incubated at $25 \pm 2^\circ\text{C}$. Since the work presented here involves study of different parameters of culture and incubation conditions such as pretreatments, media, illumination etc., the details of these conditions are given separately for each experiment. Similarly certain conditions such as pre-treatment with acid, surface sterilization with 0.1 % mercuric chloride, use of agar solidified Whites medium and dark incubation of seeds used in control sets were based on previous reports (Mathews and Rao, 1984; Chaudhary and Chandel, 1994). These conditions should be considered constant unless stated otherwise.

Effect of acid scarification on seed germination and microbial contamination: Effect of hydrochloric acid, nitric acid and sulfuric acid on germination and microbial contamination was evaluated by treating the seeds (prior to surface sterilization) with hydrochloric, nitric and sulfuric acids at 25% strength for 5 minutes followed by washing under running water. Seeds without any pre-treatments formed the control for this experiment. Treated or un-treated seeds were surface sterilized as per above.

Effect of storage duration on seed germination: The drupes of cv. Panniyur-1 were stored at 4°C in airtight containers for different durations (2, 8, 15, 30 and 60 days). The seeds were after the storage duration and then cultured.

Effect of light conditions on seed germination: Seeds of cv. Panniyur-1 cultured on White's medium were incubated in dark as well as at light intensity of $38 \mu\text{Em}^{-2}\text{s}^{-1}$ for 16 h or 24 h photoperiod.

Effect of basal media and their concentration on seed germination: Seeds of Panniyur-1 were cultured on agar solidified MS basal medium, White's medium and sterile distilled water. Modifications of White's medium with macronutrients at 1/2 and 1/4 th strength or without macronutrients were also used.

Effect of gelling agents on seed germination: was and Seeds were cultured in White's medium solidified with agar-agar (0.7%) and phytigel (0.2% Sigma) and incubated in dark.

Effect of support materials on seed germination: Three different support materials i.e. filter paper, soil and cotton sterilized by autoclaving and soaked with White's liquid medium (approximately 12 ml) were placed in disposable petridishes under sterile conditions and used for seed culture.

Effect of plant growth regulators on seed germination: Seeds were cultured on White's medium supplemented with NAA, 2,4-D, BA, TDZ and GA_3 at concentrations of 0.5, 1.0, 2.0 and 5.0 mg l^{-1} .

Effect of genotypes on seed germination: The effect of genotype was studied by culturing seeds of all 7 cultivars on White's medium (solid) in disposable petri dishes.

Germination event was marked by swelling of seeds but seeds were designated to be germinated when radicle or plumule emerged (in a few cases where plumule emerged first). The observations on germination percentage and root length were noted about 35 days from culture of seeds. In all experiments, each treatment had at least 25 seeds and 5 replicates and each experiment was repeated three times. Analysis of variance (ANOVA) for each parameter was carried out to analyze the significance of different treatments. The significance of differences between or among the treatments of the same parameter was determined by F test.

Germination vigour index was calculated by multiplying mean root length with

the mean germination percentage as reported by Chaudhury and Chandel (1994).

Germination vigour index = Mean root length x Mean germination percentage.

7.2.2. Culture of explants derived from seedlings:

Seeds of cv. Panniyur-1, Karimunda, Kuching and Kuthiravally were germinated *in vitro* in Whites medium without major elements. The seedlings were grown aseptically for 2 months and the whole seedlings as well as shoot apices and nodal explants derived from them were used for Morphogenetic studies. Whole seedlings were cultured in solid as well as liquid half MS medium with BA (0.5, 2.0 and 5.0 mg l⁻¹). Shoot apices and nodes (0.3-0.5 cm long) were cultured on filter paper floats in tubes containing liquid half MS supplemented with BA (0.0, 0.5, 2.0 and 5.0 mg l⁻¹).

The explants were subcultured after 1 month in 250 ml flasks containing liquid or solid half SH or half MS basal medium. The cultures in liquid media were placed on gyratory shaker (Steelmate, India) at 100 rpm and incubated at a light intensity of 38 $\mu\text{E m}^{-2}\text{s}^{-1}$ at 24 h photoperiod and 25 $\pm$ 2° C.

The cultures were incubated at a light intensity of 38 $\mu\text{E m}^{-2}\text{s}^{-1}$ at 16 h or 24 h (liquid shake-cultures) photoperiod provided by cool white fluorescent tubelights and 25 $\pm$ 2° C. All the experiments contained at least 10 replicates and were repeated twice.

7.2.3. Embryo culture:

Embryos were removed from acid treated and surface sterilized seeds of cv. Panniyur-1 and Karimunda soaked overnight in sterile distilled water. The embryos were suspended in sterile distilled water to remove traces of endosperm and then transferred to respective media.

Embryos were cultured in different agar-solidified basal media viz. White, SH, half SH, half MS and WPM. The embryos were also cultured on filter paper floats in liquid half SH medium with or without 2,4-D and NAA (0.5, 1.0, 3.0, 5.0 mg l⁻¹). Effect of sucrose concentration on embryo cultures was studied by using half SH medium supplemented with sucrose at 1%, 1.5%, 2% and 3% for embryo culture. Embryos were also cultured in different modifications of SH medium given in Table 7.1.

Media used for embryo culture contained 1.5 % sucrose except specified otherwise. Embryo cultures were incubated in dark and at $25 \pm 2^{\circ}\text{C}$. All the experiments contained at least 10 replicates and were repeated twice.

Table 7.1 Compositions of different modifications of SH medium used for embryo cultures of *P. nigrum* L.

Medium No.	Composition
1	SH (full strength major and microelements and vitamins)
2	1/2 SH (half strength major-and full strength microelements, vitamins)
3	1/2 SH minus (All ingredients at half strength)
4	SH minus $\text{NH}_4\text{H}_2\text{PO}_4$
5	SH minus KNO_3
6	SH minus CaCl_2 .
7	SH minus $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
8	SH minus Microelements
9	SH minus Vitamins
10	SH minus meso-inositol

7.3. Results and Discussion :

7.3.1. Whole seed culture and *In vitro* seed germination:

For optimization of conditions for *in vitro* seed germination, certain conditions such as use of acids for pre-treatment and White's medium were chosen based on previous studies (Chaudhury and Chandel, 1994; Mathews and Rao, 1984).

The seeds of cv. Panniyur-1 imbibed water and swelled within 2-3 days. The embryonal pole of the seed became prominent and could be detected without opening the seed. Usually the radicle emerged on completion of germination; though in a few seeds the plumular portion emerged while radicle was still inside the seed coat. The emergence took 15 to 50 days depending on the treatments. The different stages of seed germination are shown in figure 7.1A.

Effect of acid scarification on seed germination and microbial contamination: Of the three acids tried, hydrochloric acid and sulfuric acid were found to be effective in enhancing the Germination Percentage (GP) from 52.5% to 71% and 73.5 % in HCl and H_2SO_4 respectively (Table 7.2). The acid treated seeds germinated early (within 20 days) and the germination was uniform while the control seeds (not treated with acids) showed

scattered germination up to 60 days. The acid treatment was also useful in prevention of fungal contamination observed in control seeds (Table 7.2).

Use of acid scarification as a pre-treatment for enhancing seed germination is exemplified in several plants, including pepper (Purseglove, 1974; Chaudhury and Chandel, 1994). Pre-treatment with the acids for 5 minutes was found to be satisfactory. In contrast to Chaudhury and Chandel's observations (1994), all the acids enhanced GP and uniformity of germination (Table 7.2) though the effect of nitric acid was not significantly higher than the control. Among the three acids tested, sulfuric acid gave best results for GP and contamination control. The GP resulting from pre-treatment with hydrochloric acid was comparable to that of sulfuric acid, while GP obtained in pre-treatment with nitric acid is comparable to that of control. The increase in GP may be due to softening of the hard testa by acid inducing better water absorption.

Table 7.2. Effect of acid pretreatment on seed germination of *Piper nigrum* L. cv. Panniyur-1.

Treatment	Contamination Percent Mean $\pm$ s.d.	Germination Percent Mean $\pm$ s.d.
HCl	1.5 $\pm$ 1.0	71 $\pm$ 1 ^a
HNO ₃	5.0 $\pm$ 0.4	60 $\pm$ 3 ^b
H ₂ SO ₄	0.0 $\pm$ 0.0	74 $\pm$ 2 ^a
Control	21.7 $\pm$ 2.0	53 $\pm$ 3 ^b

Values followed by same superscript do not show significant differences at 95% confidence level.

Effect of storage duration on seed germination: It was observed that storage of drupes at 4°C in airtight containers could retain seed viability for 60 days, though GP lowered considerably after 30 days of storage (Table 7.3). However the storage did not affect root elongation and thereby germination vigour. Storage of drupes at room temperature led to rapid water loss from the seeds which dried completely within a week and did not germinate.

Recalcitrance of pepper seeds has been attributed to in short viability which is reported to vary from uncertain time period (Purseglove, 1974) to a period of 15 days (Chaudhury and Chandel, 1994). We found seeds remained viable for 60 days when

drupes were stored in airtight containers at 4° C though the GP was reduced considerably. The reduction in GP may be due to progressive desiccation of seeds and drupes (as indicated by shriveling) that could not be arrested by using airtight containers.

Effect of light conditions on seed germination: The GP was at its highest under dark conditions (81%) and was significantly higher than all the illuminations tried i.e. light intensity 38 $\mu\text{E.m}^{-2}\text{s}^{-1}$ for 16h photoperiod (59%) and 24 h photoperiod (47%) (Table 7.3). Continuous presence of light for 24 h decreased the speed of root growth resulting in low germination vigour index. Darkness supported best root elongation while 16h photoperiod gave intermediate results (Table 7.3).

Light plays dual role in seed germination where it induces germination in photoblastic seeds like *Piper auritum* (Orozco-Segovia, *et al.*, 1993) and inhibits the same in negatively photoblastic seeds such as *Nemophila insignis* (Bewley and Black, 1994). *P. nigrum* seeds were intermediate to these two types. Though dark condition was preferred to illumination, presence of light does not inhibit germination completely (Table 7.3). The continuous light affected GP most probably on account of HIR (High Irradiance Reaction) of photomorphogenesis (Bewley and Black, 1994). Since the GP reduced with the increase in duration of light the effect is quasiphotoperiodic similar to *Nemophila insignis*. (Bewley and Black, 1994). Dark conditions also facilitated better root growth than presence of light as evident from the vigour index (Table 7.3).

Effect of basal media and their concentration on seed germination: White's medium without major elements supported highest GP (83%) followed by sterile distilled water (80%), 1/2 strength (73%) and 1/4 strength (71%). White's medium though the differences among GP at all these treatments were not statistically significant. GP was significantly low at full strength of White's medium (65%) and MS medium (40%). Best results for root elongation were obtained in water followed by White's medium without Major salts and MS medium. White's medium with major salts at all the strengths (Full, 1/2 and 1/4) resulted in slow root growth as evident from vigour index (Table 7.3).

Use of White's medium for seed germination in black pepper has been reported (Mathews and Rao, 1982) whereas MS medium is the most common medium used for plant tissue culture studies. White's medium which is a low salt medium; supported

significantly higher GP than MS medium which is rich in mineral salts. Further reduction in the strength of White's medium resulted in increased GP (Table 7.3). The GP obtained in White's medium without major salts was the highest (83%). It was comparable to GP obtained in water (80%). Thus the supply of minor elements and water helped seed germination but high-salt media were inhibitory to seed germination. The rate of root growth is higher in water than White's medium without major salts as evident from the vigour index.

Effect of gelling agents on seed germination: Use of different gelling agents used to solidify White's medium also affected GP. Agar was found to be better suited than Phytigel as a gelling agent for germination medium since it supported significantly higher GP as compared to Phytigel.

Different gelling agents affect shoot multiplication. The gellan gum type gelling agents such as Gelrite and Phytigel (Sigma) are known to induce hyperhydricity (Podwyszynska and Olszewski, 1995). Though responsible for higher water availability and hyperhydricity in tissue culture, Phytigel was found to be inferior (GP 62 %) to agar (GP 79 %) as gelling agent in seed germination media (Table 7.3). The positive effect of agar on seed germination may be due to impurities in agar that acted as medium supplements (Podwyszynska and Olszewski, 1995).

Effect of support materials on seed germination: Different support materials used for seed germination affected GP considerably. Soil which was found to support seed germination *ex vitro* satisfactorily with 60-65% GP formed poor support material *in vitro* and the GP has dropped to 29% (Table 7.3). Use of cotton and filter paper gave satisfactory results with 73 and 46 % GP respectively (Table 7.3). Cotton was found to be best suited for germination among the three materials tested. The differences among the three treatments were statistically significant. Soil supported the best root growth followed by filter paper and cotton (Table 7.3).

Cotton as support material gave best GP followed by filter paper and sterilized soil respectively. Cotton could remain moist over longer periods than filter paper and thus the continuous supply of medium was available to the seeds cultured on cotton

resulting in higher GP. Medium was replenished at regular time intervals (every 4 days) in the petridishes containing filter papers, as the medium evaporated quickly. The seeds where medium was not replenished dried within 8 days and did not germinate. The inability of filter paper to retain medium for longer time rendered it unsuitable as support material for *in vitro* seed germination. Sterilized soil used for *in vitro* germination was least suitable for germination. Low GP in autoclaved soil (29%) as compared to GP in nonsterile soil used *ex vitro* (60-65%) suggests the positive effect of different biological and physico-chemical factors active in nonsterilised soil used *ex vitro*. However sterilized soil supported root growth better than cotton or filter paper (Table 7.3).

Table 7.3. Effect of different physical and chemical factors on *in vitro* seed germination of *Piper nigrum* L.

Sr. No.	Factor	Germination (%) Mean $\pm$ s.d.	Germination Vigour Index
1	<i>Storage at 4°C</i>		
	2 days	74 $\pm$ 6 ^a	297.2
	8 days	66 $\pm$ 5 ^a	283.8
	15 days	64 $\pm$ 1 ^a	243.2
	30 days	43 $\pm$ 5 ^b	172.0
	60 days	19 $\pm$ 3 ^c	86.4
2	<i>Light (38 μE.m⁻².s⁻¹)</i>		
	24 h photoperiod	47 $\pm$ 5 ^a	122.7
	16 h photoperiod	59 $\pm$ 3 ^a	194.3
3	<i>Dark</i>	81 $\pm$ 2 ^c	349.2
4	<i>Support Material</i>		
	Soil	29 $\pm$ 1 ^a	191.4
	Cotton	73 $\pm$ 11 ^b	409.9
	Filter Paper	46 $\pm$ 6 ^c	268.4
5	<i>Medium</i>		
	MS basal	40 $\pm$ 1 ^a	120.1
	White's Full strength	65 $\pm$ 2 ^b	141.7
	White's 1/2 strength	73 $\pm$ 5 ^c	146.4
	White's 1/4 strength	71 $\pm$ 2 ^c	142.6
	White's without Major	83 $\pm$ 8 ^c	249.6
	Water	80 $\pm$ 0 ^c	304.0
6	<i>Gelling Agents</i>		
	Agar agar	79 $\pm$ 6 ^a	307.7
	Phytigel	62 $\pm$ 5 ^b	186.0

Values for each factor followed by same superscript do not show significant differences at 95% confidence level

Effect of plant growth regulators on seed germination: Of the plant growth regulators tried, GA₃ was not found to promote germination at any concentration (Table 7.4). GA₃ at 0.5 and 2 mg l⁻¹ enhanced root growth, whereas at 5.0 mg l⁻¹ it reduced both GP and root growth. Effect of GA₃ on seed germination is reported in many plant species. It generally acts through breaking of dormancy. It is possible that since the seeds of black pepper

were not dormant, GA₃ was not effective in increasing germination.

The GP obtained in media with auxins 2,4-D and NAA was comparable to the control. Presence of auxins in the medium invariably induced callusing of embryos at radicle end. Supplementation of medium with 2,4-D supported root growth extremely well resulting in high germination vigour index. Root lengths in NAA containing media were comparable to the control. Multiple shoot formation was not achieved with any of the tested growth regulators. The auxins are postulated to support root induction and growth. In black pepper 2,4-D supported best root growth but NAA could not do so probably due to callus formation

Among the cytokinins, BA inhibited germination at all the concentrations. TDZ at low concentrations (0.5 and 2 mg l⁻¹) promoted germination but inhibited the same at high concentration (5.0 mg l⁻¹) as shown in Table 7.4. The root growth increase is proportional to increase in TDZ concentrations. In orchid seeds cytokinins are known to enhance germination (De Pauw, *et al.*, 1995). In black pepper however, BA inhibited seed germination at all concentrations used. It also decreased root elongation and in some instances formed callus at the root apex. The increase in root length at higher concentrations of TDZ could be due to dual nature of the growth regulator which acts as an auxin as well as cytokinin.

Thus different plant growth regulators affect seed germination in many ways (van Staden, *et al.*, 1995; De Pauw, *et al.*, 1995; Arnold, *et al.*, 1995; Raj Shekharan, *et al.*, 1985; Pancholi, *et al.*, 1995). Use of seed as an explant for shoot multiplication induced by plant growth regulators has been reported for pea, peanut and *Lathyrus* (Malik, *et al.*, 1993), peanut (Zhijian Li, *et al.*, 1993), and mungbean (Narciso, *et al.*, 1997). In the present study none of the growth regulators used could induce multiple shoots. But the growth regulators exerted distinct effects on GP or root length or both.

Table 7.4. Effect of Plant growth regulators on seed germination in *Piper nigrum* L. cv. Panniyur-1.

Plant Growth Regulator	Concentration (mg l ⁻¹)	Germination Percentage Mean $\pm$ s.d.	Germination Vigour Index
GA-3	0.5	60 $\pm$ 9 ^{a*}	666.0
	2.0	55 $\pm$ 7 ^{a*}	528.0
	5.0	35 $\pm$ 7 ^b	129.5
2,4-D	0.5	65 $\pm$ 7 ^{a*}	747.5
	1.0	50 $\pm$ 7 ^{a,b*}	730.0
	2.0	40 $\pm$ 7 ^b	560.0
	5.0	ND	ND
NAA	0.5	52 $\pm$ 2 ^{a*}	286.0
	1.0	70 $\pm$ 4 ^{a*}	301.0
	2.0	59 $\pm$ 9 ^{a*}	153.4
	5.0	ND	ND
BA	0.5	46 $\pm$ 8 ^a	115.0
	2.0	42 $\pm$ 8 ^a	84.0
	5.0	16 $\pm$ 5 ^b	14.4
TDZ	0.5	66 $\pm$ 2 ^{a*}	191.4
	2.0	76 $\pm$ 5 ^{a*}	402.8
	5.0	32 $\pm$ 0 ^b	361.6
Control	0.0	64 $\pm$ 2 [*]	268.8

Values for each plant growth regulator followed by same superscript do not show significant difference at 95% confidence level. * Values superscribed by asterisk are comparable to control at 95% confidence level.

ND : not done.

Effect of genotypes on seed germination: Cultivar specific differences were observed in germination behavior of the seeds. The lowest germinability was observed in the cultivars Valiakaniakadan and Panchami (31 and 32% respectively) among the cultivars tested whereas Panniyur-I had the highest GP (81%). Cultivars Kuthiravally (66%), Kutching (66%) and Karimunda (67%) had equal germination capacity though higher than that of cultivar Aimpiriyam (50%). The rate of root elongation was not found to differ drastically among the cultivars.

The cultivar specific germination percentage observed in the black pepper cultivars could be due to genetic or environmental factors. It is possible that some of the

cultivars may differ in the percentage of abnormal or underdeveloped embryos characteristic to the genus *Piper* (Kanta, 1961, 1963). Similarly the cultivars may require different optimum germination conditions and the conditions used in present study could be suboptimal for some of them.

Table 7.5. *In vitro* germination capacity of seeds of different cultivars of black pepper .

Cultivar	% Germination Mean $\pm$ s.d.	Germination Vigour Index
Panniyur-1	81 $\pm$ 2 ^a	349.2
Karimunda	67 $\pm$ 6 ^b	201.0
Aimpiriyam	50 $\pm$ 0 ^c	200.0
Kutching	66 $\pm$ 1 ^b	217.8
Panchami	32 $\pm$ 3 ^d	099.2
Valiakaniakadan	31 $\pm$ 2 ^d	117.8
Kuthiravally	66 $\pm$ 5 ^b	224.4

Values followed by same superscript do not show significant differences at 95% confidence level

Seed germination is considered to begin with imbibition of water by seed and end with elongation of embryo axis, usually the radicle and rarely plumule (Bewley and Black, 1994). According to this definition, 90% of the seeds of black pepper showed ability to germinate because they imbibed water. But about 20% of the total seeds which have imbibed water, could not complete the process of germination because neither of the embryonal poles emerged from them. The embryos from such seeds could not elongate into seedlings but became brown or black in colour and died. This may be due to presence of abnormal embryos arrested at different stages of differentiation in black pepper seeds (Kanta, 1961).

Presence of embryoless seeds has been reported for the genus *Piper* (Prakash, *et al.*, 1994; Kanta, 1961, 1963). Kanta, (1961, 1963) reported 32% of black pepper seeds to be embryoless. However *ex vitro* seed germination studies (Ravindran, *et al.*, 1985 and Bhat, *et al.*, 1992) as well as *in vitro* studies reported here do not corroborate with these embryological observations.

Two post-germination anomalies were prominent among the seedlings studied. In

every 100 seedlings 4 were rootless in spite of a well defined root pole (Fig. 7.1B) while 17 showed unequal cotyledonary leaves (Fig. 7.1C). These structural anomalies did not affect the growth of the seedlings. The rootless seedlings developed adventitious roots on White's basal medium.

Production of 2-4 seedlings from a single seed was sporadic (7 seeds out of 500 seeds studied) and occurred irrespective of the treatments used. In a few seeds (11 seeds out of 500 seeds cultured) indirect (Figs. 7.2A, B) or direct somatic embryogenesis (Fig. 7.2C) was observed in White's medium (full strength major elements) where the root pole produced 1-4 embryos, via callus formation or directly. The somatic embryos were separated from the zygotic embryo and could be regenerated into plants in White's medium (Fig. 7.2D).

The normal seedlings produced by seeds under different treatments appeared similar without any drastic phenotypic differences. This supports the observation of Ravindran, *et al.* (1985) for *ex vitro* seedlings.

Results presented here scored higher seed germination percentages compared to the previous reports (Ravindran, *et al.*, 1985; Anonymous, 1977) in all the varieties except for cv. Karimunda. The time required for germination has also been reduced from 60-70 days to 30 days. The parameters optimized here would help in achieving successful germination and thereby production of germfree seedlings which could be manipulated in tissue culture for shoot multiplication and genetic transformation studies. The seedlings also find application in preservation of inherent genetic diversity and also in production of novel genetic combinations by distant hybridization with other cultivated and wild species through protoplast technology and also in mutation breeding.

Fig. 7.1

A. Different stages of seed germination in cv. Panniyur-1

B. Abnormal seed germination in cv. Panniyur-1 showing rootless seedlings in spite of welldeveloped root pole (rp)

C. Abnormal seed germination in cv. Panniyur-1 showing seedlings with unequal cotyledonary leaves (c).

FIG. 7.1

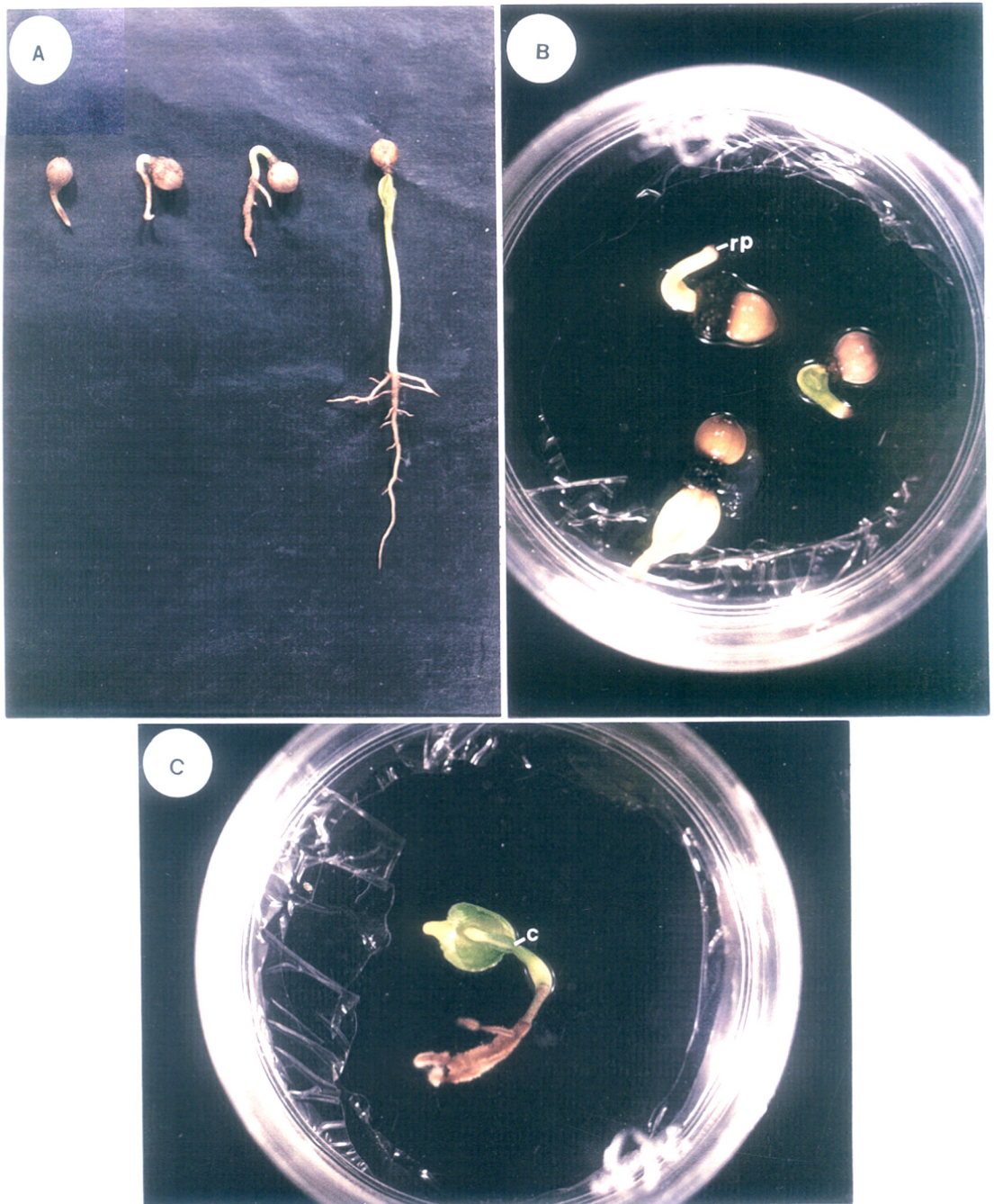


Fig. 7.2

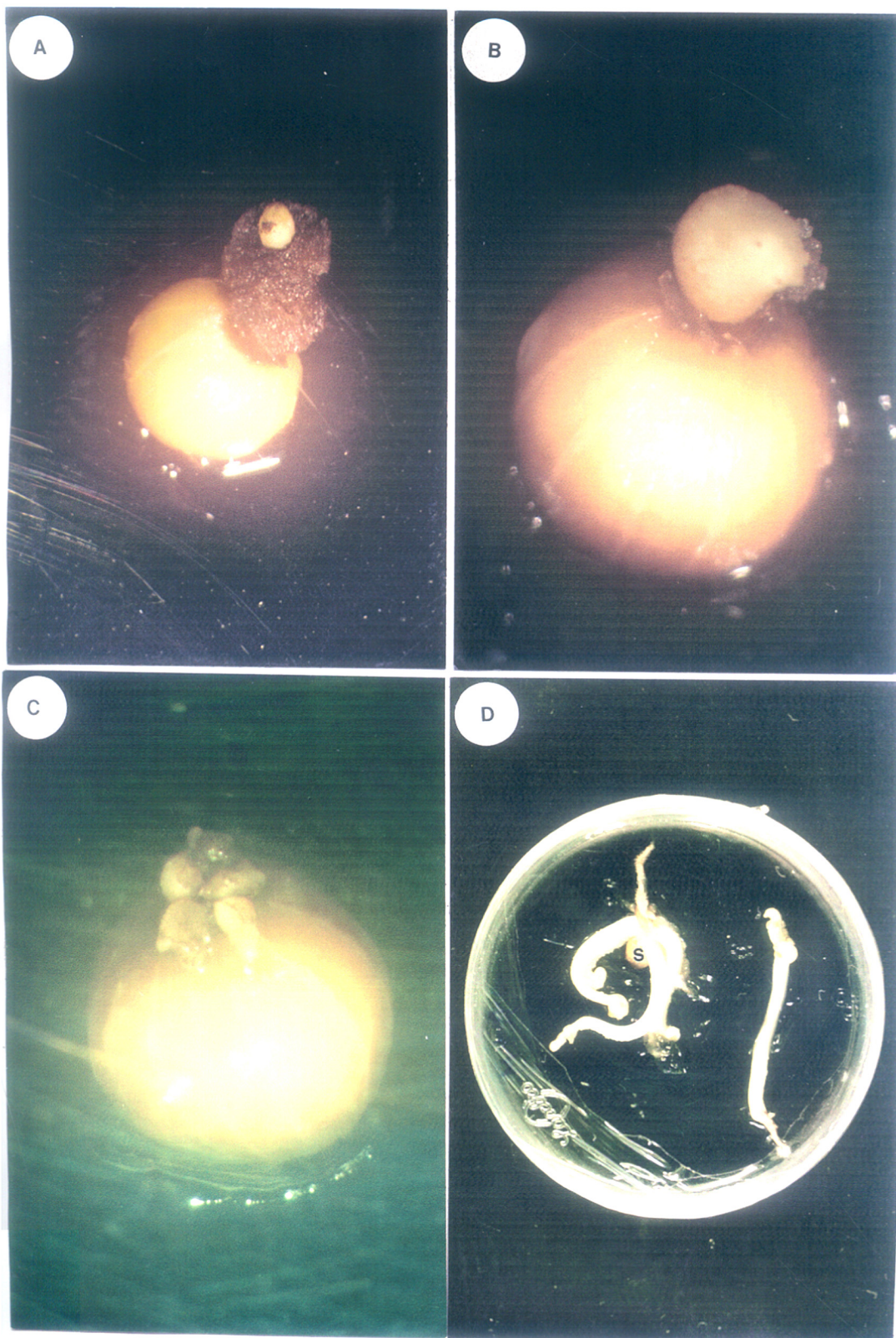
A Seed cultures of cv. Panniyur-1 in Whites medium showing callus at radicle end with a somatic embryo (e).

B. Seed cultures of cv. Panniyur-1 in Whites medium showing callus at radicle end with a somatic embryo (e).

C. Seed cultures of cv. Panniyur-1 in Whites medium showing direct somatic embryogenesis (e) at radicle end.

D. Separation of somatic embryos from the seed (S) and their germination in Whites medium

FIG.7.2



7.3.2. Culture of Seedling and Seedling derived explants

7.3.2.1 Whole Seedling Culture: Whole seedlings of cv. Panniyur-1 were cultured in liquid or solid half MS medium supplemented with BA (0.5, 2.0 and 5.0 mg l⁻¹).

Whole seedlings cultured in solid and liquid media responded differentially. After 20 d in culture, BA at the concentration of 0.5 mg l⁻¹ supported only the growth and elongation of seedlings in solid half MS medium (Fig. 7.3A), as against the multiple shoot formation at the shoot apex in liquid half MS medium with BA 0.5 mg l⁻¹ (Fig. 7.3B). In the liquid medium, BA at 2.0 mg l⁻¹ induced shoot formation at the shoot apex and callusing (Fig. 7.3C), whereas in solid medium same concentration of BA induced formation of adventitious shoot buds at the nodal region (Fig. 7.3D). BA at 5.0 mg l⁻¹ caused callus formation in liquid as well as solid media (Figs. 7.3E, F).

The shoot-buds induced in solid medium supplemented with BA at 2.0 mg l⁻¹ elongated further in the same medium after two subcultures i.e. 30 days (Fig. 7.4A). The solid medium was found to be beneficial for whole seedling culture than liquid medium because, total number of shoots produced from cultured seedlings was highest in solid half MS (Table 7.6). The number of shoots formed in solid medium at the two BA concentrations (2.0, 5.0) from nodes of whole seedlings differed significantly (Table 7.6). The quality of shoots in solid medium was better than those in liquid medium. The shoots in liquid medium were vitreous and weak. Culture of whole seedlings was proven to be advantageous because of rapid morphogenetic response and ease in handling the explants. Further it did not require rooting step.

Table 7.6 Effect of physical state of medium and BA on regeneration of shoots from whole seedling explants of Panniyur-1.

Medium	BA (mg l ⁻¹)	Explants responding (%) Mean ± s.d.		No. of shoots per explant Mean ± s.d.	
		Shoot apex	Node	Shoot apex	Node
Half MS (Solid)	2.0	-	60.0 ± 10.0	-	6.1 ± 1.9 ^a
	5.0	-	80.0 ± 10.0	-	3.6 ± 1.3 ^b
Half MS (Liquid)	2.0	90.0 ± 10.0	-	5.5 ± 1.5 ^{ab*}	-
	5.0	100.0 ± 0.0	-	-	-

Values followed by same superscript do not show significant differences at 95% confidence level

*: vitrified shoots. ND: not done.

Fig. 7.3

A. Elongation of the seedling of cv. Panniyur-1 in half MS solid medium.

B. Shoot multiplication at the tip resulting in rosette-like structure in whole seedling culture of cv. Panniyur-1 in half MS liquid medium

C. Formation of nodal structures (Arrow) in solid half MS with BA 2.0 mg l^{-1}

D. Vitrification of shoot apical structures and callus induction in liquid half MS with BA 2.0 mg l^{-1}

E. Dedifferentiation of nodal structures in solid half MS with BA 5.0 mg l^{-1}

F. Dedifferentiation of nodal structures in liquid half MS with BA 5.0 mg l^{-1}

FIG.7.3

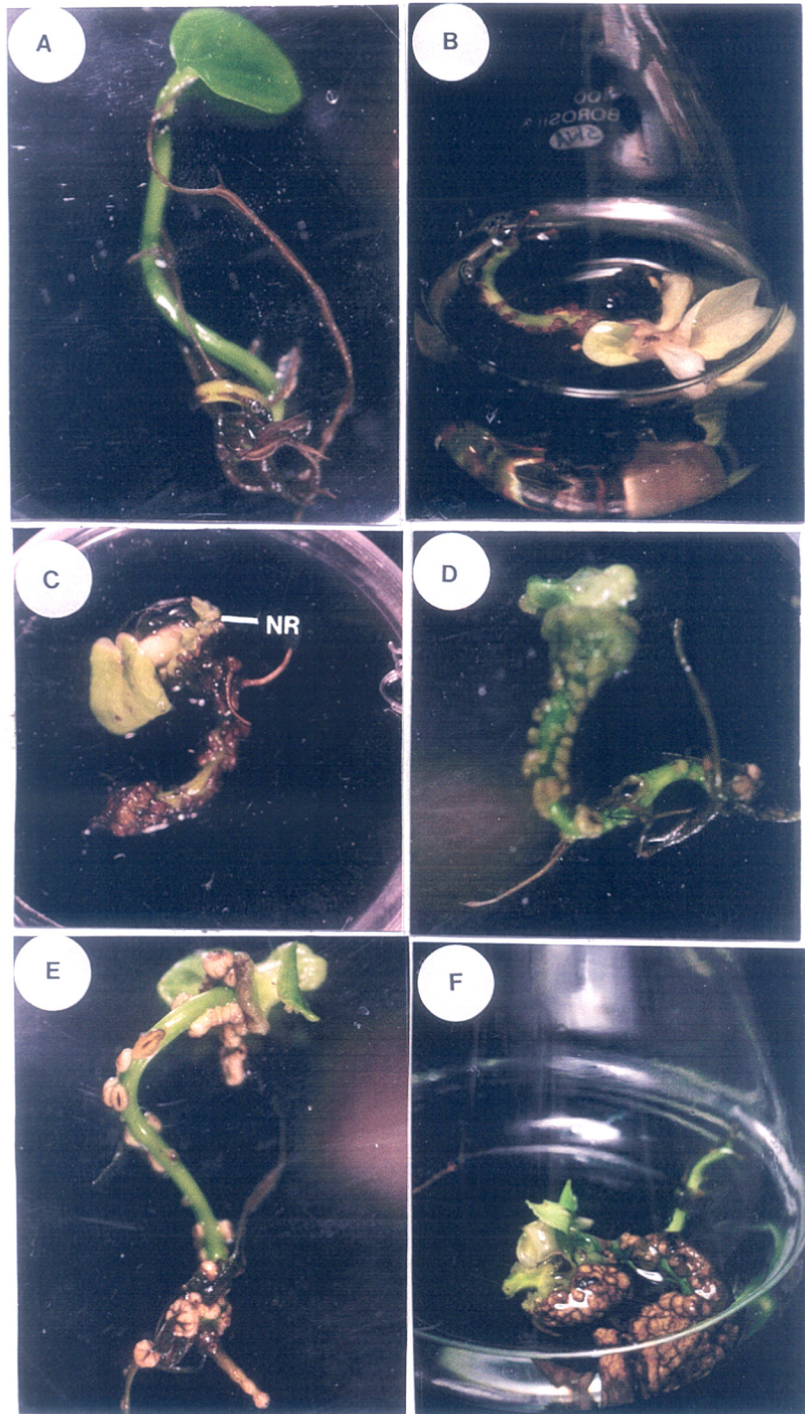


Fig.7.4

A. Elongation of nodal multiples in solid half MS medium

FIG.7.4



7.3.2.2 Culture of shoot apex explants: Shoot apices belonging to cv. Panniyur-1, Karimunda, Kuthiravally and Kuching were excised from seedlings and cultured in liquid half MS with BA (0.0, 0.5, 2.0 and 5.0 mg l⁻¹).

The shoot tip explants (Fig. 7.5A) cultured in half MS with BA (0.0 and 0.5 mg l⁻¹) resulted in single shoot formation (Fig. 7.5B) as against the results in whole seedling explants. The shoot apices cultured in half MS medium with BA at 2.0 and 5.0 mg l⁻¹ formed multiple shoots in all the four cultivars (Figs. 7.5C, D; Table 7.7). The highest number of shoots was obtained in cv. Kutching in BA 3.0 mg l⁻¹ whereas in cv. Kuthiravally, the lowest number of shoots was obtained in BA 5.0 mg l⁻¹. But BA at 5.0 mg l⁻¹ was found to inhibit shoot formation in cv. Kutching. Thus the shoot tip explants of this cultivar appeared to be very sensitive to the BA concentration. Among the four cultivars, the differences in number of shoots formed were not significant except Kutching in BA 3.0 mg l⁻¹. The differences within cultivars for number of shoots formed at the two BA treatments were insignificant for cv. Panniyur-1 and Karimunda. But significant differences were observed between the two BA treatments for number of shoots formed in cv. Kuthiravally (Table 7.7).

The multiple shoots induced in half MS with BA 2.0, 5.0 mg l⁻¹ were elongated in half MS basal medium. These shoots were separated and used for further culture work and not rooted or hardened.

Fig. 7.5

A.Shoot apex explant of cv. Panniyur-1.

B. Single shoot elongation from shoot apex explant in half MS of cv. Panniyur-1.

C. Induction of shoot buds at the apex in half MS with BA 5.0 mg l^{-1} in cv. Kuthiravally.

D. Induction of shoot buds at the apex in half MS with BA 5.0 mg l^{-1} in cv. Kuching

FIG. 7.5

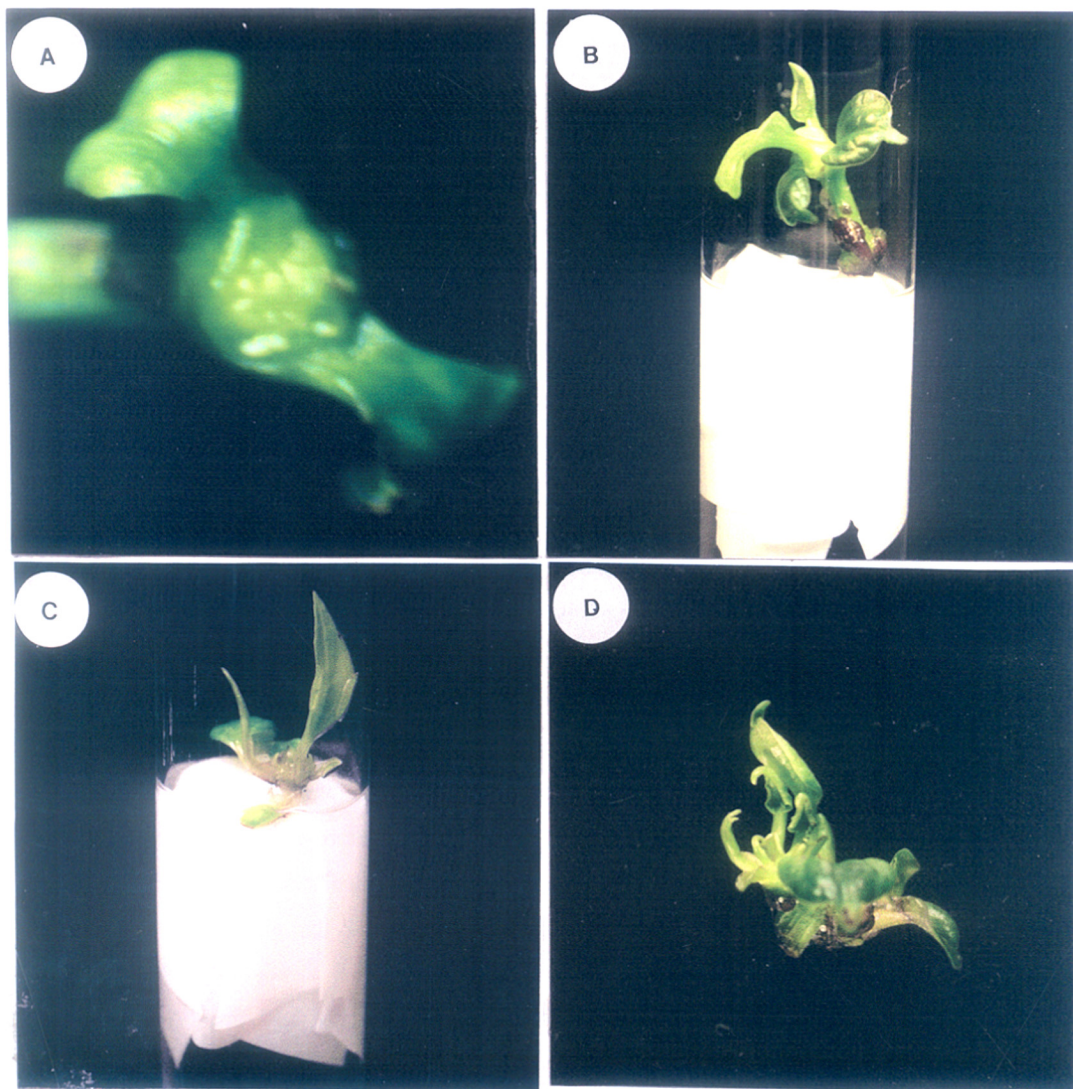


Table 7.7 Effect of BA on shoot tip explants of four cultivars of *P. nigrum* cultured in half MS medium.

Cultivar	BA (mg l ⁻¹)	% Response for shoot induction Mean $\pm$ S.D.	No. of shoots/explant Mean $\pm$ S.D.
Panniyur-1	2.0	66.6 $\pm$ 12.0	5.3 $\pm$ 2.8 ^a
	5.0	23.3 $\pm$ 4.7	4.3 $\pm$ 1.5 ^a
Karimunda	2.0	53.3 $\pm$ 4.7	4.3 $\pm$ 1.6 ^a
	5.0	24.3 $\pm$ 10.2	4.2 $\pm$ 1.3 ^a
Kuthiravally	2.0	50.0 $\pm$ 4.8	5.5 $\pm$ 2.1 ^a
	5.0	25.0 $\pm$ 4.0	2.6 $\pm$ 0.7 ^b
Kuching	2.0	30.0 $\pm$ 10.0	7.1 $\pm$ 2.1 [#]
	5.0	-	-

Differences significant at 95% confidence level in number of shoots formed per explant in 2 concentrations of BA in the same cultivar are denoted by different letters.

[#] Differences in number of shoots formed per explant among cultivars are significant at 95% confidence level., - no response.

7.3.2.3 Culture of nodal explants: The nodal explants were belonging to seedlings of cv. Panniyur-1, Karimunda, Kuthiravally and Kuching were excised and cultured in liquid half SH and liquid half MS media supplemented with BA (0.0, 0.5, 2.0 and 5.0 mg l⁻¹). The nodal explants cultured in half SH medium supported shoot bud induction in presence as well as absence of BA.

All the concentrations of BA supported shoot bud formation in form of a ring at the nodal region (Figs. 7.6A, B, C; Figs. 7.7A, B, C, D; Table 7. 8). For half MS the BA at 0.0 and 0.5 mg l⁻¹ supported only the single shoot elongation whereas shoot bud induction was restricted to BA at 2.0 and 5.0 mg l⁻¹.

The shoot buds originated from nodal explants in 6 media (Table 7.8) when subcultured in half SH medium after 4 weeks converted into shoots (Figs. 7.8A, B, C) within 5-8 weeks. But only the shoot buds from half SH medium with BA at (0.0, 0.5 mg l⁻¹) and half MS with BA 2.0 and 5.0 mg l⁻¹ could be converted into shoots on subculture.

The percentage response of cv. Karimunda for shoot formation was the lowest in half SH basal medium. But the highest number of shoots per nodal explant was obtained in the same cultivar though it was not significantly higher than the other cultivars (Table 7.8). Intravarietal comparison showed that each cultivar scored higher number of shoots

Fig. 7.6

A. Formation of nodal structures in cv. Kuthiravally in half SH basal medium

B. Formation of nodal structures in cv. Panniyur-1 in half SH medium with BA 0.5 mg l^{-1} .

C. Formation of nodal structures in cv. Panniyur-1 in half MS medium with BA 3.0 mg l^{-1} .

FIG. 7.6

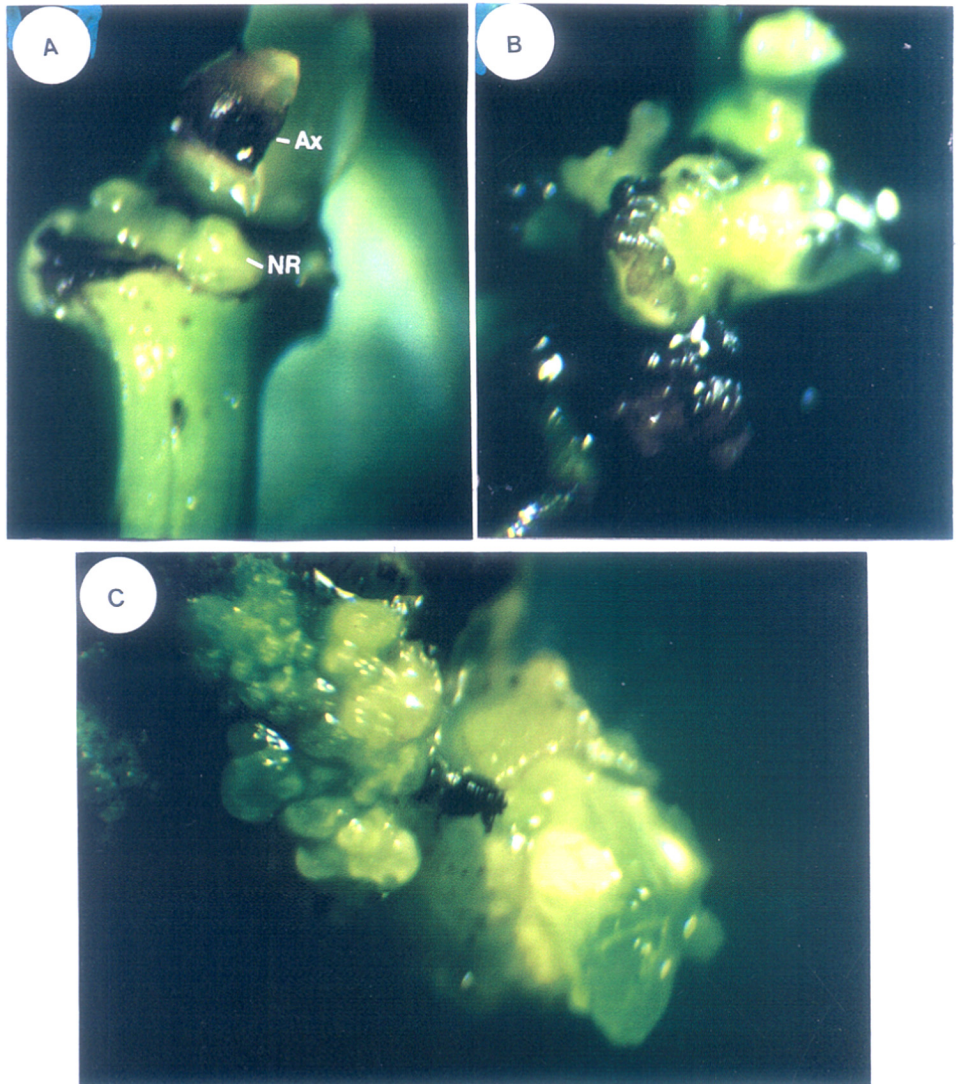


Fig. 7.7

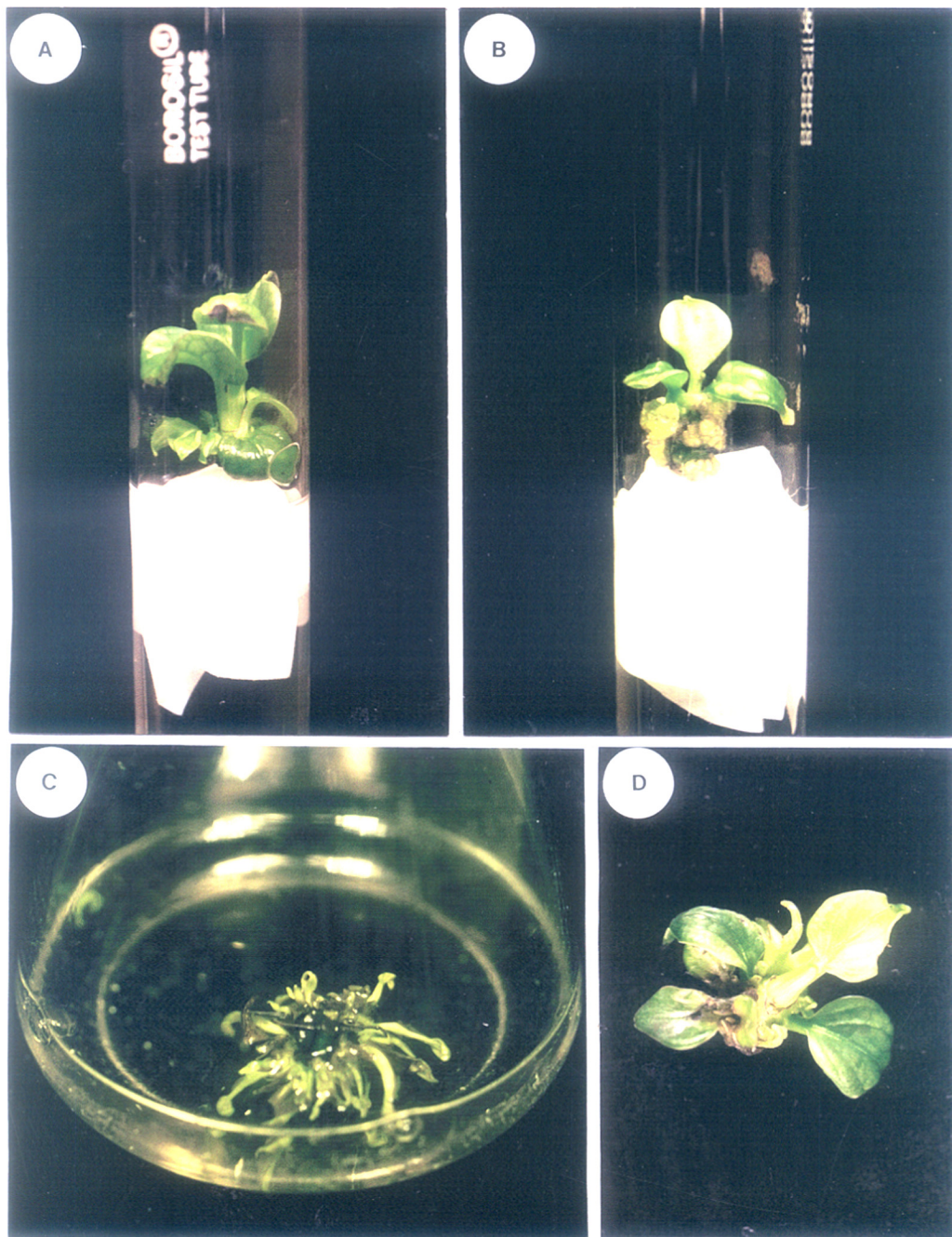
A. Conversion of nodal structures into shoots in half SH medium in cv. Karimunda

B. Conversion of nodal structures into shoots accompanied by callus formation in half SH medium in cv Panniyur-1.

C. Conversion of nodal structures into shoots in half SH medium in cv. Kuching

D. Conversion of nodal structures into shoots in half SH medium in cv. Kuthiravally.

FIG.7.7



in half SH supplemented with BA 0.5, than in half SH basal. The same was found to be true for percentage response except cv. Kuching. The differences among the cultivars for number of shoot formed per explant were not significant (Table 7.8).

In half MS with BA 2.0 mg l⁻¹, the number of shoots formed per explant was uniform in all cultivars and it was significantly higher compared to medium with BA at 5.0 mg l⁻¹ for all cultivars but for cv. Karimunda BA at 5.0 mg l⁻¹ was suboptimal for number of shoots formed per explant. This was reflected from the reduction in percentage response for shoot formation as well.

Thus the nodal explants responded differentially pertaining to the Basal media and concentrations of BA. Relationship of media composition and plant growth regulators with respect to morphogenetic induction has been discussed by Preece, (1995). In this article he has suggested that medium components can substitute for the plant growth regulators at least partially if not completely and *vice-versa*. He has supported this assumption with numerous examples where choice of basal medium influenced regeneration rather than plant growth regulators at different concentrations and in combinations did.

Results presented in Table 7.8 are in agreement with this assumption. In all the 4 cultivars of black pepper half SH basal medium was competent for shoot bud induction in nodal explants. Supplementation of half SH medium with of BA at all concentrations (0.5, 2.0 and 5.0 mg l⁻¹) enhanced the number of shoots per explant. But BA at higher concentrations though produced numerous buds, inhibited elongation. However, half MS medium was not sufficient for shoot bud induction and this deficiency in action of was complemented by BA at higher concentrations (2.0, 5.0 mg l⁻¹) that resulted in shoot bud induction comparable to half SH with BA (0.0, 0.5 mg l⁻¹).

Table 7.8 Effect of basal media and BA on adventitious shoot induction in nodal explants of 4 cultivars of *P. nigrum* L.

Basal Medium	BA (mg l ⁻¹)	Cultivar	Explants responding (%)	No. of shoots per explant
Half SH	0.0	Panniyur-1	23.3 ± 10.0	4.2 ± 1.1 ^a
		Karimunda	13.3 ± 8.0	5.8 ± 1.3 ^{ab}
		Kuthiravally	30.0 ± 10.0	3.6 ± 1.2 ^a
		Kuching	33.3 ± 6.0	3.0 ± 0.6 ^a
	0.5	Panniyur-1	47.0 ± 3.0	6.2 ± 1.1 ^{ab}
		Karimunda	50.0 ± 0.0	9.6 ± 2.5 ^b
		Kuthiravally	57.0 ± 3.0	6.2 ± 1.1 ^{ab}
		Kuching	31.5 ± 1.5	9.2 ± 1.6 ^b
	2.0	Panniyur-1	37.6 ± 5.5	-
		Karimunda	24.3 ± 10.0	-
		Kuthiravally	20.0 ± 0.0	-
		Kuching	23.3 ± 4.7	-
	5.0	Panniyur-1	75.0 ± 5.0	-
		Karimunda	59.0 ± 9.0	-
		Kuthiravally	56.6 ± 4.0	-
		Kuching	29.0 ± 7.0	-
Half MS	0.0	Panniyur-1	-	-
		Karimunda	-	-
		Kuthiravally	-	-
		Kuching	-	-
	0.5	Panniyur-1	-	-
		Karimunda	-	-
		Kuthiravally	-	-
		Kuching	-	-
	2.0	Panniyur-1	35.0 ± 5.0	9.2 ± 1.6 ^b
		Karimunda	24.4 ± 2.2	9.6 ± 1.0 ^b
		Kuthiravally	45.0 ± 5.0	8.0 ± 2.2 ^b
		Kuching	46.6 ± 3.3	10.0 ± 1.0 ^b
	5.0	Panniyur-1	25.0 ± 5.0	3.4 ± 1.0 ^a
		Karimunda	24.9 ± 8.3	5.8 ± 1.1 ^{ab}
		Kuthiravally	26.1 ± 1.1	4.6 ± 1.0 ^a
		Kuching	14.4 ± 2.2	3.2 ± 0.7 ^a

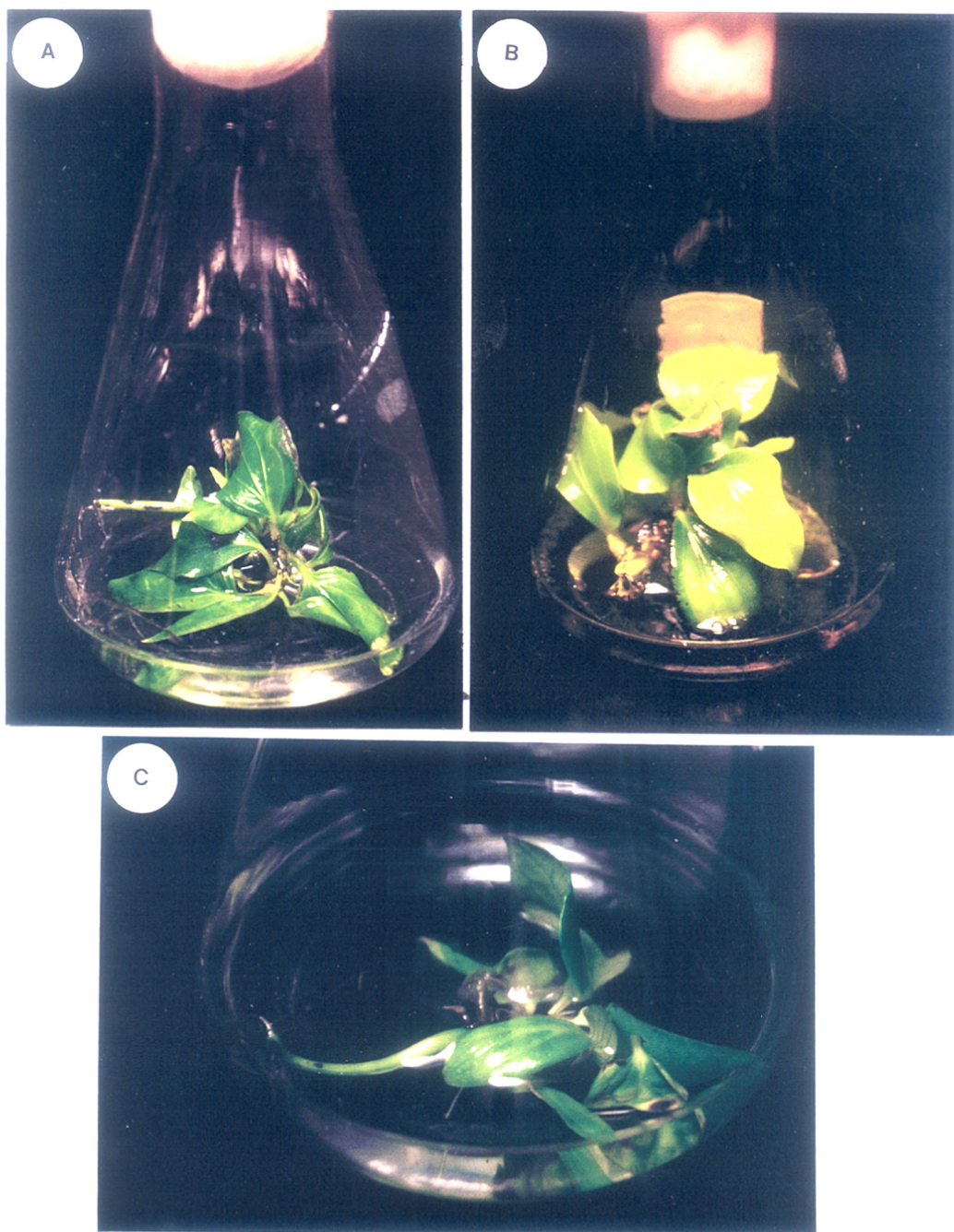
Values followed by same superscript do not show significant differences at 95% confidence level.

-: no response

Fig. 7.8

A-C Elongation of shoots in half SH in cv. Kuching, Kuthiravally, Karimunda respectively

FIG. 7.8



The shoot buds were elongated into shoots in half SH medium. (Fig. 7.8A, B, C). The phase of medium not only influenced the shoot bud induction but also the elongation of shoots. The nodal explants showing conversion shoot buds into shoots were transferred to liquid as well as solid half SH medium for elongation. The elongating shoots in liquid medium showed two-fold increase in height within 5 weeks as compared to the buds in solid medium (Figs. 7.9A, B; Table 7.9).

Table 7.9. Effect of physical state of subculture medium on elongation of *in vitro* shoots in 4 cultivars of *P. nigrum* L.

Medium	Height of plantlets (cm) Mean $\pm$ s.d.			
	Panniyur-1	Karimunda	Kuthiravally	Kuching
Half SH (Liquid)	7.5 $\pm$ 1.7 ^{a*}	7.5 $\pm$ 0.5 ^{a*}	8.25 $\pm$ 1.0 ^{a*}	7.0 $\pm$ 1.6 ^{a*}
Half SH (Solid)	3.25 $\pm$ 0.6 ^b	3.5 $\pm$ 0.4 ^b	3.1 $\pm$ 0.6 ^b	3.5 $\pm$ 0.9 ^b

* Differences between treatments for each cultivar were significantly higher at 95% confidence level. Values followed by same letter denote that differences among the cultivars for shoot length for each treatment were not significant at 95% confidence level.

Suitability of the liquid medium for shoot induction could be attributed to better uptake of BA in liquid medium (Bornmann and Vogelmann, 1987) whereas the better elongation in the liquid media could be due to higher availability of the medium through increased surface area exposed to medium.

The elongated shoots rooted in the same medium (half SH) within 4 weeks and were transferred to soil after reaching height of 4-8 cm. (Fig. 7.9C) and were acclimatized using the standardised procedure (Chapter 2).

Histology: The histology of nodal explants forming shoots in all the cultivars established the direct pathway of organogenesis. The sections passing through the nodal explant (Figs. 7.10 A, B) showing nodal ring formation in cv. Panniyur-1 and Kuthiravally respectively, represent the histological characters of all the four cultivars. Fig. 7.10A shows the asynchronous development of shoot buds without any callus formation. Fig. 7.10B shows an axillary sprout along with small adventitious shoot buds, all showing distinct vascular connection with the explant. The origin of adventitious shoot buds is subepidermal. The growth of adventitious shoot buds is asynchronous exhibiting buds in different stages of elongation.

Fig. 7.9

A-B. Effect of solid and liquid half SH medium on elongation of *in vitro* shoots in cv. Panniyur-1.

C. Acclimatization of cv. Panniyur-1 shoot.

FIG. 7.10

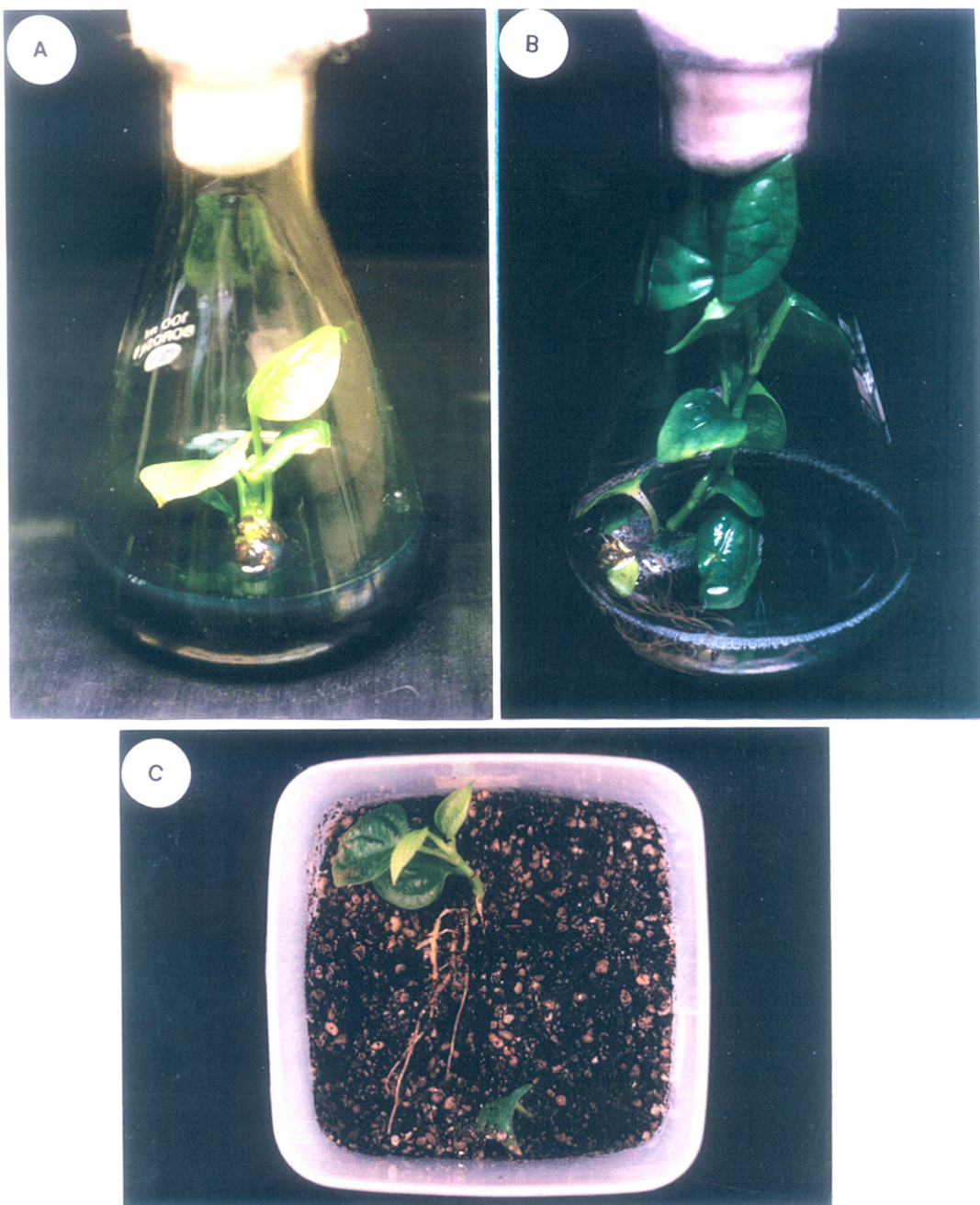
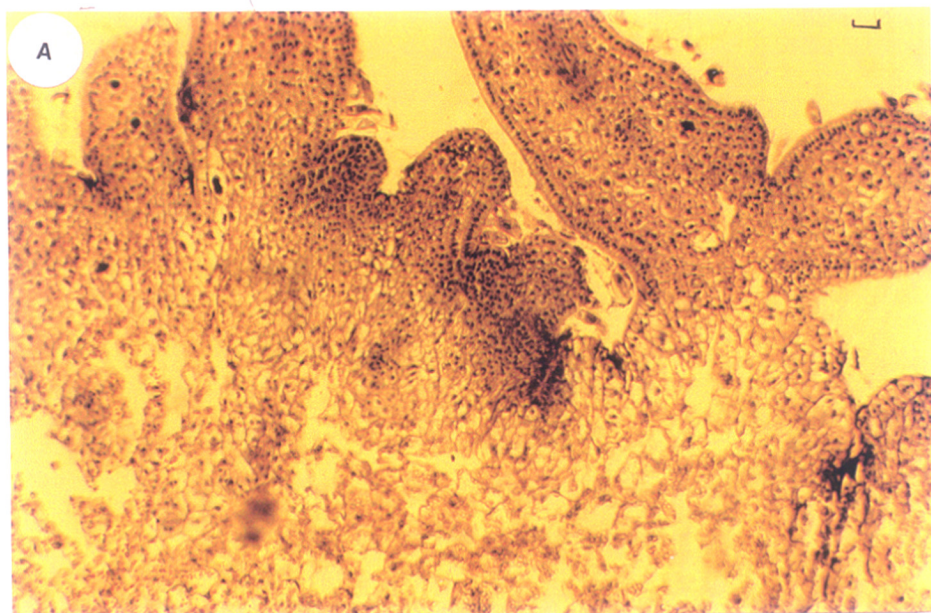


Fig. 7.10.

A. T.S. passing through nodal ring of cv. Panniyur-1 showing asynchronous development of shoot buds Bar = 0.16 mm

B. A part of V. S passing through de novo shoot developed in cv. Kuthiravally.
Bar = 0.2 mm

FIG.7.9



7.3.3 Embryo culture: Based on the previous report (Joseph, *et al.*, 1996) of somatic embryogenesis in cv. Panniyur-1 and Karimunda, embryos excised from seeds of these two cultivars were cultured in half SH in solid as well as liquid phase. The effect of half SH on embryonal explants was compared with other media i.e. Full SH, WPM, Whites medium, B5 and half MS medium. Liquid half SH enhanced the percentage of callus formation considerably.

The protocol for somatic embryogenesis reported by Joseph, *et al.*; (1997) for cv. Panniyur-1 and Karimunda could not be implemented in our laboratory since half SH with 1.5% sucrose did not support formation of somatic embryos. However we achieved somatic embryogenesis in both the cultivars in full SH and Whites medium. All the other basal media namely, half SH, B5, WPM, and half MS after 6-8 weeks in culture, supported either germination of the zygotic embryos (Fig. 7.10A) or non-embryogenic callus formation or both (Figs. 7.10B, C) in embryonal explants of both cultivars with the exception of B5 for cv. Karimunda (Table 7.10).

The number of explants forming somatic embryos in either full SH or Whites medium (Table 7.10) was very low as compared to Joseph *et al.* (1997). The embryonal explants responding for somatic embryogenesis formed yellowish callus in 6-8 weeks after culture. This callus in the medium formed globular structures (Fig. 7.11A). These globular structures converted into cotyledonary stage structures in 12-15 days (Fig. 7.11B). The number of globular embryos converted into cotyledonary structures was very less (Table 7. 10). The cotyledonary structures turned green if incubated in light at the intensity of $38 \mu\text{E m}^{-2}\text{s}^{-1}$ and at 16 h photoperiod (Fig. 7.11C) Well developed cotyledonary structures were transferred to fresh medium after 2 weeks where they germinated to form plantlets under dark as well illuminated conditions mentioned above (Fig. 7.12 A, B).

Table 7.10 Effect of different basal media on *in vitro* response of embryos of cv. Panniyur-1 and cv. Karimunda of *P. nigrum* L.

Medium	Response	Mean Percentage response	
		Panniyur-1	Karimunda
Half SH (solid)	Callus	63.55	79.4
	Somatic embryos	-	-
Half SH (liquid)	Callus	28.9	23.3
	Somatic embryos	-	-
Full SH (solid)	Callus	50.0	48.0
	Somatic embryos	8.0 (2/25)	15.0 (3/20)
WPM (solid)	Callus	40.0	30.0
	Somatic embryos	-	-
B5 (solid)	Callus	20.0	-
	Somatic embryos	-	-
Half MS (solid)	Callus	25.0	10.0
	Somatic embryos	-	-
White's (solid)	Callus	35.0	50.0
	Somatic embryos	15.0 (3/20)	12.0 (3/25)

* Figures in parenthesis represent number of explants responding for somatic embryogenesis.

Different modifications of SH medium were used for induction of somatic embryogenesis in zygotic embryos of cv. Panniyur-1 and cv. Karimunda. All these modifications also resulted in formation of only callus and not somatic embryogenesis. The callus formation some times occurred in the whole embryo or only at root pole (Figs. 7.13A, B) The percentage of callus formation differed in different media. Percentage response for callus formation in cv. Panniyur-1 was significantly low in combination 5 i.e. in absence of potassium nitrate. All the other modifications of SH medium yielded comparable results. In cv. Karimunda combinations 5, 8 and 9 exhibited significantly low percentage of callus formation. Thus absence of potassium nitrate, microelements and vitamins resulted in decreased callus formation in cv. Karimunda.

Fig. 7.11.

A. Globular stage somatic embryos developed from embryonal explants in cv. Panniyur-1 in Whites medium.

B. Cotyledonary stage somatic embryos developed from embryonal explants in cv. Panniyur-1 in half SH medium.

C. Cotyledonary stage somatic embryos developed from embryonal explants in cv. Karimunda in half SH medium.

FIG. 7.11

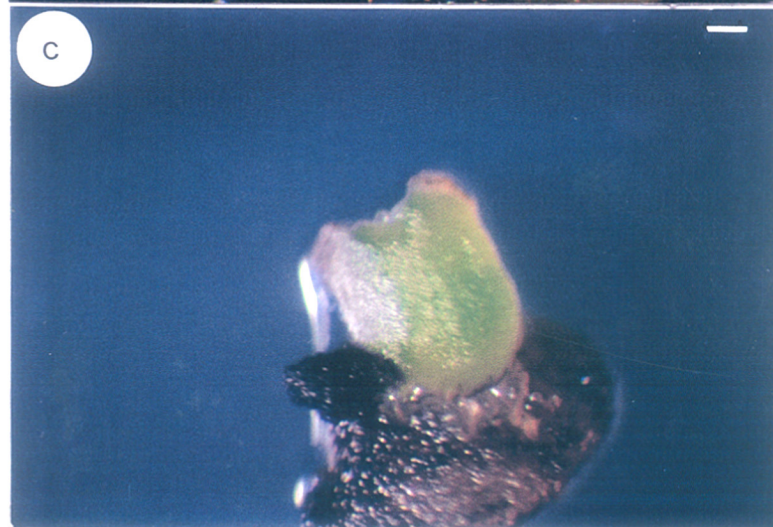
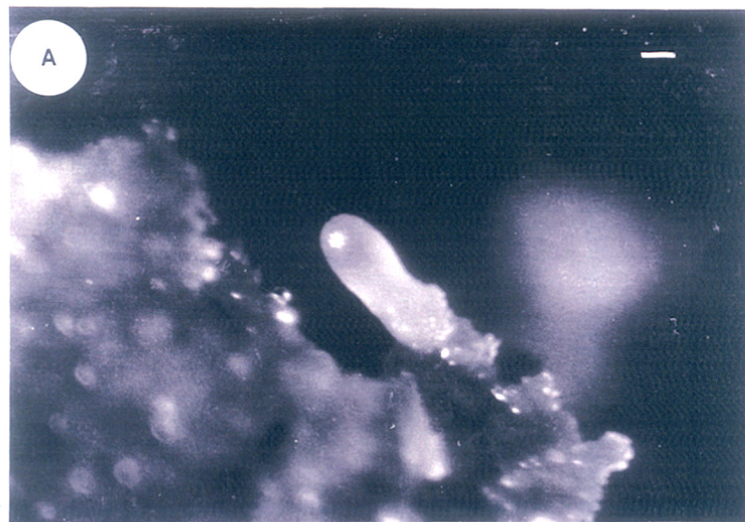


Fig. 7.12

A. Germination of somatic embryos developed from embryonal explants in cv. Panniyur-1 in half SH medium.

B. Germination of somatic embryos developed from embryonal explants in cv. Karimunda in half SH medium

FIG. 7.12

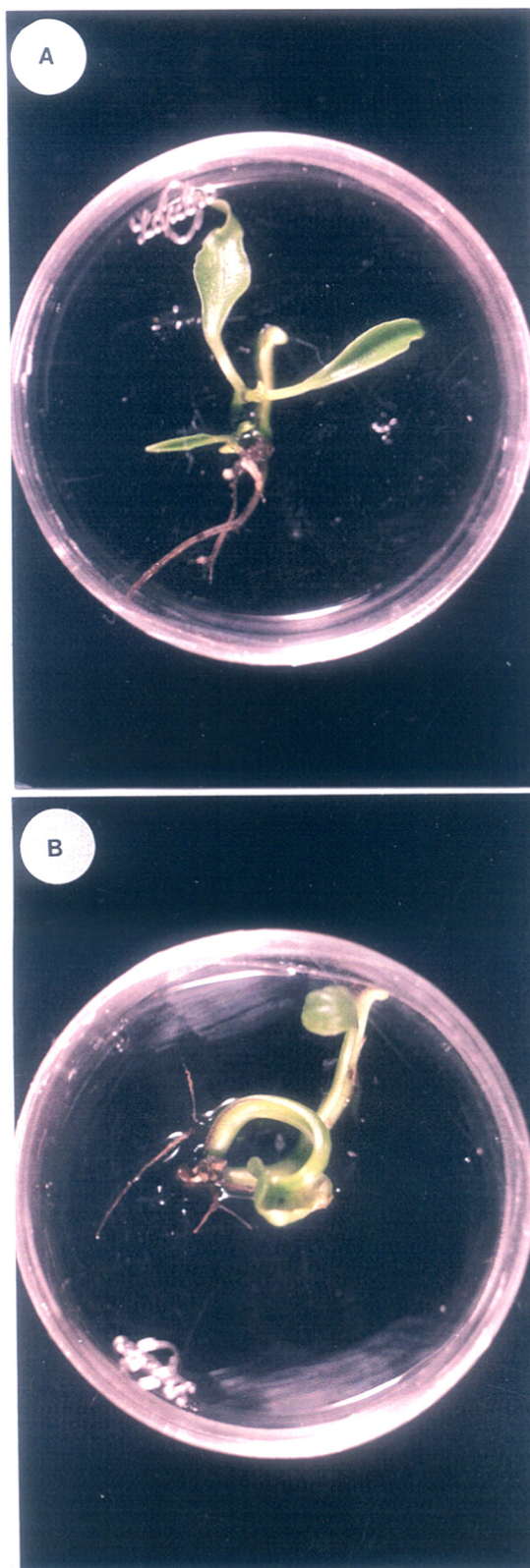


Fig. 7.13.

A. Germination and elongation of zygotic embryo of cv. Panniyur-1

B-C. Callus induction in the germinated zygotic embryos in cv. Panniyur-1 and cv. Karimunda.

FIG. 7.13

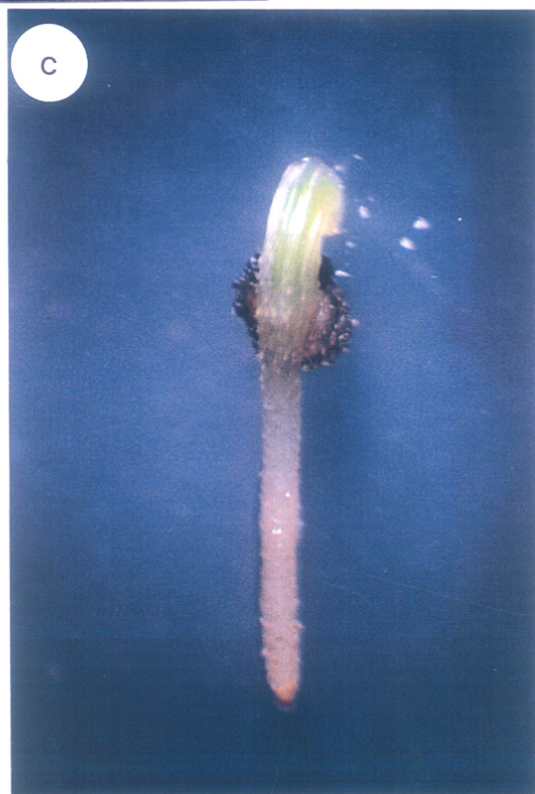
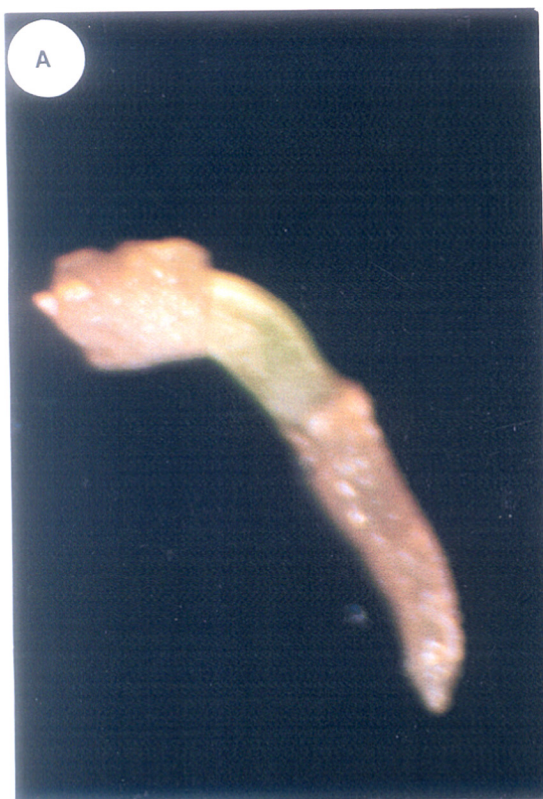


Table 7.11 Effect of different modifications of SH medium on embryo culture in cv. Panniyur-1 and cv. Karimunda of *P. nigrum* L.

Medium No.	Explants forming callus (%)	
	Panniyur-1	Karimunda
1	40.0 ± 0.0	65.0 ± 10.0
2	43.3 ± 10.0	48.9 ± 11.0
3	50.0 ± 10.0	50.0 ± 10.0
4	60.0 ± 10.0	40.0 ± 0.0
5	30.0 ± 1.0	30.0 ± 0.0
6	50.0 ± 10.0	50.0 ± 0.0
7	50.0 ± 10.0	70.0 ± 10.0
8	50.0 ± 10.0	30.0 ± 10.0
9	50.0 ± 10.0	20.0 ± 0.0
10	60.0 ± 0.0	50.0 ± 10.0

The positive effect of higher sucrose concentration on somatic embryogenesis is well documented (Thorpe, 1994; Lu *et al.*, 1983) that could be due to osmotic stress (Litz, *et al.*, 1986; Kamada, *et al.*, 1986). But Joseph, *et al.*, (1997) have reported 1.5% sucrose to be optimal for embryogenesis in black pepper. We found that increase in sucrose concentration of half SH resulted in decrease in percentage of callus formation in cv. Karimunda and Panniyur-1 (Table 7.12). Subsequently, it increased the number of germinating zygotic embryos.

Table 7.12 Effect of sucrose concentration on callus induction in zygotic embryos of cv. Panniyur-1 and cv. Karimunda of *P. nigrum* L.

Sucrose Concentration (%)	Explants forming callus (%)	
	Panniyur-1	Karimunda
0.5	70.0 ± 10.0	90.0 ± 10.0
1.5	50.0 ± 10.0	40.0 ± 0.0
3.0	25.0 ± 5.0	30.0 ± 0.0
6.0	15.0 ± 5.0	25.0 ± 5.0

Fig. 7.14

A-B Callus formation in embryonal explants of cv. Panniyur-1 and cv. Karimunda in half SH medium

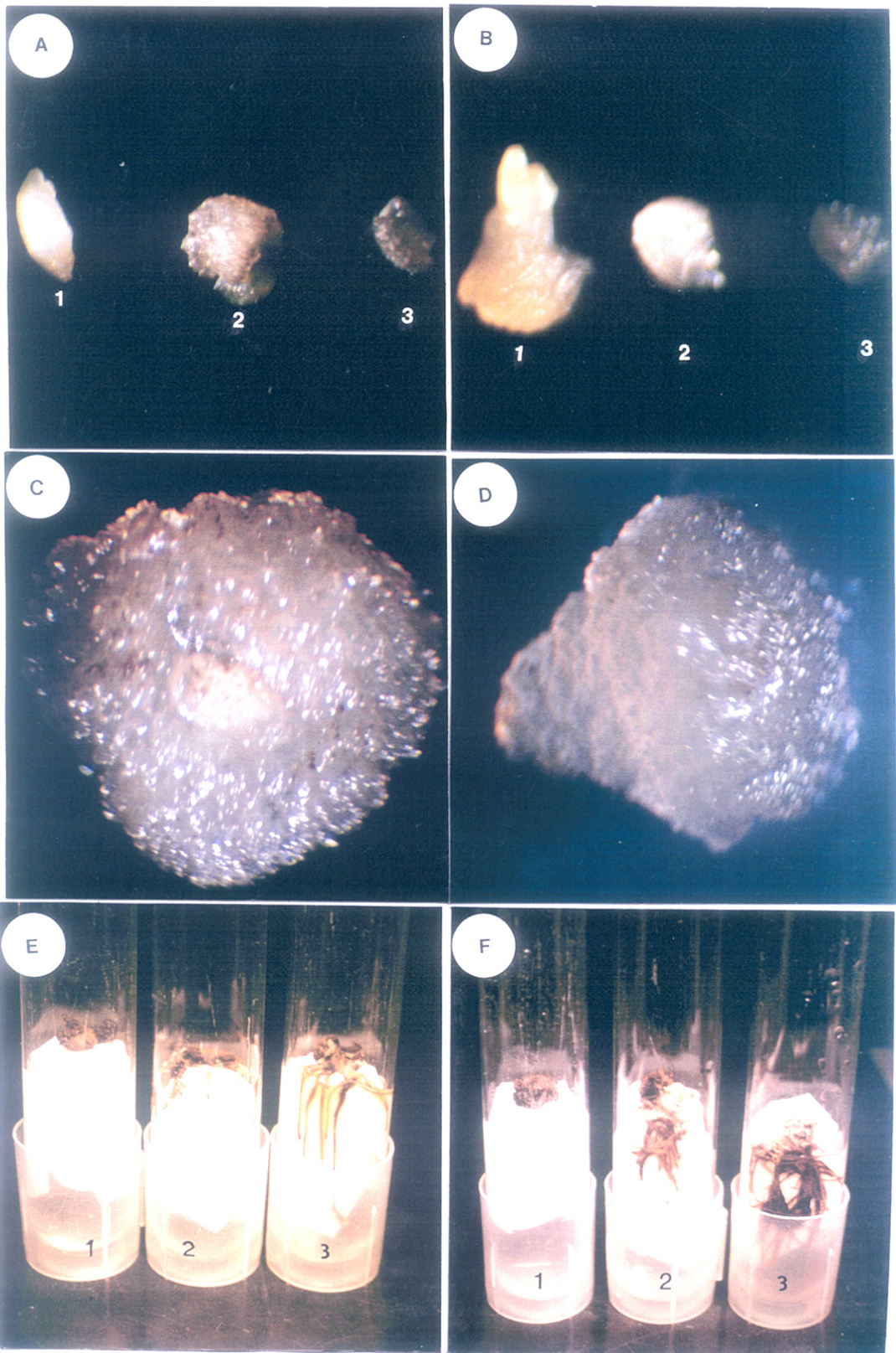
C. Callus formation in embryonal explants of cv. Panniyur-1 in half SH with NAA 1.0 mg l^{-1}

D. Callus formation in embryonal explants of cv. Panniyur-1 in half SH with 2,4-D 1.0 mg l^{-1}

E. Rhizogenesis in embryonal explants of cv. Panniyur-1 in half SH with NAA 0.5 and 1.0 mg l^{-1} Absence of root formation in control.

F. Rhizogenesis in embryonal explants of cv. Karimunda in 1/2 SH with 2,4-D 0.5 and 1.0 mg l^{-1} Absence of root formation in control.

FIG. 7.14



Auxins are considered to play important role in control of somatic embryogenesis (Halperin and Wetherell, 1964; Evans, *et al.*, 1981; Sagare, *et al.*, 1995). However in black pepper embryos NAA as well as 2,4-D (0.5, 1.0 mg l⁻¹) induced only callus formation in both the cultivars, Karimunda and Panniyur-1 (Figs.7.14C, D Table 7.12). The callus formed in these two hormones proliferated within one week and in two weeks time the calli turned brown and profuse root formation occurred in the calli (Figs.7.14E, F). The two cultivars showed different preferences to the two auxins. Auxin 2,4-D at both the concentrations evoked higher callus formation response than NAA in cv. Karimunda. In contrast, NAA induced higher percentage of callus formation than 2,4-D in embryonal explants of cv. Panniyur-1 (Table 7.12). Joseph, *et al.*, (1997) reported callus formation in auxin containing media, but the rhizogenesis was not reported.

Table 7.12 Effect of auxins on callus induction in embryos of cv. Panniyur-1 and Karimunda of *P. nigrum* L.

Auxin	Conc (mg l ⁻¹)	Explants forming callus (%)	
		Panniyur-1	Karimunda
2,4-D	0.5	58.0 ± 10.2	100.0 ± 0.0
	1.0	40.0 ± 07.9	100.0 ± 0.0
NAA	0.5	78.8 ± 13.4	70.0 ± 10.0
	1.0	86.6 ± 11.8	92.0 ± 8.0

The emblings derived from somatic embryos however were lost in contamination and could not be hardened.

7.4. Conclusions: The conditions were standardized for *in vitro* seed germination in black pepper and it was found that dark incubation, Whites medium without major elements or sterile distilled water, use of cotton as support material or agar as gelling agent supported higher percentage of *in vitro* seed germination. Intervarietal differences were observed for the parameters of *in vitro* seed germination optimized for cv. Panniyur-1 seeds. The culture of seeds did not instigate response of multiple shoot formation but in rare cases resulted in somatic embryogenesis.

Solid half MS medium was found to be beneficial for culture of whole seedlings. In solid half MS medium supplemented with BA 2.0 and 5.0 mg l⁻¹ resulted in formation of multiple shoots whereas in half MS with BA at 0.0 and 0.5 mg l⁻¹ single shoots were formed. The liquid medium though supported multiple shoot formation, the shoots were vitreous.

Culture of shoot apices in half MS supplemented with BA (2.0 and 5.0 mg l⁻¹) resulted in formation of multiple shoots.

A part of this Chapter is communicated as a paper entitled 'In vitro seed germination in black pepper, *Piper nigrum* L.' by SM kelkar and KV Krishnamurthy to the journal 'Current Science'.

SUMMARY

Diseases and pests have remained the major threat to agro-based economy due to their potential for parallel evolution and versatile genomes. They cause extensive yield and crop loss that could result in historical phenomena like 'Great Potato Famine' caused by the fungus *Phytophthora infestans* in 1845. For effective control of diseases and pests, the conventional methods must be supplemented with modern techniques. The use of biotechnology would provide an opportunity to bring about improvement in a large number of perennial crops that have been relatively inaccessible to the conventional techniques. though the woody perennials are difficult to improve, owing to their economic importance, the attempts could be particularly rewarding.

Black pepper (*Piper nigrum* L) is one of such perennial crops which is valued for its culinary as well as medicinal properties. The cultivation of black pepper supports economy of tropical countries such as India, Brazil, Indonesia, Malaysia and Sri Lanka. However its susceptibility to diseases like slow wilt and quick wilt and pests like nematodes has posed a serious threat to the pepper economy all over the world. The lack of intrinsic variation for disease and pest resistance among genotypes of black pepper has forced to obtain variability from outside sources such as related species. Extensive screening of *Piper* germplasm has discovered the multiple resistance potential of *Piper colubrinum*, a wild species native to Brazil. This species is totally resistant to the pathogen complex comprised of *Phytophthora capsici* causing quick wilt, *Fusarium solani* cf. *piperi* causing slow wilt and the plant parasitic nematodes *Meloidogyne incognita* and *Radopholous similis*. Acquisition of wilt resistance in *Piper nigrum* by conventional methods like hybridization or use of *Piper colubrinum* as a root stock has not been successful. The application of modern techniques such as protoplast mediated distant hybridization provide an alternative strategy to transfer multiple resistance trait/s from wild pepper to black pepper or developing disease and pest resistant black pepper by introducing alien genes. The work presented here was initiated to fulfill some of basic pre-requisites of biotechnological endeavors in black pepper improvement. These pre-requisites include protocols for *in vitro* regeneration and standardization of parameters for protoplast and transformation studies.

In vitro culture of different explants of *P. spp* was initiated with the aim of

developing regeneration systems. However severe contamination by fungi and bacteria hampered all the initial attempts to establish sterile cultures especially in *P. nigrum* cultivars. Browning of explants and media due to phenolics resulted in further increase in mortality percentage of the cultures in both the species. Hence systematic study was carried out to identify the contaminants, to define the source of contaminants, and to control the contaminants. Most of the contaminants isolated were of plant origin. Hence the control measures were applied at the level of the plant and explant. It was found that use of 1% bavistin (aq. solution) for 2h as a pretreatment in combination with Gentamicin (200 mg l^{-1}) followed by 10 min surface sterilization with 0.1% mercuric chloride with or without vacuum reduced fungal as well as bacterial contamination in nodal explants of *Piper* spp from 80-90% to 30-40 %. Same procedure for half the time duration gave satisfactory results for leaf and shoot apex explants. The use of MS medium at half strength of major elements was the most successful strategy followed for control of phenolics.

After standardizing the surface sterilization protocol, morphogenetic potential of different explant types of wild pepper (*P. colubrinum*) was assessed by culturing root, inflorescence, leaf and internode explants in different basal media and different plant growth regulators. Most of the concentrations and combinations of Auxins (NAA, 2,4-D, 2,4,5-T, Dicamba and Picloram) and cytokinins (BA, Kinetin , TDZ) resulted in formation of non-morphogenetic calli or roots. The root and inflorescence explants could not be induced for shoot formation. The leaf explants cultured in half MS with BA ($1, 2$ and 5 mg l^{-1}) with NAA or 2,4-D ($0.5, 1 \text{ mg l}^{-1}$) or with alone TDZ ($0.5, 1 \text{ mg l}^{-1}$) formed organogenetic calli which on subculture in half MS with BA formed shoot buds. The shoots were elongated and rooted in half MS with or without IAA or IBA ($0.5, 1 \text{ mg l}^{-1}$) and hardened. The internode explants on pulse treatment for 60 and 120 min with BA ($5, 10, 20 \text{ mg l}^{-1}$) formed shoots without callus formation. These shoots were elongated and rooted in half MS basal medium. The pre-existing apical and axillary meristems cultured in half MS with BA $0.5, 2.0$ and 5.0 mg l^{-1} produced multiple shoots from callus or directly. All the shoots were rooted *ex vitro* and hardened.

In vitro plants raised from leaf calli of wild pepper were further manipulated for

morphogenesis. Root, internode, leaf and petiole explants excised from these plants and cultured in half MS with BA (0.2, 0.5, 1, 5 and 10mg l⁻¹) formed adventitious shoots. These shoots were elongated in half MS with or without BA and rooted *ex vitro*. In another experiment it was found that reduction in culture period in medium with BA did not affect regeneration efficiency. These plants were used for protoplast isolation and transformation work. Mannitol (8%), an enzyme mixture of cellulase R-10 (2%) and macerozyme R-10 (0.5%) and incubation period for 12 h was found to be optimum for protoplast isolation from leaf explants of wild pepper. Under these conditions, a yield of 20-30 x 10⁴ protoplasts per gm leaf tissue with approximately 70 % viability was obtained. These protoplasts however did not divide after culture and degenerated eventually.

The leaf, internode and nodal explants derived from *in vitro* plants of wild pepper were subjected to Kanamycin sensitivity test. The internode explants were highly susceptible to adverse effects of Kanamycin were bleached at whereas Kanamycin at 50 mg l⁻¹. The leaf and node explants could survive Kanamycin upto 400 and mg l⁻¹ respectively. Co-cultivation with *Agrobacterium* however resulted in overgrowth of the bacteria which was fatal to the explants. Reduction in co-cultivation time did not result in control of bacterial growth. Thus genetic transformation could not be achieved.

In the cultivated species, *P. nigrum*, leaf, internode, node and shoot tip explants from 6-8 year old plants of three high yielding cultivars were cultured in different basal media with or without plant growth regulators. It was found that use of WPM for cultures controlled shoot tip necrosis.. For nodal explants there was no preference for basal medium. The shoot multiplication was achieved only in shoot tip and nodal explants whereas culture of leaf and intrnode explants resulted only in callus formation. In the shoot apex explants as well as in nodal sprouts, BA induced enhanced axillary sprouting and subsequent multiple shoot formation. In addition, in the nodal explants, BA induced adventitious shoot formation. TDZ also could induce adventitious shoot buds but the shoots were stunted and vitrified. Hence BA was found to be superior to TDZ. All the 3 cultivars showed uniform responses in culture. Half MS was found to be optimum for elongation of the shoots. The shoots were rooted *ex vitro* and hardened.

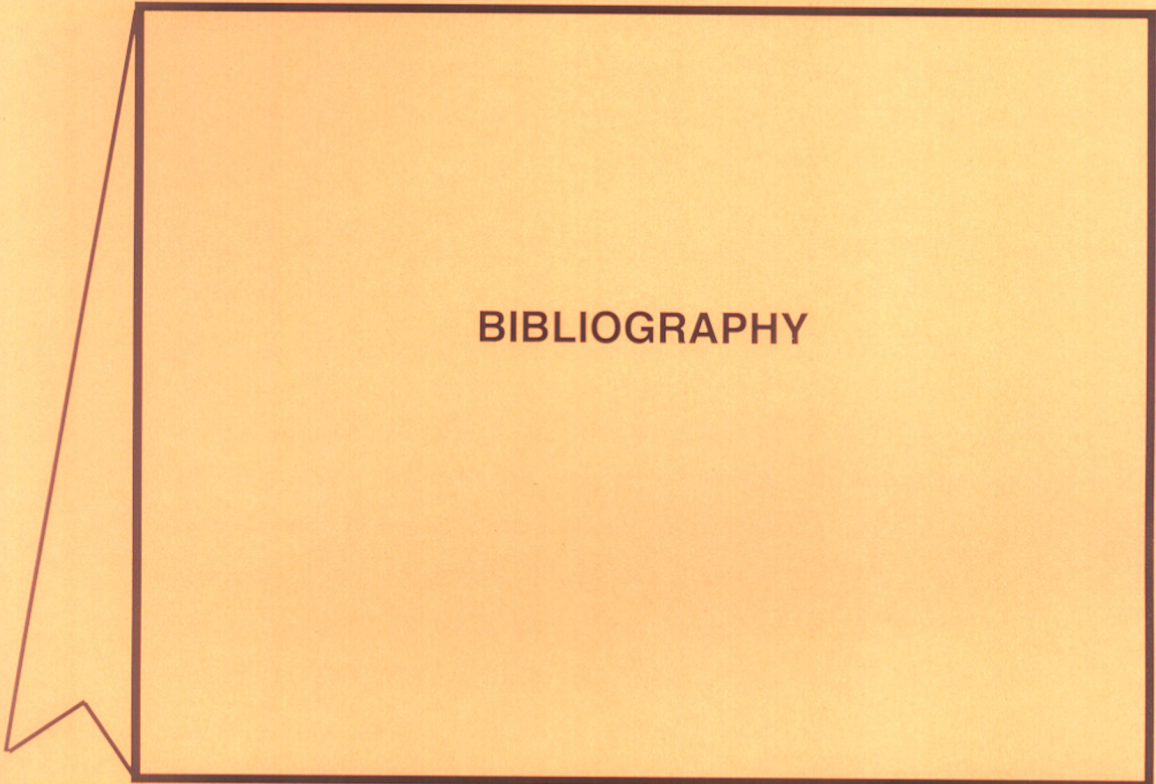
Seed culture is a potential device of maintenance of available genetic variation and also of introducing new variations. Further the juvenile explants have less contaminants and more regenerability. Hence ripe seeds of seven cultivars of black pepper were germinated *in vitro*. The parameters such as storage period, medium, gelling agent, support material, illumination during incubation were standardized for obtaining high germination percentage. Acid pre-treatment of the seed was found to be essential for uniform germination. The seed cultures of cv. Panniyur-1 resulted in sporadic somatic embryogenesis of direct or indirect origin but multiple shoot formation could not be induced in seeds cultured in media supplemented with different growth regulators.

Further manipulations for *in vitro* multiplication were carried out in only four cultivars due to availability constraints. . Whole seedlings cultured in half MS medium with BA resulted in multiple shoot formation. Solid medium was found to result into healthy multiple shoots whereas in liquid media, vitrification occurred. The shoot tip explants cultured in medium with BA 2 and 5 mg l⁻¹ resulted in multiple shoots in all four cultivars. The culture of nodal explants in half SH with or without BA resulted in formation of multiple shoots. But when half MS was used, BA was essential for induction of multiple shoots. Similar to composition, the physical state of basal medium also influenced the induction as well as elongation of shoots. Subculture in liquid half SH medium resulted in two-fold increase in height of the shoots and also facilitated rooting. Rooted shoots were hardened but died due to sudden bacterial contamination.

Embryo cultures were carried out for cv. Panniyur-1 and Karimunda. Based on previous report, Half SH and its different modifications were used for induction of somatic embryogenesis. However very few embryos responded for somatic embryogenesis. In contrast to previous report, half SH did not support somatic embryogenesis. Embryogenic calli were developed in full SH medium and in White's medium. In media containing auxins, the embryos formed non-embryogenic calli.

The work presented in this thesis forms basic frame work for biotechnology - aided breeding programme in black pepper. Further the shoot multiplication protocols standardised herein could be used for mass multiplication needed to support the huge

demand for planting material of black pepper. Protocols developed for callus formation could be utilized for *in vitro* large scale production of Piperine. In addition, the calli produced in *P. colubrinum* could be analysed for presence of novel phytochemicals as the presence of valuable pharmaceuticals especially anti-cancer drugs is being detected in several unexplored species of Piperaceae.



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Direct Differentiation in Wild Pepper, *Piper colubrinum**

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ABSTRACT

A single step high frequency regeneration protocol has been standardised for the *Phytophthora* resistant wild pepper *Piper colubrinum*. *In vitro* grown callus derived plantlets were used as the source of explants. The shoot tips and axillary buds when cultured on Murashige and Skoog (MS) basal medium supplemented with BAP ($1.0-10.0 \text{ mg.l}^{-1}$) produced multiple shoots. Other explants such as internode, petiole, leaf and root produced shoot buds directly in basal medium containing BAP (0.1 to 10.0 mg.l^{-1}). These shoot buds were elongated on a medium containing lower concentrations of BAP. The elongated shoots were directly rooted in soil and hardened in the greenhouse.

Abbreviations: BAP, 6-benzylaminopurine; MS, Murashige and Skoog medium.

Key words: Disease resistance, *in vitro* plantlets, Direct organogenesis.

INTRODUCTION

Black pepper, *Piper nigrum* L. is the most important spice of India but is susceptible to *Phytophthora capsici* Leonian causing foot/root rot disease. The estimated crop loss is 20–30%. *Piper colubrinum*, a wild species of the same genus and native of South America has been identified to be resistant to *P. capsici* and also to plant parasite nematodes *Meloidogyne incognita* Chitwood and *Radopholus similis* (Cobb.) Thorne. (Alconero *et al.* 1968). Hybridization between *P.*

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nigrum and *P. colubrinum* was not successful and the use of *P. colubrinum* as a rootstock for *P. nigrum* was also unsuccessful due to graft incompatibility (Purseglove *et al.* 1981, Nambiar and Sarama 1977). The transfer of genetic resistance of *P. colubrinum* to high yielding cultivars of black pepper, *P. nigrum* by asymmetric hybridization should be investigated. For asymmetric hybridization, the introduction of genetic marker through genetic transformation preferably in the donor line and development of a protoplast regeneration system are essential. An efficient *in vitro* regeneration of one of the parental species involved in asymmetric hybridization experiments is a basic prerequisite. With this objective high frequency direct regeneration protocol was developed for *Piper colubrinum*.

MATERIALS AND METHODS

Plant Material

In vitro raised 30 days old plantlets, derived from leaf derived callus, were used as source plants. The explants such as shoot apices, nodes, internodes, leaves, petioles and roots were treated with 0.1% HgCl_2 for 2 min. and rinsed with sterile distilled water 5–6 times.

Medium and Culture Conditions

Shoot apices and nodal explants were cultured on half strength MS medium supplemented with BAP (1.0, 5.0, 10.0 mg l^{-1}). The other explants such as internode (3.0–5.0 mm length), leaf (5.0 mm^2 bits), petiole (2.0 mm length) and root (2.0–3.0 mm length) were cultured on half strength MS medium containing BAP (0.1, 0.2, 0.5, 1.0, 5.0, 10.0 mg l^{-1}). The induced shoots were elongated on half strength MS medium containing 0.2 mg l^{-1} BAP.

All the media contained 3% sucrose and 0.6% agar. The pH was adjusted to 5.8 before autoclaving. All experiments were repeated thrice, treatments were replicated 20 times and the cultures were incubated at $25 \pm 2^\circ\text{C}$, 16 h photoperiod (30 $\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) provided by white fluorescent tubes.

The *in vitro* shoots were rooted directly in the soil and sand (1:1) mixture and hardened in the greenhouse.

RESULTS AND DISCUSSION

The concentration of BAP was found to be the key factor governing the *in vitro* response of all the explants tried.

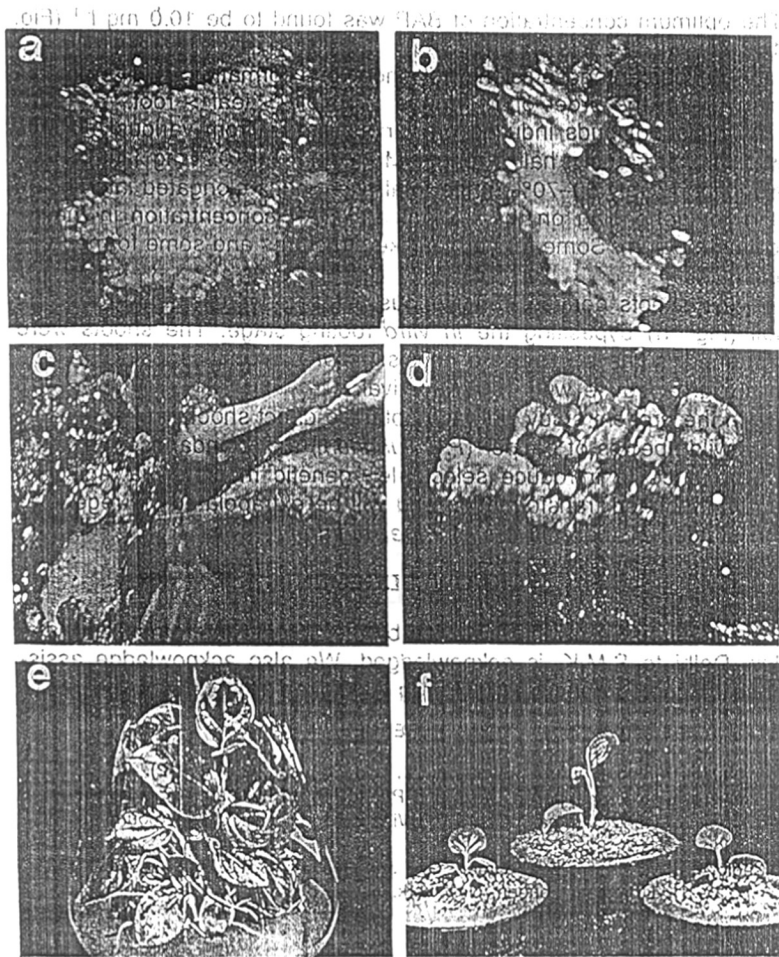


Fig. 1 *In vitro* responses of various explants of *Piper colubrinum*, a. *In vitro* leaf showing shoot bud induction; b. *In vitro* petiole showing shoot bud induction; c. *In vitro* internode showing shoot bud induction; d. *In vitro* root showing shoot bud induction; e. Elongation of shoot buds; f. Hardening of *in vitro* shoots.

4. Root

The roots showed a gradual increase in percentage of shoot bud formation with the increase in BAP concentration from 0.5 to 10.0 mg l⁻¹.

The optimum concentration of BAP was found to be 10.0 mg l^{-1} (Fig. 1d).

The percentage efficiency of shoot bud formation of different explants is in the order of: internode > petiole > leaf > root.

The shoot buds induced on various media from various explants were transferred to half strength MS containing 0.2 mg l^{-1} BAP for elongation. Only 50–70% of the total shoot buds elongated into shoots (Fig. 1e) depending on the explant and BAP concentration in the initiation medium. Some formed leaflike structures and some formed callus.

The shoots derived from various explants were directly rooted in soil (Fig. 1f) bypassing the *in vitro* rooting stage. The shoots were hardened in the soil mixture consisting of soil and sand in 1:1 ratio in the greenhouse with 100% survival.

In the present study we have obtained direct shoot morphogenesis from wild species of pepper (*P. colubrinum*) and standardised protocol will be used to introduce selectable genetic markers via *Agrobacterium* mediated transformation and will be extrapolated for regeneration studies of the cultivars of *Piper nigrum*.

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Adventitious shoot regeneration from root, internode, petiole and leaf explants of *Piper colubrinum* Link.

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Abstract A single-step, high-frequency regeneration protocol has been standardised for *Phytophthora*-resistant wild pepper, *Piper colubrinum* (Link) using root, internode, leaf and petiole explants derived from in vitro plantlets. The effect of BA on shoot-bud induction and elongation was assessed by supplementing half-strength MS medium (macronutrients at half the concentration) with different concentrations of BA, i.e. 0.2–10 mg l⁻¹ in induction media and 0, 0.2 and 0.5 mg l⁻¹ in subculture media. The interaction between culture period and BA concentration was studied by culturing the explants for 8, 15 and 30 days before the first subculture. The elongated shoots were rooted directly in soil and hardened in the greenhouse. The developed protocol would be useful in marker-assisted asymmetric hybridisation programmes involving wild-type *Piper colubrinum* and the cultivated species *P. nigrum*.

Key words *Piper colubrinum* · Plant regeneration

Abbreviations *MS*, Murashige and Skoog medium (Murashige and Skoog 1962) · *BA* 6-benzyladenine · *2,4-D* 2,4-dichlorophenoxyacetic acid · *NAA* 1-naphthaleneacetic acid · *IM* induction medium · *SM* subculture medium

Introduction

Piper colubrinum Link., a wild plant belonging to family Piperaceae, is valuable for its unique genetic make-up that

renders it resistant to the *Phytophthora*-nematode pathogen complex causing quick wilt disease in 38 other *Piper* spp., including the widely cultivated black pepper, *Piper nigrum* L., the king of spices (Turner 1971). Thus, *P. colubrinum* has immense potential as the donor parent in breeding programmes for the improvement of the cultivated species *P. nigrum*.

Conventional hybridisation and grafting of *P. colubrinum* and *P. nigrum* have not been successful as yet (Alconero et al. 1972). Biotechnological methods, such as asymmetric hybridisation and genetic transformation, are attractive alternatives for the transfer of quick wilt resistance from *P. colubrinum* to *P. nigrum* and other economically important *Piper* spp. As a pre-requisite for these biotechnological techniques, we have already standardised an in vitro plant regeneration protocol for callus-mediated regeneration in *P. colubrinum* (Kelkar et al. 1996). No other reports of plant regeneration in this species are to be found in the literature.

The success of genetic transformation and protoplast technology in perennials largely depends on the efficiency of the in vitro regeneration systems (Dandekar 1992; Yepes and Aldwinkle 1994). In order to increase regeneration efficiency, root, internode, leaf and petiole explants derived from in vitro-raised plantlets were used in the present study. The use of in vitro-raised plants provides juvenile explants that often have a better regenerability than explants derived from mature tissue (Thorpe 1993; Harding et al. 1996). Further, the explants had the advantage of being free of endophytic bacterial contaminants commonly observed in tissue cultures of *Piper* spp. (Fitchet 1990; Philip et al. 1992; Bhat et al. 1992, 1995; Aminuddin et al. 1993; Meyer et al. 1992; Kelkar et al. 1996).

Materials and methods

Plant material

In vitro plantlets were raised from leaf calli of greenhouse-grown *P. colubrinum* as previously reported (Kelkar et al. 1996). The plant-

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Table 1 Effect of BA concentration on shoot-bud formation in different explant types of *Piper colubrinum*

BA (mg l ⁻¹)	Explants forming shoot buds (%)				Number of shoots per explant (mean±SD)			
	Root	Petiole	Internode	Leaf	Root	Petiole	Internode	Leaf
B 0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B 0.2	0.0	55.6	95.6	0.0	0.0	7.2±2.6	11.0±3	0.0
B 0.5	15.8	78.3	97.5	26.4	2.0±0.8	6.6±2.7	9.5±2.3	5.3±1.5
B 1.0	21.6	93.1	94.8	79.6	2.5±0.7	5.9±2.2	8.8±2.5	6.1±1.9
B 5.0	83.5	93.0	91.1	97.6	3.3±1.0	2.6±1.0	5.9±1.2	4.6±1.4
B 10.0	86.7	93.0	93.1	88.6	3.5±1.1	2.3±1.0	2.7±0.8	3.6±1.6

lets, all of which at the four- to six- node stage and maintained for 8–10 weeks on half-strength MS medium (containing macronutrients at half the concentration) without plant growth regulators, were used as the source of explants. Different explants were aseptically cut into pieces of appropriate sizes, i.e. root (2–3 mm in length), internode (3–5 mm in length), petiole (2–3 mm in length) and leaf (5–8 mm²), and used for culture.

Media and culture conditions

Aseptically cut root, internode, petiole and leaf explants were cultured on half-strength MS medium supplemented with either BA (0.2, 0.5, 1.0, 5.0 and 10.0 mg l⁻¹) alone, or in combination with 2,4-D (0.5, 1.0 mg l⁻¹) or NAA (0.5, 1.0 mg l⁻¹) in the pilot experiments. Further, the effect of BA on shoot-bud induction and elongation was studied in three parallel experiments:

- 1) To study the effect of BA concentration on shoot-bud induction we cultured all four explant types on a range of IMs which comprised half-strength MS medium with BA (0.2, 0.5, 1.0, 5.0 and 10.0 mg l⁻¹) or without BA. The explants which showed shoot-bud induction were transferred to different SMs after 30 days.
- 2) To study the effect of the continued presence of BA in the IM on the elongation of shoot buds and regeneration efficiency, i.e. number of shoots per explant, the root, internode, petiole and leaf explants with shoot buds from experiment 1 were subcultured on half-strength MS medium with or without BA (0.0, 0.2 and 0.5 mg l⁻¹). Further subcultures were done at monthly intervals. The number of shoots more than 1 cm in height were counted after two subcultures.
- 3) In the pilot experiments a high concentration of BA in the IM was found to lower the number of shoots regenerated per explant in all of the explant types except root. In order to analyse the interaction between culture period and high BA (5.0, 10.0 mg l⁻¹) concentration in the IM on multiple shoot formation, explants from all four explant types were cultured on IMs containing BA (5.0, 10.0 mg l⁻¹) for different time intervals (8 and 15 days) and then subcultured on half-strength MS medium without growth regulators. Further subcultures were carried out at monthly intervals. The number of shoots (more than 1 cm in length) regenerated per explant was counted, and results were compared against results of experiment 2 where the culture period was 30 days.

All of the regenerated shoots from the three experiments were further elongated on half-strength MS without growth regulators for 2 months and then transferred for ex vitro rooting and hardening.

All media contained 3% sucrose and 0.6% agar (Qualigens, India). The pH was adjusted to 5.8 before autoclaving. Each treatment had at least ten replicates, and each experiment was carried out at least thrice. The cultures were incubated at 25±2°C, 16-h photoperiod (30 µE m⁻² s⁻¹) provided by cool-white fluorescent tubes.

Acclimatization

Twenty shoots at the three- to five-node stage from each explant type were transferred to a sand:soilrite mixture (1:1 ratio) where they rooted and were established.

Statistical analysis

The number of shoots per explant were compared by ANOVA. The Student t-test was applied to compare individual treatments. (Wardlaw 1985).

Results and discussion

Tissue culture studies carried out so far in *Piper* spp. have used BA for shoot bud induction (Mathews and Rao 1984; Fitchet 1990; Philip et al. 1992; Bhat et al. 1992, 1995; Aminuddin et al. 1993; Meyer et al. 1992; Babu et al. 1994; Kelkar et al. 1996). Based on these reports, we chose to use BA for shoot-bud induction in the current experiments. The use of half-strength MS basal medium was based on our own previous experiments (Kelkar et al. 1996).

The combination of BA with the auxins 2,4-D and NAA (0.5 and 1.0 mg l⁻¹) induces organogenesis in mature leaf explants of *P. colubrinum* (Kelkar et al. 1996). Consequently, in the pilot experiments, auxins were used in addition to BA (0.2, 0.5, 1.0, 5.0, 10.0 mg l⁻¹). However, as this combination resulted in callus formation as well as other forms of organogenesis described by Tran-Thanh-Van (1981) such as rhizogenesis and formation of leaflike structures and trichomes (data not shown), only BA was used in further experiments.

When used alone in the medium, BA influenced organogenetic induction (Table 1) as well as the continuation of the organogenetic process (Table 2). The inhibition of organogenetic induction caused by a high concentration of BA in IM could be traced to an early stage when the explants were exposed to different culture periods (Table 3). The effect of BA on different phases of organogenesis can be explained as follows.

Effect of BA concentration of IM on shoot-bud induction

The use of half-strength MS basal medium alone as the IM did not evoke any response other than occasional rhizogenesis (Table 1). However, the presence of BA in the IM supported shoot-bud formation in different explants at various frequencies depending on the concentration (Table 1). The threshold concentration of BA required for induction of organogenesis and optimal response differed for the different explant types.

Table 2 Effect of BA concentration of SM on the number of shoots formed per explant in different explant types of *Piper colubrinum*

BA concentration (mg l ⁻¹)		Number of shoots per explant (mean±SD)			
IM	SM	Root	Petiole	Internode	Leaf
0.2	0.5	0.0	8.3±1.6	10.8±3.2	0.0
	0.2	0.0	8.6±2.2	13.5±2.6	0.0
	0.0	0.0	4.1±1.5*	7.5±1.7*	0.0
0.5	0.5	2.0±0.8	7.9±1.8	10.0±2.3	5.7±1.2
	0.2	2.0±1.0	7.2±2.4	10.2±2.0	6.0±1.3
	0.0	2.0±1.0	3.1±1.1*	7.7±2.1*	3.8±1.4*
1.0	0.5	2.8±0.8	7.0±1.3	9.4±1.8	6.4±1.5
	0.2	2.3±0.8	6.0±2.2	10.2±1.9	7.4±1.6
	0.0	2.3±0.5	3.9±2.2*	6.7±1.9*	4.2±1.4*
5.0	0.5	2.9±1.3	3.1±1.3	5.6±1.0	4.7±1.2
	0.2	3.4±0.9	2.5±1.0	5.8±1.4	5.0±1.6
	0.0	3.6±0.8	1.9±0.9	6.3±1.0*	4.2±1.4*
10.0	0.5	3.0±1.0	2.8±1.1	2.3±0.6	3.5±1.2
	0.2	3.7±1.0	2.4±0.9	2.8±0.7	4.0±1.8
	0.0	4.0±1.2	1.3±0.5	3.0±0.9	3.1±2.1

* Differences among the subculture treatments for each induction treatment are significant at the 95% confidence level

Root

After 2 weeks of culture, the roots became intense green and swollen in all of the IMs tried, except for the medium containing 0.2 mg l⁻¹ BA. The incubation period for initiation of shoot-bud induction was relatively long, i.e. 4 weeks for roots as against the 2–3 weeks required by the other explant types. By the end of the 4th week, small nodule-like structures appeared predominantly on the proximal end (Fig. 1a) and rarely on the distal end. Such a polarisation of morphogenetic response in root explants is common in other plants (Rybczynski et al. 1995). The percentage response for shoot-bud induction increased with increasing concentration of BA, the lowest being 15.8% at 0.5 mg l⁻¹ BA and the highest being 86.7% at 10.0 mg l⁻¹ BA.

Internode

The percentage response of internode explants to form shoot buds was more or less similar in all the concentrations of BA tried (Table 1). The shoot buds appeared after 2–3 weeks of culture (Fig. 1b), first at the cut ends and later on the central portion. The medium with 0.5 mg l⁻¹ BA supported highest (97.5%) percentage of explants forming shoot-buds whereas the lowest percentage response (91.1%) was observed at 5.0 mg l⁻¹ BA (Table 1).

Leaf

Leaf explants formed shoot buds at all concentrations of BA except at 0.2 mg l⁻¹. The buds were induced at the cut

Table 3 The effect of induction period on number of shoots formed per explant in root, internode, leaf and petiole explants in *P. colubrinum*

IM BA (mg l ⁻¹)	Induction period (days)	Number of shoots per explant (mean±SD)			
		Root	Petiole	Internode	Leaf
5.0	30	3.6±0.8	1.9±0.9	6.3±1.0	4.2±1.4
	15	3.8±0.9	3.0±0.6	6.8±1.2	4.6±1.8
	8	3.1±0.6	2.0±1.0	6.0±0.6	3.0±1.0
10.0	30	4.0±1.2	1.3±0.5	3.0±0.9	3.1±2.1
	15	3.0±0.7	2.1±0.9	4.0±1.5	4.0±1.5
	8	3.0±0.6	1.3±0.5	2.8±0.7	3.1±1.0

ends after 2–3 weeks (Fig. 1c). The response increased gradually along the concentration gradient from 26.5% at 0.5 mg l⁻¹ BA to 97.6% at 5.0 mg l⁻¹ BA but decreased to 88.6% at 10.0 mg l⁻¹ BA (Table 1). BA induced callusing at higher concentrations (5 and 10 mg l⁻¹) and also caused blackening of the explants.

Petiole

Petiole explants showed shoot-bud induction at all concentrations of BA. The lowest percentage of explants, i.e. 55.6%, forming shoot buds was noted at 0.2 mg l⁻¹ BA. It increased with increasing concentrations of BA up to 5.0 mg l⁻¹ (Table 1). The shoot-buds were induced only at the cut ends after 2–3 weeks of culture (Fig. 1d).

Effect of BA concentration of the IM on number of shoots regenerated per explant

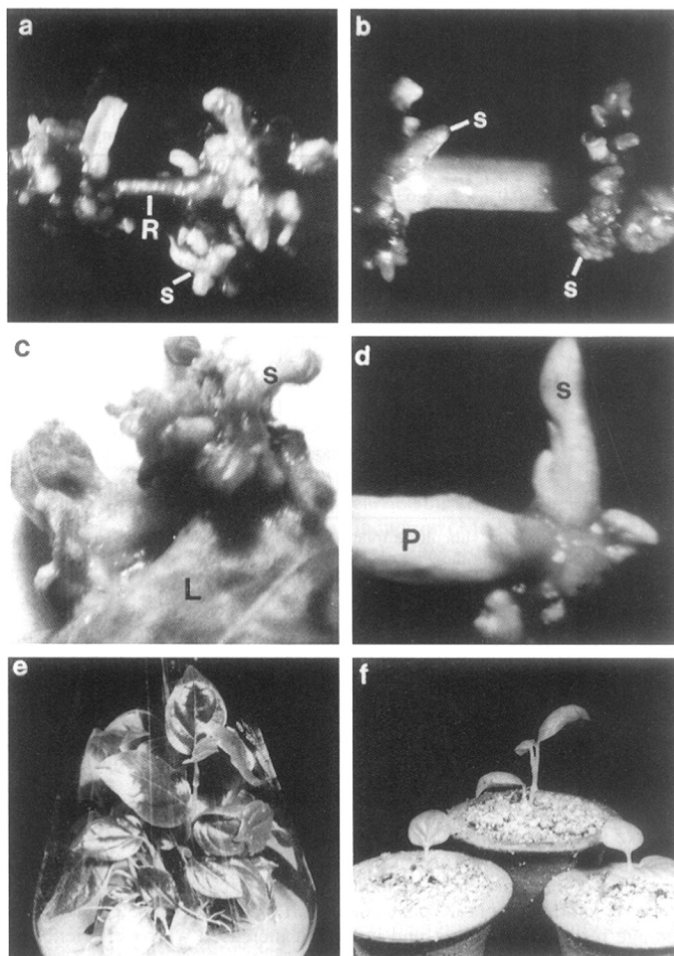
The concentration of BA in the IM markedly affected the efficiency of regeneration. Each explant type showed a differential preference for maximal response.

For root explants, the increase in number of shoots per explant was directly proportional to the increase in BA concentration. This relationship reversed in petiole and internode explants, where the number of shoots decreased with increasing BA concentrations (Table 1). The regeneration frequency for leaf explants gradually increased with an increase in BA concentration, reaching its maximum at 1.0 mg l⁻¹ BA and then decreased with any further increase in BA concentration (5.0, 10.0 mg l⁻¹). In internode and petiole explants the maximal response was achieved at 0.2 mg l⁻¹ BA whereas in root explants it was at 10.0 mg l⁻¹ BA (Table 1).

Effect of BA concentration of the SM on number of shoots regenerated per explant

The continued presence of BA in the SM was not necessary for continuing growth and elongation of the shoot buds (Fig. 1e, Table 2). However, the continued use of BA in

Fig. 1a-f Shoot regeneration from root, internode, leaf and petiole explants of *Piper colubrinum* Link. **a** Root explant (*R*) showing shoot-bud induction (*s*). **b** internode explant showing shoot-bud induction (*s*). **c** leaf explant (*L*) showing shoot-bud induction (*S*). **d** petiole explant (*P*) showing shoot-bud induction (*s*). **e** elongation of shoot buds on half-strength MS medium, **f** hardening of in vitro shoots



the SM had a positive effect on the number of shoots formed per explant depending on the explant type and the BA concentration used in the IM. In the preliminary experiments, all the concentrations of BA used in the IM were also used in the SM. However, the presence of BA at concentrations of 1.0, 5.0 and 10.0 mg l⁻¹ in the SM caused darkening of the leaf explants and callusing in other explant types. Therefore, only 0.2 and 0.5 mg l⁻¹ BA were used in the SMs.

In internode, petiole and leaf explants the interaction of BA concentration in the SM and IM followed a definite pattern. The presence of BA in the SM had a significant advantage over its absence when the IM contained lower concentrations of BA (0.2, 0.5, 1.0, 5.0 mg l⁻¹). However, the differences between SM with or without BA were rendered insignificant when the IM contained 10.0 mg l⁻¹ BA (Table 2).

The combined action of low BA (0.2 mg l⁻¹ for internode and petiole; 1 mg l⁻¹ for leaf) in the IM and in SM (0.2 and 0.5 mg l⁻¹) was optimal for high number of shoots

per explant (Table 2). But the differences between the regeneration response at these two levels of BA, i.e. 0.2 and 0.5 mg l⁻¹, were not found to be statistically significant except for internode explant (Table 2). High levels of BA (5.0, 10.0 mg l⁻¹) in the IM gave a low response irrespective of the concentration of BA used in the SM (Table 2).

In root explants the interaction between the SM and IM seemed to differ. The absence of BA in the SM had a promoting effect when the IM contained high levels of BA (5.0, 10.0 mg l⁻¹), but the difference was not found to be statistically significant.

From the results presented in Tables 1 and 2, it can be concluded that the regeneration efficiency of the internode explants is the best among the four explant types tested, followed by petiole, leaf and root in consecutive order. In general, the level of organogenetic competence of the explants was defined by the BA concentration in the IM itself, whereas the SM had a secondary effect on elongation of the shoots formed on the IM.

Effect of culture period on number of shoots formed per explant

For internode, petiole and leaf explants high BA in the IM resulted in a high percentage of shoot-bud induction but lowered the number of shoots per explant irrespective of the SM used (Table 1). So to investigate the relationship between high BA (5.0, 10.0 mg l⁻¹) and the culture period on the IM, we cultured explants for 8 and 15 days on IM with BA (5.0, 10.0 mg l⁻¹) and then subcultured them on half-strength MS medium without any plant growth regulators. The results were compared to those of experiment 2 where the culture period was 30 days.

The reduction in culture period from 30 days to 15 and 8 days caused no statistically significant increase in regeneration frequency in any of the explant types (Table 3). This suggests that the inhibitory action of higher doses of BA (5.0, 10.0 mg l⁻¹) was operative within 8 days of exposure of the internode, petiole and leaf explants. For root explants, an 8-day culture period was sufficient to induce an optimum response. The gradual increase in number of shoots formed per explant with increase in culture period was not found to be statistically significant.

All the regenerated shoots (more than 1 cm in height) from all the treatments were further elongated on half-strength MS medium without growth regulators for 7–8 weeks (Fig. 1e).

Acclimatization and ex vitro rooting

A total of 20 elongated shoots at the four- to five-node stage were selected randomly from the regenerants of each explant type and transferred to the sand: soilrite mixture (1:1 ratio) for hardening. Adventitious roots were formed and the plantlets were established in 4–5 weeks (Fig. 1f). The degree of hardening success differed considerably for the shoots regenerated from different explants. The shoots derived from roots exhibited 100% hardening, whereas shoots regenerated from internode explants showed 88.3% hardening, followed by petiole explants (87.2%) and leaf explants (85.0%).

The present paper presents an efficient single-step plant regeneration method from root, petiole, internode and leaf explants which will be useful for studying the basic aspects of organogenesis as well as applied aspects such as genetic transformation and protoplast technology involving wild as well as cultivated species of pepper.

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