"*IN-VITRO* PLANT REGENERATION AND COMPARATIVE STUDY OF SECONDARY METABOLITE FROM TRANSFORMED AND NONTRANSFORMED PLANT OF *PIPER SPP.*"

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This is to certify that the thesis entitled "***In vitro* plant regeneration and comparative studies of secondary metabolite from transformed and nontransformed plants of *piper spp.***" submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science in Agriculture (Agricultural Biotechnology)** to the Orissa University of Agriculture and Technology is an authentic record of bonafide research work carried out by **Sri Kirath Singh (Adm. No. 05ABT/12)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that the evidence and help obtained by him from various sources during the course of investigation has been duly acknowledged.

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ABSTRACT

The genus *Piper* (piperaceae) is largely distributed in tropical and sub-tropical regions of the world. It is famous as the spices king due to its pungent quality. The genus *Piper* has more than 1000 species but the most well known species are *P. nigrum, P. longum* and *P. betle*. In *P. nigrum*, about 51 cultivars have been reported from the tropical and sub-tropical regions of the India. *P. nigrum* fruits are also used to produce white pepper and green pepper and valued due to presence of Piperine. It has high amount of secondary compounds such as unsaturated amides, flavonoids, lignans, long and short chain esters, terpenes, steroids and alkaloids (Parmer *et al.*, 1997,1998; Navickiene *et al.* 2000; Facundo *et al.*, 2005) and also having insecticidal activity (Boll *et al.* 1994). On the view of above fact, the present investigation is to standardize the *in vitro* protocol on plant regeneration from different explants of *Piper* species and comparative analysis of secondary metabolite associated in different explants as well as transformed and non-transformed callus and its impact on microbial activity. The shoot multiplication was achieved in MS medium supplemented with 1.5 mg/l BAP, 0.5 mg/l IAA and 100 mg/l adenine sulphate in *Piper longum*. However, the same composition did not effect any regeneration efficiency in other species like *P.betle* and *P.nigrum*. Among the three cytokinins used, it was observed that BAP was most suitable for shoot proliferation & multiplication. Inclusion of 0.5 mg/l IAA in the culture medium showed higher percentage of shoot proliferation (83.3%) and multiplication within 8 weeks of culture. The number of multiple shoots per explant varied from 1.0 – 3.4 on different culture medium. Callus induction and proliferation was achieved from leaf and

stem explants on MS media supplemented with 2.5 mg/l 2, 4-D within 4 weeks of culture. Sub culturing was made every 4 weeks interval to enhance the production of multiple shoots. The elongated microshoots were separated and transferred to different rooting media for induction of root. The maximum percentage of rooting was achieved on ½ strength MS medium supplemented with 0.25 mg/l IBA and 2% (W/V) sucrose. The rooted plantlets were successfully transferred to greenhouse and grown normally. Further, the green friable calli developed from leaf tissues were used for transformation study to compare the Piperine content in transformed and non-transformed calli, fruits, leaf and root through TLC anf HPTLC method. *Agrobacterium* strain A4 harbouring root inducing plasmid (Ri- plasmid) was used for *in vitro* transformation. The percentage of Piperine content in transformed callus was 1.35% more than the non-transformed calli. The results also indicate that the root having more Piperine content than leaf and fruits. The phytochemical constituents such as alkaloid, steroid, Triterpenoids, glycosides, Flavonoids, Tannins and carbohydrates were present in the *Piper* species. But, Saponin and proteins were absent on the basis of chemical test. Further, the crude extracts of *P.betle*, *P. longum* and *P.nigrum* were used for antimicrobial activity. The positive zone of inhibition was observed in bacteria like *Micrococcus luteus*, *Streptomyces epidermidis*, *E.coli* and fungus like *Aspergillus flavus* and Rhizoctonia *solani.* Further work is necessary to standardize the protocol on propagation of elite clone of *Piper* species and enhance the secondary metaboliote by using biotechnological tools.

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LIST OF ABBREVIATIONS

µM	:	micromolar
2, 4-D	:	2, 4-dichlorophenoxy acetic acid
Ads	:	adenine sulphate
ANOVA	:	analysis of variance
Au	:	area unit
BAP	:	benzyl aminopurine
BC	:	before Chirst
cm	:	centimeter
C-TAB	:	cetyltrimethylammonium bromide
cv	:	cultivar
Dw	:	double distilled water
DMSO	:	dimethylsulfoxide
dw	:	dry weight
E.Coli	:	*Escherichia coli*
Fig.	:	figure/figures
FW	:	fresh weight
FYM	:	Farm yard manure
g	:	gram/grams
h	:	hours
ha	:	hectare
HCL	:	hydrochloric acid
Hpt	:	Hygromycin phosphotransferase
HPTLC	:	high performance thin layer chromatography
IAA	:	indole-acetic acid
IBA	:	indole-3- butyric acid
Kn	:	kinetin
L	:	litre
LB	:	lauria bertani
Luc	:	Luciferase
M	:	metric
M	:	molar
M.W	:	molecular weight
MAPs	:	medicinal and aromatic plants
mg	:	Milligram
mg/l	:	milligram per Litre
mg/l	:	milligram per Litre
Min.	:	minute
ml	:	millilitre
mm	:	millimetre
MS	:	Murashige and Skoogs medium (1962)
MTCC	:	Microbial Type Cell Cultures
N	:	normal
NA	:	nutrient agar

NAA	:	α-napthalene acetic acid
NaOH	:	sodium hydroxide
nm	:	nanometer
Npt	:	neomycin phosphotransferase
OD	:	optical density
OUAT	:	Orissa University of Agriculture and Technology
pH	:	hydrogen ion concentration
ppm	:	parts per million
Rf	:	retardation factor
rpm	:	revolution per minute
TBE	:	tris –boric acid -EDTA buffer
T-DNA	:	transferred DNA
TDZ	:	thidiazuron
TLC	:	thin layer chromatography
v/v	:	volume/volume
Vir	:	virulence
w/v	:	weight/volume

CHAPTER- 1

INTRODUCTION

Plants have been used since ancient times to heal and cure diseases and to improve health and well being. Despite ancient nature of the tradition, medicinal plants still form the basis of traditional or indigenous health systems and are reported by the World Health Organization (WHO) to still be used by the majority of the populations in most developing countries. Medicinal and aromatic plants (MAPs) play a significant role in meeting the demands of the traditional medicine markets which are found both domestically in the producing and in overseas markets. Traditional medicine, for example, which is related to Traditional Chinese medicine (TCM), Indian Ayurveda and Arabic Unani medicine and to various forms of indigenous medicine, as well as the complementary or alternative medicine utilized in industrialized countries, is growing credibility in many parts of the world. The last three decades, it has been that the substantial growth in herb and herbal product in markets across the world. Rapidly rising exports of medicinal plants during the past decade attests to worldwide interest in these products as well as in traditional health systems. According to the Secretariat of the Convention on Biological Diversity, global sales of herbal products totalled an estimated US$60 000 million in 2002. At present, 80 percent of the population in developing countries rely largely on plant-based drugs for their health care needs, and the WHO has estimated that in coming decades a similar percentage of the world population may well rely on plant-based medicines. Thirty percent of the drugs sold worldwide contain compounds derived from plant material. As a result of the expanding interest in medicinal and aromatic plants, new income generating opportunities are opening up for rural populations. With many of the MAPs (medicinal and aromatic plants) gathered from the wild, the collection and sale of MAPs is providing a complementary source of cash for many extremely poor rural households.

1.1. Status of medicinal crops

Nature always stands as a golden mark to exemplify the outstanding phenomena of symbiosis. Natural products from plant have been the basis of the

treatment of human disease. Today estimate that about 80% of people in developing countries still relays on traditional medicine based largely on species of plants for their primary health care. Herbal medicines are currently in demand and their popularity is increasing day by day. About 500 plants with medicinal use are mentioned in ancient literature and around 800 plants have been used in indigenous systems of medicine. India is a vast repository of medicinal plants that are used in traditional medical treatments (Chopra *et al*, 1956). The various indigenous systems such as Siddha, Ayurveda, Unani and Allopathy use several plant species to treat different ailments (Rabe and Staden, 1997). The use of herbal medicine becoming popular due to non-toxicity and no side effects as compare with allopathic medicines. This led to sudden increase in the number of herbal drug manufactures. Herbal medicines as the major remedy in traditional system of medicine have been used in medical practices since antiquity. The practices continue today because of its biomedical benefits as well as place in cultural beliefs in many parts of world and have made a great contribution towards maintaining human health.

In India, around 20,000 medicinal plant species have been recorded but more than 500 traditional communities use about 800 plant species for curing different diseases (Kamboj, 2000). Currently, 80% of the world population depends on plant-derived medicine for the first line of primary health care for human alleviation because it has no side effects. Plants are important sources of medicines and presently about 25% of pharmaceutical prescriptions in the United States contain at least one plant derived ingredient. In the last century, roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources.

India has a rich culture of medicinal herbs and spices, which includes about more than 2000 species and has a vast geographical area with high potential abilities for Ayurvedic, Unani, Siddha traditional medicines but only very few have been studied chemically and pharmacologically for their potential medicinal value. Human being used plants species for the treatment of diverse ailments for thousands of years. According to the World Health Organization, most populations still rely on traditional medicines for their psychological and physical health requirements (Rabe and Van Stoden, 2000), since they cannot afford the products of Western pharmaceutical industries, together with their side effects and lack of healthcare facilities. Rural areas

of many developing countries still rely on traditional medicine for their primary health care needs and have found a place in day-to-day life. These medicines are relatively safer and cheaper than synthetic or modern medicine (Ammara *et al.*, 2009). People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products to maintain human health, but they may not understand the science behind these medicines, but knew that some medicinal plants are highly effective only when used at therapeutic doses (Maheshwari *et al.*, 1986).

Recent estimates suggests the over 9,000 plants have known medicinal applications in various countries, and this is without having conducted comprehensive research amongst several indigenous and other communities (Farnsworth *et al.*, 1976). Medicinal plants are used at the household level by women taking care of their families at the village level by medicine men or tribal shamns, and by the practitioners of classical traditional systems of medicine such as Ayurveda, Chinese medicine, or the Japanese kampo system. According to the world Health Organization, over 4.3 billion people rely upon such traditional plant based systems of medicine to provide them with primary health care (Attisso *et al.* 1983). Allopathic medicine too owes a tremendous debt to medicinal plants; one in four prescriptions filled in a country like the U.S are either a synthesized form or derived from plant materials (Srivastava et al, 1995). According to the International Trade Centre, the total value of imports of starting materials of plant origin for the pharmaceuticals and cosmetics industry was one of the order of USD 53.9 million (Attisso *et al.* 1983) . India was the largest supplier by far, with 10,055 tons of plants and 14 tons of vegetables alkaloids and their derivative (Lewington *et al.* 1993). However, it is only during the last decade that the real significance of the medicinal plants sector has begun to be realized by mid 1980s, there was a renewed interest in natural materials and approaches to health care, coupled with recognition that technology alone could not solve the pressing health care needs of the world's population. The participation of various companies in the market also attests to its new strength and importance.

By 1990, some 223 major companies worldwide were reportedly screening plants for new leads; the figure had been low in 1980 (Aryial *et al.* 1993). In response to the overwhelming interest in alternative therapies, many of the prestigious

allopathic medicinal institutions have also recognized their importance: an example is the National Institute of Health which created the Office of Alternative medicine in 1991 to provide the public with information on alternative treatments and to assess those therapies which have been proven successful. In 1992, about 74 species of medicinal plants were being commercially traded in the global market (Handa *et al.* 1992).

1.2. Distribution of medicinal plants in India

In India, several medical systems have evolved and prominent among these systems are Ayurveda, Siddha and the Unani Systems of Medicine. In different civilizations the contribution of floral biodiversity to health care has been well documented (Posey, 1999). According to Schippmann *et al.* (2002), more than 50000 species are used for medicinal purposes worldwide, of which almost 13% are flowering plants. Over 8000 plant species are used in traditional and modern medicine in India and 90-95% collection of medicinal plants was from the wild, of which more than 70% collection involves destructive and unscientific extraction. Over exploitation of trade species, destructive way of collection, vulnerability due to anthropogenic pressure are some of the major threats to medicinal plants. In order to achieve sustainable harvest of medicinal plants and other non timber forest products (wild edibles), fuel, fodder, timber, making agricultural tools, fiber, religious and various other purposes), a multi-disciplinary approach must be considered which include ecological, biological, socio-cultural and economical aspects of the species (Ghimire *et al.*, 2004). Around 70% of Indias medicinal plants are found in tropical areas mostly in the various forest types spread across the western and Eastern Ghats, Chota Nagpur plateau, Vindyas, Aravalis and Himalayas. Although less than 30%of medicinal plants are found in the temperate and alpine areas and higher altitudes they include species of high medicinal values. Over 800 species of medicinal plants are used in the production of traditional medicines by industry. Of these about 90% are collected from the wild. Less than 20 species of plants are under commercial cultivation.

Paradoxically, there are hardly any reliable data available on area, production and productivity of cultivated medicinal species since these are not recorded at the grass root level for want of adequate knowledge and interest by the record keeping

authorities. All species are considered as one commodity and sometime some data are taken by some states. The area of 272.150 ha presented here for some of the important medicinal plants are on the basis of consolidation made from the fragmented information available in literatures. National Medicinal Plant Board has identified some important medicinal plants need to be cultivated (Table 1.1).

Table 1.1 Area under cultivation of important medicinal plants.

Sl. no.	Common name	Botanical name	Area (ha)	Cultivating states
1	Psyllium	*Plantago ovata*	55,000	RAJ. & GUJ.
2	Opium poppy	*Papaver somniferum*	20,000	M.P, U.P, RAJ.
3	Senna	*Cassia senna*	20,000	T.N, RAJ, UP
4	Cinchona	*Cinchona spp.*	8,000	Darjeeling(W.B.). T.N.
5	Ashwaganda	*Withania somnifera*	5,000	M.P, RAJ, U.P
6	Safed musali	*Chlorophytum spp.*	5,000	M.P, GUJ, U.P
7	Periwinkle	*Catharanthus roseus*	4,000	A.P, KTK, M.H
8	Khai katari	*Solanum spp*	4,000	M.H.
9	Sarpaganadha	*Rauvolfia serpentina*	2,500	M.P.
10	Bael, Billi	*Aegle marmemari*	3,000	Bihar, W.B,U.P, M.H
11	Kalmegh	*Andrographis paniculta*	2,000	Many states
12	Neem	*Azadiachta indica*	2,000	GUJ, M.H, A.P, RAJ, KTK, T.N
13	Pipal, pipli	*Piper longum*	1,500	M.H, Kerala, T.N, Assam, W.B.
14	Guggal	*Commiphora wightii*	2,000	GUJ, M.P, M.H, RAJ.
15	Kaunch	*Mucuna pruriens*	1,000	A.P, KTK, H.P, GUJ, M.H
15	Tulsi	*Ocimum sanctum*	1,000	U.P, J&K, KTK

Source: Kumar, S. (1997) CIMAP-records

T.N – Tamilnadu, U.P – Uttar Pradesh, W.B- West Bengal, A.P- AndraPradesh, H.P-Himachal Pradesh, J&k- Jammu and Kashmir, KTK- Karnataka, GUJ.-Gujarat, RAJ.-Rajasthan

1.3. Importance of medicinal plants

In spite of great advances of modern scientific medicine, traditional medicine is still the primary form of treating diseases of majority of people in developing countries including India; even among those to whom western medicine is available, the number of people using one form or another of complementary of alternative medicine is rapidly increasing worldwide. Increasing knowledge of metabolic process and the effect of plants on human physiology has enlarged the range of application of medicinal plants. According to the report by the World Bank in 1997, it is apparent that the significance of plant based medicines has been increasing all over the world. Nearly 50% of medicines in the market are made of natural basic materials. Interestingly, the market demands for medicinal herbs are likely to remain high because many of the active ingredients in medicinal plants cannot yet be prepared synthetically (Thomas *et al,* 1996). The universal role of plants in the treatment of disease is exemplified by their employment in all major systems of medicine irrespective of the underlying philosophical premise.

In recent years, the use of herbal medicines worldwide has provided an excellent opportunity to look for therapeutic lead compounds from an ancient system of therapy, i.e. Ayurveda, which can be utilized for development of new drug. Over 50% of all modern drugs are of natural product origin and they play an important role in drug development programs of the pharmaceutical industry. Epidemiological evidence suggests that dietary factors play an important role in human health and in the treatment of certain chronic diseases including cancer. Some dietary sources contain antitumor compounds (Rogers *et al,* 1993) and such compounds are candidates for chemo preventive agents against cancer development (Dorai *et al.,* 2004). The anticancer property of nutrients derived from plants as well as non nutritive plant derived constituents have been proved in different *in vitro* and *in vivo* models, which had led to an increased emphasis on cancer prevention strategies in which these dietary factors are utilized (Barnes *et al,* 1995). Dietary measures and traditional plant therapies as prescribed by Ayurvedic and other indigenous systems of medicine are used commonly in India (Singh *et al,* 2003).

Among various medicinal plants, piper species under family Piperaceae has significant role in human welfare. It contains about 2,000 species of shrubs, herbs,

and lianas. many of which are keystone species in their native habitat. The diversification of this taxon is of interest to understanding the evolution of plants.

1.3.1. *Piper nigrum*

Piper nigrum is famous as the spices king due to its pungent quality. Piper nigrum *(P.nigrum)* L. is a member of family Piperaceae. The genus piper has more than 1000 species but the most well known species are *P.nigrum, P.longum and P.betle;* 51 cultivars of p. nigrum have been reported from the tropical and subtropical regions of India. *P.nigrum* fruits are also used to produce white pepper and green pepper and are valued due the presence of piperine including its different isomers. Black pepper can be used for different purposes such as human dietaries, as medicine, as preservatives, as bio-control agents. This plant and its active component piperine can stimulate the digestive enzymes of pancreas and intestines and also increases biliary bile acid secretion when orally administrated. Some reports have been demonstrated that black pepper consumption in humans increased orocecal transit time. Piperine prevents and minimize diarrhea produced by various oil and chemicals and also reduce intestinal fluid accumulating in mouse intestine. The active agents of *P. nigrum* activates the epithelial cells in rat jejunum to permeates the uptake of various amino acids through the activation of membranes, enhance the production of proteins which are later used for the formation of cytoskeleton system due to surface adsorption property. This valuable species has also the power to minimize different mutations like ethylcarbamte induced mutation in *Drosophila.*

As compare to mutation, black pepper also reduced tumour formation in mice such as Ehrlich ascites tumour and Doltons lymphoma cells. Other related activities included Anti-inflammatory activity, thermogenic action, growth stimulatory activity, anti-thyroid activity and chemo preventive. Secondary, metabolites from *P. nigrum* play defensive role against infections by microbes, insects and animals Piperamides extracted from *P. nigrum* had shown insecticidal activities caryophyllene showed anesthetic activity. Nero idol is very famous secondary metabolite of *P. nigrum*, used to control mites. Another important component of pepper volatile oil is pipene, which is a famous odorants. Black pepper is important for its medicinal value. Medicinally black pepper can be used for digestive disorders like large intestine toxins, different gastric problems, diarrhea, and indigestion and also can be used against respiratory

disorders including cold, fever, and asthma. The West African Black pepper, (*P. guineense*), important as flavorant and its different parts are used as internal medicine for curing bronchitis, gastric ulcer, rheumatism and as antiviral agent. Recently, an interesting study has shown effects of piperine on mood and cognitive disorder. Notwithstanding, it has been shown that enhances the bioavailability of various nutrients including vitamins: carotene and selenium. Most of the plant species including piper produced secondary metabolites which help in animal metabolism and also used as defence system against various agents such as insect feeding plants and animals. Recently scientists from different biological fields screen plants for identification of various secondary metabolites which can be used for preparation of medicines and bio control agents.

1.3.2. *Piper longum*

Long pepper *(Piper longum)*, (Pippali), sometimes called Indian long pepper, is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spice and seasoning. Long pepper has a similar, but hotter, taste to its close relative Piper nigrum - from which black, green and white pepper are obtained. The plant is distributed from Central Himalayas to Assam, Lower hills of Bengal, evergreen forests of Western Ghats, Nicobar Islands, Uttar Pradesh and Nepal. The Indonesian or Malaysian long pepper is from Piper retrofractum. Pepper long is cultivated on a large scale in lime stone soil and in heavy rainfall areas where relative humidity. Pepper long is the dried fruit of Piper longum which is a slender, aromatic plant with creeping jointed stems and perennial woody roots. The leaves are 5-9 cm long, 3-5 cm wide, ovate, cordate with broad rounded lobes at the base. Female spikes are cylindrical, male spikes are larger and slender. Female spikes are 1.3-2.5 cm long, 4.5 mm diameter, fruits ovoid, yellowish orange, minute, and drupe and are sunk in the fleshy spike. The spike is red when ripe. It is used as a spice and also in pickles and preserves. The fruits and roots are used as medicine for respiratory disease and as counter irritant and analgesic for muscular pains and inflammation. It has carminative, haematinic and anti-helminhic properties are high.

1.3.3. *Piper nigrum*

Black pepper *(Piper nigrum)* is a flowering vine in the family Piperaceae, cultivated for itsfruit, which is usually dried and used as a apice and seasoning. The fruit, known as a peppercorn when dried, is approximately 5 millimetres (0.20 in) in diameter, dark red when fully mature, and, like all drupes, contains a single seed. Peppercorns, and the ground pepper derived from them, may be described simply as pepper, or more precisely as black pepper (cooked and dried unripe fruit), green pepper (dried unripe fruit) and white pepper (unripe fruit seeds).

Black pepper is a stout-stemmed liana (woody vine) that may typically grows to 4 m (13 ft), and may send out roots from leaf nodes if they touch the ground. The leaves, which may be rather different on climbing than on flowering stems, are typically large and heart shaped, 12.5 to 18 cm (5 to 7 in) long, with 5 to 7 prominent palmate veins. The small flowers, which are usually monoecious (with separate female and male flowers) but may be polygamous (with individual inflorescences that contain both male and female flowers), are borne on spikes that are about as long as the leaves. The fruits are small globose drupes (a fleshy fruit containing a seed with a hard, stony covering), 3 to 4 mm (less than 0.25 in) in diameter, that ripen to red. Pepper plant grows easily in the shade and requires little maintenance until harvest, so they are frequently cultivated for supplemental income on even small farms. Pepper is used in various forms. The fresh unripe green fruits may be harvested, then pickled or freeze-dried, to make green pepper. For "black" pepper, the fruits are harvested green, but then sun-dried; the skin of the unripe fruits turns black when dry. The fruits may also be allowed to ripen, after which the red skin is removed, and the stony seed sun-dried to make white pepper. Pepper is used in diverse dishes around the world. Before Europeans brought Capsicum peppers from the New World, black pepper was the primary seasoning in many Indian and south-eastern Asian dishes (Bailey *et al,* 1976)

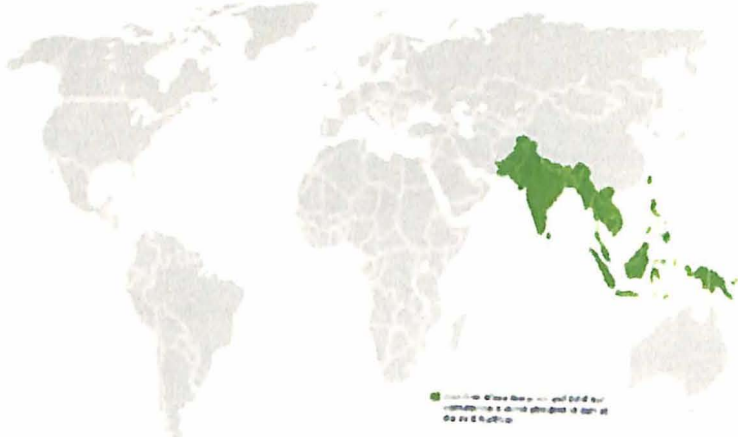
This spice with its characteristic pungency and flavour is an ingredient in many food preparations. For the common Indians, pepper is spice as well as a medicine. White pepper of commerce is also a product from the same pepper plant, produced by removing the pericarp from the ripe pepper fruits. In India black pepper is grown over area 2, 25, 327 hectares producing about 72, 465 tonnes per annum.

The productivity of black pepper in India is the lowest (322 Kg/ha) in 2007 (Directorate of Arecanut and Spice Development, Calicut, Kerala, India). Crop loss caused by Phytophthora is one of the major production constraints in black pepper. *Piper nigrum* or Black pepper oil can be used to help in the treatment of pain relief, rheumatism, chills, flu, colds, increase circulation, exhaustion, muscular aches, physical and emotional coldness, nerve tonic and fevers. It furthermore increases the flow of saliva, stimulates appetite, encourages peristalsis, tones the colon muscles and is a general digestive tonic. Sometimes it is used in place of cubebs for gonorrhoea. As a gargle it is valued for relaxed uvula, paralysis of the tongue. On account of its stimulant action it aids digestion and is especially useful in atonic dyspepsia and turbid condition of the stomach. It will correct flatulence and nausea. It has also been used in vertigo, paralytic and arthritic disorders. It has also been advised in diarrhoea, cholera, scarlatina and in solution for a wash for tinea capititis. Externally it is used for its rubefacient properties and as a local application for relaxed sore throat and some skin diseases. Its oleoresin has bacteriostatic and fungistatic properties. Black pepper is native to south India, and is extensively cultivated there and elsewhere in tropical regions. Currently Vietnam is the world's largest producer and exporter of pepper, producing 34% of the world's *Piper nigrum* crop as of 2008. In about, 1930 Japanese immigradents who travelled through Southeast Asia introduced the plant into Para state of northern Brazil, where it became a major crop. In India, Malaysia and Indonesia there is tradition of commercial cultivation by smallholders.

1.3.4. Piper betle

Betel *(Piper betle)* is the leaf of a vine belonging to the Piperaceae family, which includes pepper and kava. It is valued both as a mild stimulant and for its medicinal properties. Betel leaf is mostly consumed in Asia and elsewhere in the world by some Asian emigrants, as betel quid or in paan, with or without tobacco, in an addictive psycho-stimulating and euphoria-inducing formulation with adverse health effects. Betel is notable for staining the teeth of regular users. The betel plant is an evergreen and perennials creeper, with glossy heart-shaped leaves and white catkin. The betel plant originated from South and South East Asia There are around 2000 species of Piper betle distributed worldwide, of which 10 species are available in Nepal. Piper betle is currently distributed in Africa, western Asia, Himalaya, India,

Southeast Asia, Malaysia, China, Nepal, and Sri Lanka (Press et al, 2000). In Nepal, the plant is widely cultivated at altitudes of 150-1400 m. Piper betle is a perennial, climbing vine that has a deep green heart-shaped leaf. It is one of the most famous spices in Nepal, India, and China, and is cultivated for chewing and for traditional aesthetical uses. Piper betle is Yellowish green to dark green in colour with glossy upper surface. The betel leaves are aromatic with varied taste, ranging from sweet to pungent due to the presence of essential oils. The betel leaf is a heart shaped with different size. The size of the leaf varies with different cultivar from 7-15cm in length and 5-14cm in width. Betel leaves are simple alternate stipulate petiolate with 0.75 to 3.8cm, ovate oblong broadly ovate cordate or obliquely elliptic entire glabrous coriaceous 10 to 18 cm long and 5 to 10 cm broad acuminate oblique and rounded base (Lakshmi et al,2010). According to Ravindran (2000), most of the *Piper* species occur in the evergreen and moist deciduous forests of the Western Ghats of India. Kerala supplies about 97% of the country's pepper production and 16 Piper spp. have been recorded from the various forest ranges of the state. Leaves are carminative, stomachic, tonic, laxative, stimulant, astringent and antiseptic; used for the cure of indigestion, colic, diarrhoea, pulmonary catarrh, laryngitis, headache and cough. Leaf stalks dipped in mustard oil is applied as suppository for rectal evacuation in infants. Fruit with honey is a remedy for cough. Root is reported to develop permanent sterility in women (Yusuf *et al.*, 2009). Ethanolic extract of the leaf possesses significant activities against wide range of pathogenic bacteria and phytopathogenic fungi (Anwar *et al.*, 2007).



As biotechnological tools play important role for harnessing the production of secondary metabolites. Agro bacterium rhizogenes has been used regularly for gene transfer in many dicotyledonous plants (Tepfer *et al.* 1990). Plant infection with this bacterium induces the formation of proliferative multibranched adventurous roots at the site of infection, so called hairy roots (Chilton, 1982). In search for alternative to production of desirable medicinal compound from plants, biotechnological approach specially tissue culture are found to have potential as a supplement to agriculture in the industrial production of bioactive plan metabolites (Ramchandra Rao and Ravishankar, 2002)

On the view of the above fact, the aim of present study is in-vitro plant regeneration and comparative studies of secondary metabolites from transformed and untransformed plants of Piper species with the following objectives:

Objectives

1. To standardize the protocol for regeneration of plant through *in vitro.*
2. To standardize the protocol on genetic transformation to enhance the secondary metabolite production.
3. To estimate the different chemical compounds and piperine content in different *Piper* species by use of TLC and HPTLC method.
4. To analyse the biochemical characterization of different species of *Piper* and transformed and non-transformed plant of *Piper betle.*
5. To characterise the antimicrobial activity of the crude extract derived from leaf, root, transformed and non-transformed calli of *Piper* species.

CHAPTER-11

REVIEW OF LITERATURE

The genus *Piper* belongs to the Piperaceae and has over 2000 species distributed in both hemispheres. They are Erect or scan dent herbs, shrubs or infrequently trees. The Piper species have high commercial, economical and Medicinal importance. Economically the Piperaceae is important for the pepper in the worldwide spice markets. The ripened fruit of *P. nigrum* is the source of white pepper, while the unripe fruit of the same species is the source of black pepper. A narcotic beverage is produced in Oceania from the roots of *P.methysticum*. Several species of *Piper* are grown domestically as house plants for their foliage. Piper species, widely distributed in the tropical and subtropical regions of the world are used medicinally in various manners. Plants belonging to the genus Piper are reputed in the Indian Ayurvedic system of medicine for their medicinal properties (The wealth of India, 1969) and in folklore medicine of Latin America and West Indies. Chloroform extract of the stems of *P.* aborescens was found to display significant activity against a KB cell culture system and a P.388 lymphocytic leukaemia system in cell culture (Geran *et al,* 1972). In Jamaica, of the eleven *Piper* species known, *P.aduncum* and *P.hispidum* are listed as remedies for stomach aches and as insect repellents (Asprey et al, 1954). P. amalago, distributed from Mexico to Brazil is used to alleviate chest pains and as anti-inflammatory agent. *P.syfvaticum* roots are used as an effective antidote to snake poison in the indigenous system of Indian medicine. *P. chaba* roots and fruits find numerous applications in medicine. In particular they are useful in asthma, bronchitis, fever, pain in abdomen, as stimulant and in haemorrhoidal afflictions (Kirtikar *et al,* 1933). P. brachystachyum shows insecticidal properties (Jacobson *et al,* 1971), whereas *P. futokadsura* is a medicinal plant that grows in Fuchein and Taiwan Provinces. The stem of *P. futokadsura*, known as haifengteng is widely used in the Chinese herbal medicinal prescriptions for the treatment of asthma and arthritic conditions; the benzene extract of its leaves showed anti-feedant activity against the larvae of Spodoptera fitura. The West African black pepper (*P. guineense*) is a woody climber distributed throughout West Africa, it fruits have been used as a flavorant, while preparations of leaves, roots and seeds have been

used internally as medicinal agents for the treatment of bronchitis, gastrointestinal diseases, venereal diseases and rheumatism. The preparations obtained from the seed kernels have been used for their counter irritant and insecticidal properties (Irvine *et al,* 1961). An extract of the black pepper shows carcinogenesis in mice. The evidence of malignant tumours and of multiple tumours was greater in the pepper treated mice than in vehicle treated mice (Concon *et al,* 1979). The petrol and dichloromethane extracts of the leaves and stems of P. falconeri have shown insecticidal activity against Musca domestica (flies) and Aedes aegyptii (mosquitoes).The dichloromethane extract of *P. acutisleginum* has also been reported to show insecticidal activity against Musca domestica and Aedes aegyptii. The extract of P. betle has shown antihypertensive activity and that of P. acutisleginum has shown activity as inhibitor of aflatoxin B,-DNA binding (unpublished results from our Laboratories). The chemistry of *Piper species* has been widely investigated and the phytochemical investigations from all parts of the World have led to the isolation of a number of physiologically active compounds viz. Alkaloids amides, propenylphenols, lignans, neolignans, terpenes, steroids, kawapyrones, piperolides, chalcones, dihydrochalcones, flavones and flavanones. A lot of work is going on phytochemical investigations of Indian Piper species with an aim to develop biodegradable insecticidal compounds and antitumor drugs.

2.1. *Piper longum*

Piper longum (piperaceae) is accepted source of drugs pippali and pippalimulam throughout the country. Pippali is the fruit and pippalimulam is the root of this plant (Sivarajan and Balachandran, 1994). The plant is a dioecious slender aeromatic climber with woody root or perennial creeping under shrub. Fruit spikes cylindrical berries red or black when ripe globose with aeromatic order and pungent taste.

Piper longum L., the Indian long pepper or pipli, is the third most economically important species of the genus Piper after *P. nigrum* and *P. betle.* The plant grows extensively in the Western Ghats and north eastern parts of India and is rich in two major alkaloids, piperine and piperidine (Kanaki *et al,* 2008). In traditional and Ayurvedic medicines, mature spikes of the female plants (long pepper), thick stems, roots and leaves are used against bronchial diseases, dyspepsia, worms and

amoebiosis (Ghosal *et al,* 1996). The spikes have also been effectively used as an anti- inflammatory, memory enhancing and aphrodisiac agent (Mujumdar *et al,* 1999). When used in conjunction with a drug piperine has been shown to improve the drug's bioavailability. *P. longum* is also gained significance in processing of essential oils and oleoresins that are used as food additives, in pharmaceuticals and as insecticide.

2.1.1. Uses and benefits of long pepper

It has various uses human beings, as it helps in expelling out the mucus accumulated in the respiratory tract. It strengthens the nervous system, improves the gastrointestinal condition and normalizes the peristaltic movements and the herb serves as a good digestive agent. Its oil and paste is applied on wounds and skin-related ailments that help in suppressing pain and reducing inflammation. The herb helps maintain the normalcy of the digestive tract and tones up the urinary tract. Its fruits are used for respiratory tract diseases like cough, bronchitis, asthma and also benefits in anorexia, indigestion, flatulence, abdominal pain, hyperacidity, piles, paralysis of the tongue, diarrhea, cholera, chronic malaria, viral hepatitis, diseases of the spleen and tumours. It is given with amalaki, to treat anaemia. The decoction of the plant is used in sciatica and hemiplegic. The herb is mixed with honey to control hiccups. Long pepper is used as an aphrodisiac, since it boosts the reproductive system. It is used as a sedative in insomnia and epilepsy. The infusion of the herb's root is used after childbirth, to induce the expulsion of the placenta.

2.1.2. Chemical compositions

Other reported beneficial effects of *piper longum* include analgesic and diuretic effects, relaxation of muscles tension and alleviation of anxiety (Sunila, 2004) Piper extracts and piperine possess inhibitory activities on prostaglandin and leukotrienes COX-1inhibitory effect and thus exhibit anti inflammatory activity (Stohr et al, 2001) Recently, biochemical activities of some important medicinal plants including Piper species and their metabolites have been described (Nongyao Sawangjaroen et al, 2004). However, very little is done to elucidate the possible targets of its action. The fruits of Piper longum have been widely used since time immemorial in household spices and also in various traditional systems of medicine.

According to Ayurvedic system of medicine, P. longum fruits are anathematic, antiasthma tic, alterative, and used to treat pain, piles, insomnia, and epilepsy that is has anticonvulsant and bioavailability-enhancing properties of the drug. The fruits contain 1.0–2.5% volatile oil, 5–9% alkaloids, of which the major ones are piperine, chavicine, piperidine, and piper tine, and a resin (Nongyao Sawangjaroen et al, 2004) most of the pharmacological properties of *P. nigrum* fruits are attributed to a piperidine alkaloid, piperine, which is present in the fruits in amounts of 1.7–7.4%.. Pharmacological and clinical studies have revealed that piperine has CNS depressant, antipyretic, analgesic, anti-inflammatory], antioxidant (Sorbera et al, 2005), and hepatoprotective (Van den Brock, et al, 1996) activities. Piperine has also been shown to enhance the bioavailability of several drugs, for example sulfadiazine, tetracycline, streptomycin, rifampicin, pyrazinamide, ionized, ethambutol, and phenytoin.

2.2. *Piper nigrum*

Black pepper known as the "king of spices□, originated from Western Ghats of India is the most important and most widely used spice in the world. The black pepper of commerce is the dried, mature fruits (called berries) of the tropical, perennial climbing plant *Piper nigrum* L. It belongs to the family Piperaceae. Black pepper is a woody climber, grown in the south western region of India, comprising the states of Kerala, parts of Karnataka, and Tamil Nadu, the entire region once known as Malabar, a name now used restrictively to mean only the northern parts of Kerala. It is grown as a pure crop trained on live tree supports of *Erythrina indica or Garuga pinnata* and also as a mixed crop in coffee plantations and also on coconut and areca nut trunks.

2.2.1. Uses of black pepper

Many plant-derived molecules have shown a promising effect in therapeutics (Lokhande *et al.,* 2007). Among the plants investigated to date, one showing enormous potential is the pepper family otherwise known as Piperaceae. The fruit contain 1% volatile oil, resin, a waxy alkaloid. It is used for several medicinal properties. It has much pharmacological action such as antifungal, anti- inflammatory, antioxidant and anti cancer effect (Atal *et al.,* 1985) and it is known to have insecticidal activity against mosquitoes and flies. The plant grows all over India, in

evergreen forests and is cultivated in Assam, Tamil Nadu and Andhra Pradesh. *P. nigrum* (black pepper) is a monocious or decorous climbing vine native to Southern India and Sri Lanka and is extensively cultivated there and elsewhere in tropical regions. The short climbing stem are very flexible with leathery blackish green leaves, they are widely cultivated in the tropics. They have several uses such as they help in pain relief, rheumatism, chills, flu, colds, muscular aches and fever. Externally it is used for its rubefacient and as a local application for relaxed sore, throat and some skin disorder. It has antimicrobial ant mutagenic, antioxidant and radical scavenging property and inhalation of black pepper oil increase the reflexive swallowing movement (Vijayakumar *et al.*, 2004). *P. cubeba* (Java pepper or tailed pepper) the berries of *P. cubeba* are commonly known as cubeb. It is mostly grown in Java and Sumatra. This is a perennial plant, with a climbing stem, round branches, about as thick as a goose-quill, ash colored and rooting at the joints. The leaves are from four to six and a half inches long by one and a half to two inches broad, ovate-oblong, acuminate, and very smooth. Flowers arranged in spikes at the end of the branches; fruit, a berry rather longer than that of black pepper. It is used to treat gonorrhea, dysentery, syphilis, abdominal pain and asthma and has also inhibitory effect on hepatitis-C virus protease. Choi and Hwang (2000) demonstrated anti inflammatory and analgesic activity of methanol extract from the fruit of P. cubeba it accumulates lignans and essential oil in a relatively high amount. The alkaloids, of which some 5,500 are known, comprise of the largest single class of secondary plant substance. Alkaloids are often toxic to man and many have dramatic physiological activities; hence their wide use in medicine. They are usually colourless, often optically active substances; most are crystalline but a few (e.g. nicotine) are liquids at room temperature. Recently, many bacterial pathogens are becoming resistant to existing antibiotics due to their indiscriminate use in the treatment of infectious diseases (Davis, 1994). Therefore, there is exigency to discover new and efficient antimicrobials from other source such as plant. In the present study an attempt was made to screen different extracts prepared from dried fruit of *P. nigrum, P. longum and P. cubeba* for its antimicrobial action against gram positive and negative bacteria. The alkaloid piperdine was purified for further studies for antitumor activity.

2.2.2. Chemical composition of *Piper nigrum*

The maturity of black pepper has been studied and reported by various workers (Sumathykutty *et al*, 1983). The major constituents of black pepper were reported as starch, crude fibre and fat, but the most significant ones from the point of view of quality was reported to be the pungent principle piperine and essential oil. Sumathykutty *et al* (1983) have analysed different grades of four black pepper cultivars i.e. 'Panniyur', 'Kalluvally', 'Karimunda' and 'Kottanadan'. The volatile oil constituents of different cultivars of black pepper have been studied by many authors. (Menon *et al.*, 2000) identified 55 compounds in the oils of 4 pepper cultivars namely, 'Karimunda', 'Kulluvally', 'Arakulammunda' and 'Thommankodi' by gas chromatography (GC) and mass chromatography (MS). The main components reported were α-pinene (2.4-11.4%), β-pinene (2.0-15.27%), δ-2 carene (0.1-21.0%), limonene (9.4-21.9%) and β-caryophyllene (19.8-45.3%). Kurian *et al,* (2002) reported 5% essential oil and 9.4% oleoresin in 'Panniyur-5'. Menon et al (2002) reported α-pinene, β-pinene, δ-3- carene, limonene and β-caryophyllene as major compounds in 'Karimunda' oil. They had reported up to 45% β-caryophyllene. Menon *et al* (2003) identified 55 compounds in the oil of four major black pepper cultivars namely, 'Thevanmundi', 'Poonjaranmunda', 'Valiakaniakkadan' and 'Subhakara' and reported β-caryophyllene (20–34%), sabinene (4.5-16%), limonene (14.9-15.8%) and α-pinene (3-6.5%). Radhakrishanan et al (2004) studied 7 black pepper cultivars namely, 'Panniyur-2', 'Panniyur-3', 'Panniyur-4', 'Sreekara', 'Subhakara', 'KS-88' and 'Neelamundi' for yield and constituents such as piperine, oleoresin and essential oil. 'Panniyur-4' recorded highest yields of 2,101 kg/ha, the lowest oleoresin of 9.2%, piperine of 4.4% and contained 2.2% of essential oil. Singh Gurdip et al (2004) reported 49 components in pepper oil. Major components were β-caryophyllene (24%), limonene (16.8%), sabinene (13%), β-bisabolene (7.6%) and α-copaene (6.3%).

2.3. *Piper betle*

Piper betle Linn. is a perennial dioecious, semi woody climber. It is cultivated in Sri Lanka, India, Malay Peninsula, Philippine Islands and East Africa. The chief constituent of the leaves of this plant is a volatile oil known as betel oil. The volatile oil is bright yellow to dark brown liquid possessing a clove like flavour and consists

of terpenes and phenols. Leaves of Piper betle possess several bioactivities and are used in traditional medicinal systems. The ant diabetic property of this plant has been tested in normoglycaemic and strepozotocin induced diabetic rats. The water extract in normoglycaemic rats has shown significant lowering of the blood glucose level.

2.3.1. Uses of *Piper betle*

The family of Piperaceae belonging to super order Nymphaeiflloraea, order Piperales and *genus Piper* of family Piperaceae commonly known as pan comprises about 10 genera, 2000 species. The Genus *Piper (Piperaceae)* is largely distributed in tropical and subtropical regions of the world *(WealthAsia, 1997).* Over 700 species of Piper betel has been distributed in both of the hemispheres of world of these, 30 species have been recorded in India, 18 in Srilanka and 3 are endemic. *Piper betel* is cultivated in India, Srilanka, Malaysia, Indonesia, Philippine Islands and East Africa (Parmar *et al*, 1997). Different plant parts leaves, roots, stems, stalks and fruits have been utilized. *Piper betel* has light yellow aromatic essential oil, with sharp burning taste. Leaf posses activity like antidiabetic, antiulcer, antiplatelet aggregation, antifertility, cardiotonic, antitumour, antimutagenic, respiratory depressant and a ntihelminthic (Majumdar *et al,* 1999). *Piper betel* is used to treat alcoholism, bronchitis, asthma, leprosy and dysepsia. Earlier, antiulcerogenic activity of *Piper betel* was attributed to its antioxidative property. A preliminary study has reported *Piper betel* leaves extracts contains large numbers of bioactive molecules like polyphenols, alkaloids, steroids, saponins and tannins (Koff *et al,* 1971). The leaves extract of have also been reported to exhibit biological capabilities of detoxication, ant oxidation, and antimutation that suggested the chemo preventive potential of extracts against various ailments including liver fibrosis and carcinoma (Shun *et al,* 2007).

Free radicals play a vital role in most major health problems like cancer, rheumatoid arthritis, cardiovascular diseases, Alzheimer's disease and other neurodegenerative disorders. Antioxidant that scavenges these free radicals proves to be beneficial for these disorders as they prevent damage against cell proteins, lipids and carbohydrates (Beris, 1991). Antioxidant activity includes free radical scavenging capacity, inhibition of lipid peroxidation, metal ion chelating ability and reducing capacity. *Piper betel* leaves are also known to

contain significant amount of anti-oxidants like hydroxychavicol, eugenol, ascorbic acid and b-carotene.

2.3.2. Chemical composition

Chemical studies carried out on Brazilian Piperaceae species have revealed the occurrence of pyrones, lignoids and chromenes besides various amides bearing isobutyl, pyrrolidine, dihydropyridone and piperidine moieties. These amides have generated interest as a result of their potent insecticidal and antifungal properties. The structures of the several antifungal amides N-[7-(30, 40-methylenedioxyphenyl)-2(Z), 4(Z) heptadienoyl] pyrrolidine, (3Z, 5Z)-N-isobutyl- 8-(30, 40- ethylenedioxyphenyl) - heptadienamide isolated from leaves of Piper hispidum and 8(Z)-N- (12, 13, 14-trimethoxycinnamoyl)-3-pyridin-2-one from Piper tuberculatum besides eight known antifungal amides have already been reported. Two new amides which are N-[10-(13,14-methylenedioxyphenyl)-7(E),9(Z)- pentadienoyl]- pyrrolidine, arboreumine and nine known antifungal amides N-[10- (13,14- methylenedioxyphenyl)- 7(E)-pentaenoyl]-pyrrolidine , its derivative N-[10-(13,14 methylenedioxyphenyl)-pentanoyl]- pyrrolidine and N-[10-(13,14 methylenedioxyphenyl)- 7(E),9(E)-pentadienoyl]-pyrrolidine; besides pellitorine , abdihydropiperine , piplartine, dihydropiplartine, cis-piplartine (or 8(Z)-N-(12,13, 14- trimethoxycinnamoyl)-3-pyridin-2- one) and fagaramide have also been reported recently. In addition to these amides two cinnamoyl derivatives, methyl 6, 7, 8-trimethoxydihydrocinnamate and methyl trans-6, 7, 8- trimethoxycinnamate have also been isolated (Bernard, et al 1999) he amides isolated from leaves of Piper arboreum and their hydrogenated derivatives are active against the fungus Cladosporium sphaerospermum, and the compounds isolated from seeds and leaves of *Piper tuberculatum* are also active against the fungi *Cladosporium sphaerospermum and C. Cladosporioides.*

2.4. Piperine content

Biological source of piperine is dried unripe fruit of *Piper nigrum, piper longum and piper clusii* and also in the root of bark of *Piper geniculatum* belonging to family Piperaceae. The dried unripe fruits are extracted with ethanol in a Soxhlet apparatus till extraction is complete. The solvent is evaporated under vacuum in a Rotary Thin Film Evaporator. The residue of the alcoholic extract is digested with

dilute alkali to affect saponification, when piperine remains unaffected. The residue, thus obtained is decanted and washed with distilled water several times. The resulting product is dissolved in hot ethanol and on cooling the crystalline *piperine* separates out. It is obtained as monoclinic prisms from alcohol having mp 130°C and is tasteless at first, but has a burning aftertaste. Its dissociation constant pK (18°C) is 12.22. It is soluble in ethanol, chloroform, ether; freely soluble in acetic acid and benzene; and almost insoluble in water (40 mg/L at 18°C), and petroleum ether.

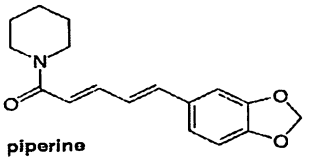


Figure 2.1 Chemical structure of *piperine*

In biosynthesis of piperine, the piperdine ring forms part of a tertiary amide moiety which is incorporated via piperidine itself i.e., the reduction product of D1-piperiedine as shown under. Interestingly, the piperic acid residue in obtained from a cinnamoyl-COA precursor. The extension of chain is caused by virtue of acetate/malonate and ultimately combines as its precursor with the previously obtained piperdine nucleus.

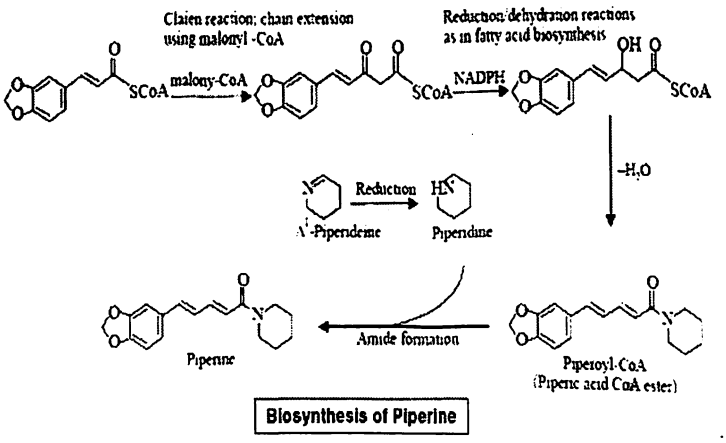


Figure 2.2 Biosynthetic pathway of *piperine*

2.5. *In vitro* plant regeneration

Plant tissue culture refers to the *invitro* culture of plants from different plant parts (tissues, organs, embryos, single cells, protoplasts, etc.) on nutrient media under aseptic conditions. In vitro cultures are now being used as tools for the study of various basic problems in plant sciences. It is now possible to propagate all plants of economic importance in large numbers by tissue culture. In vitro propagation of plants holds tremendous potential for the production of high quality plant based medicines. This can be achieved through different methods including micro propagation Plant tissue culture is widely used to produce clones of a plant in a method known as Micro propagation. Micro propagation of many plants including medicinal plants have been reported (Murashige, 1962).Tissue cultures are started from pieces of whole plants. The small organs or pieces of tissue that are used are called explants. Explants can therefore be consisting of many different kinds. Plant grow in external environment are easily susceptible to many diseases. The correct choice of explants material can have an important effect on the success of tissue culture. When cultured in vitro, all the needs of the plant cells, both chemical and physical, have to met by the culture vessel, the growth medium, and the external environment (light, temperature, etc.). The growth medium has to supply all the essential mineral ions required for growth and development. In many cases (as the biosynthetic capability of cells cultured in vitro may not replicate that of the parent Plant), it must also supply additional organic supplements such as amino acids and Vitamins. Many plant cell cultures, as they are not photosynthetic, also require the addition of a fixed carbon source in the form of a sugar (most often sucrose). One other vital component that must also be supplied is water, the principal biological solvent. Physical factors, such as temperature, pH, the gaseous environment, light (quality and duration), and osmotic pressure, also have to be maintained within acceptable limits. Numerous factors were reported to influence the success of *in vitro* propagation of different medicinal plants (Hussey *et al,* 1997).

2.5.1. Effect of Plant growth regulator on callus initiation

Callus is defined as a mass of undifferentiated loosely arranged parenchymatous cells, which are usually rapidly dividing. The explants undergo certain period of unorganized growth (callus) prior to shoot differentiation. Callus

tissue is usually produced as a result of wounding or initially the explants swelled and callus was initiated at the cut ends and spread all over the surface of explants. The first report of viable callus culture was reported by Gautheret (1939) and White (1939) in tobacco and carrot, respectively. After the ground breaking discovery that callus can be generated artificially *in vitro* (Gautheret, 1939) and that the balance between two plant hormones, auxin and cytokinin, determines the state of differentiation and dedifferentiation (Skoog and Miller), callus has been widely used in both basic research and industrial applications (George and Sherrington, 1984; Bourgardet al., 2001).

Exogenous application of auxin and cytokinin induces callus in various plant species. Generally speaking, an intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin-to-cytokinin or cytokinin-to-auxin induces root and shoot regeneration, respectively (Skoog and Miller, 1957). Since the discovery of this regeneration system, it has been widely used, for example, in the propagation of economically important traits and the introduction of transgenes. Other hormones, such as brassinosteroids or abscisic acid, also induce callus and in some species may substitute auxin or cytokinin in callus formation. However, auxin and cytokinin have been by far the most extensively used and studied hormones in the context of callus formation and subsequent organ regeneration. Mandolino and Ranalli (1999) reported occasional shoot regeneration from callus. Successful regeneration and propagation has been achieved in other fibrous plants such as sisal (Hazra *et al.*, 2002) and flax (Rakousky' *et al.*, 1999). Conventional breeding and biotechnological approaches, including tissue culture and transformation procedures, could be extended to hemp breeding.

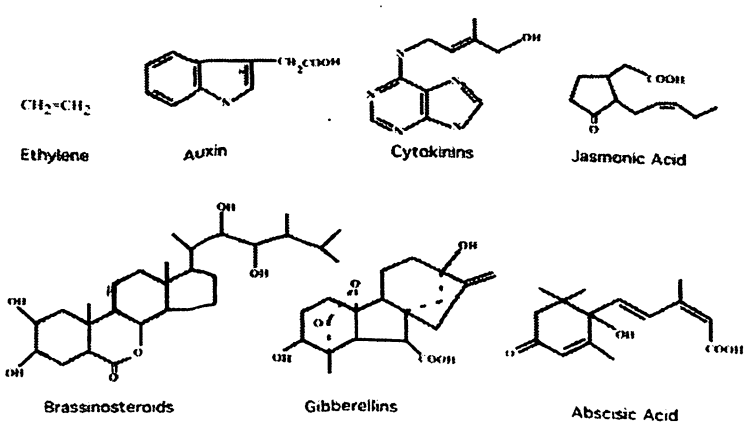


Figure 2.3. The structure of different hormone used for *in vitro* culture of plants

2.5.2. *In vitro* regeneration of *Piper* species

Conventional propagations beset with problems of poor seed viability, low percentage of germination and scanty, delayed rooting of vegetative cuttings. Therefore, there is a need for alternative propagation methods (Sarasan *et al.*, 1993). Tissue culture techniques might be applied to generate large number of clonal propagules (true to type). Not much tissue culture work has been done in this plant except some reports on regeneration (Sarasan and Nair, 1991; Sarasan *et al.*, 1993; Bhat *et al.*, 1995; Philip *et al.*, 2000). The rapid propagation of field grown plants by shoot multiplication is reported. In vitro establishment of Piper is greatly hampered by the high incidence of bacterial and fungal contamination (Fitchet, 1990; Philip *et al.*, 1992; Anand *et al.*, 1997). Regeneration protocols for many cultivated species of Piper have been reported, including callus culture and somatic embryogenesis in betel vine (Aminduddin *et al.*, 1993), micropropagation of black pepper through shoot tip cultures (Mathews and Rao, 1984; Philip *et al.*, 1992) and plant regeneration from various explants of cultivated Piper species (Bhat *et al.*, 1995). Micropropagation of *P. barberi* through shoot tip cultures raised in vitro on Woody Plant Medium (Lloyd and McCown, 1981) was described earlier by Babu *et al.* (1996).

Development of a tissue culture protocol for mass proliferation and conservation could be a viable alternative. Regeneration protocols for this plant have been reported earlier from shoot tips, root, leaf, node and internodes (Anand and Rao, 2000; Bhat *et al.* 1992; Philip *et al.* 2000; Soniya and Das, 2002). However, none of the reported protocols have been used for mass proliferation or for any conservation programme. Repeated attempts at maintaining cultures for long term has met with failure because of reappearance of bacterial infection in apparently clean cultures. Entophytes are an important source for the appearance of bacterial infection in established cultures in several plants (Thomas, 2007). Endophytes are bacterial or fungal microorganisms that colonize the intercellular spaces in the plants without causing any apparent damage to the host (Reiter *et al,* 2002). However, under in vitro conditions these bacteria may outgrow the tissue cultures causing serious loss (Thomas, 2007). Therefore, a mass proliferation protocol for this plant could not be established because of the need of initiating fresh cultures every time. This necessitates inclusion of a regular step of culture indexing using bacteriological media

for covert bacterial detection in conservation or a micro propagation programme (Panicker *et al.*, 2007).

Plant tissue culture refers to the In vitro culture of plants from plant parts (tissues, organs, embryos, single cells, protoplasts, etc.) on nutrient media under aseptic conditions. In vitro cultures are now being used as tools for the study of various basic problems in plant sciences. It is now possible to propagate all plants of economic importance in large numbers by tissue culture. Black pepper is also an important traditional medicine and used to treat asthma, chronic indigestion, colon toxins, obesity, sinus, congestion, fever (Ravindran, 2000), intermittent fever, cold extremities, colic, gastric ailments and diarrhoea. The plant grows extensively in the Western Ghats and north eastern parts of India (Prasad *et al.* 2009) and is rich in two major alkaloids, piperine and piperidine.

The germplasm of *P. nigrum* conserved in natural repositories is under serious threat from various environ- mental stresses. In addition, germplasm conservation in a seed bank is not pragmatic due to heterozygous nature induced through cuttings (Nair and Gupta, 2003). In vitro propagation methods provide powerful tools for the mass multiplication and germplasm conservation of this economically important species. To date, there have been no reports on the successful in vitro regeneration of P. nigrum from leaf explants of potted plants. However, several protocols for regeneration and somatic embryogenesis in P. nigrum from shoot tips and nodal explants have been published (Philip *et al,* 1992; Bhat *et al,* 1995; Nair and Gupta, 2003). Kapoor *et al,* 2009 recently demonstrated that the protective effect of piperine is most likely due to its anti- oxidant activity. Although a few reports on the antioxidant activity of cultivated P. nigrum are available in the literature (Aziz *et al.* 2009), there seems to be no mention of the influence of organogenesis on antioxidant activity in P. nigrum. In the study reported here, we established an in vitro system for the production of P. nigrum from leaf explants of potted plants and conducted 1, 1-diphenyl-2- picrylhydrazyl (DPPH8)-based antioxidant assays to evaluate the antioxidant activity of the main secondary metabolites in different in vitro-derived tissues. Leaves were collected from potted plants of P. nigrum maintained in the greenhouse. Leaves were sterilized according to the method of Abbasi *et al.* (2010). Briefly, the leaves were immersed first in 70% (v/v) ethanol for

60 s and then in a 0.2% (w/v) mercuric chloride ($HgCl_2$) solution for approximately 2 min, followed by three rinses with sterile distilled water. These surface-sterilized explants were placed on MS (Murashige and Skoog 1962) medium containing 30 g/l sucrose, and solidified with 8 g/l agar. Different plant growth regulators (PGRs) were added to the medium, and the pH was adjusted to 5.8. All media were autoclaved at 121^0c for 20 min. All cultures were maintained in a growth room at 25 $\pm 1^0$c under a 16/8-h light/dark photoperiod with light provided by cool-white fluorescent tubes (Abbasi *et al.* 2010). For regeneration, leaf explants were cut into reasonably sized pieces, and placed onto MS medium supplemented. There has been mention of endogenous microbial contamination causing severe setbacks to the in vitro establishment of aseptic cultures of P. nigrum (Philip *et al,* 1992; Bhat *et al,* 1995). To avoid such issues, we used the valuable protocol of Abbasi *et al,* (2010) to decontaminate leaf explants and observed no contamination in subsequent experiments. The application of this decontamination protocol significantly decreased the levels of contamination by approximately 75% (B5%; data not shown). In many of the protocols used for decontaminating leaf explants, the explants have shown sensitivity to the decontaminating agents. However, we did not observe any inhibitory effect of the decontamination protocol on regeneration. The effects of various PGRs, such as BA, GA_3, 2, 4-D alone or BA in combination with 1 mg /l GA_3 or 1 mg/l NAA on indirect organogenesis were evaluated. The leaf explants of P. nigrum used in our study responded to all of the PGRs used. The best callus induction was recorded on MS medium supplemented with 0.5 mg/l BA alone (93%) and with 1.5 mg/l BA, 1.0 mg/l NAA. Callus induction on medium containing only 2,4-D (42%) or GA_3 (32%) was significantly lower than that in medium containing the other PGRs ,and no callus induction was observed on MS medium. However, the addition BA to GA3-containing medium enhanced callus induction (70%) to levels comparable to those obtained with 1.0 mg/l BA alone and 2.0 mg/l BA and 1 mg/l NAA. In a recent study on Silybum, we reported that the addition of NAA to medium containing BA/GA_3 enhanced callus induction (Abbasi *et al.* 2010).

Callogenesis is considered to be a significant feature of indirect organogenesis and essential for research on bio- logically active molecules in medicinal plant species (Abbasi *et al,* 2007, 2010). Data on organogenesis was determined after 5 weeks of subculture. The best percent- age shooting was recorded

for explants cultured on medium containing the combination of 1.5 mg/l BA and 1 mg/l GA_3 (85%). However, the combination 2.0 mg/l BA and 1 mg/l GA_3 produced a shooting percentage of 78%, which was similar that obtained on medium containing 0.5 mg/l BA (75%). In contrast, the addition of NAA to medium already containing BA significantly inhibited shooting. Similar values have been reported for Capsicum species (Rubluo and Barroso, 1992). However, in another study on Silybum, Abbasi *et al.* (2010) made different observations regarding the incorporation of auxin in cytokinin-containing medium.

2.6. Production of secondary metabolites

Plant secondary metabolism produces products that aid in the growth and development of plants but are not required for the plant to survive. Secondary metabolism facilitates the primary metabolism in plants. This primary metabolism consists of chemical reactions that allow the plant to live. In order for the plants to stay healthy, secondary metabolism plays a pinnacle role in keeping all the of plants' systems working properly. A common role of secondary metabolites in plants is defence mechanisms. They are used to fight off herbivores, pests, and pathogens. Although researchers know that this trait is common in many plants it is still difficult to determine the precise role each secondary metabolite. Secondary metabolites are used in anti-feeding activity, toxicity or acting as precursors to physical defence systems.

Studies on plant secondary metabolites have been increasing over the last 50 years. These molecules are known to play a major role in the adaptation of plants to their environment, but also represent an important source of active pharmaceuticals. Plant cell culture technologies were introduced at the end of the 1960s as a possible tool for both studying and producing plant secondary metabolites. Different strategies, using in vitro systems, have been extensively studied with the objective of improving the production of secondary plant compounds. Undifferentiated cell cultures have been mainly studied, but a large interest has also been shown in hairy roots and other organ cultures. Specific processes have been designed to meet the requirements of plant cell and organ cultures in bioreactors. Despite all of these efforts of the last 30 years, plant biotechnologies have led to very few commercial successes for the production of valuable secondary compounds. Compared to other biotechnological

fields such as microorganisms or mammalian cell cultures, this can be explained by a lack of basic knowledge about biosynthetic pathways. More recently, the emergence of recombinant DNA technology has opened a new field with the possibility of directly modifying the expression of genes related to biosyntheses. It is now possible to manipulate the pathways that lead to secondary plant compounds (Bourgaud *et al,* 2001).

2.6.1. Strategies for the improvement of metabolite production in plant cell cultures

Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as plant leaves or stems. Strain improvement, methods for the selection of high-producing cell lines, and medium optimizations can lead to an enhancement in secondary metabolite production. However, most often trials with plant cell cultures fail to produce the desired products. In such cases, strategies to improve the production of secondary metabolites must be considered. One of the main problems encountered is the lack of basic knowledge of the biosynthetic routes, and mechanisms responsible for the production of plant metabolites. Where the productivity of the desired metabolites is limited by the lack of particular precursors, biotransformation using an exogenous supply of biosynthetic precursors may improve the accumulation of compounds. Feedback inhibition of metabolic enzymes as well as inhibition of membrane transport can be eliminated by the accumulation of synthesized products in a second phase introduced into the aqueous medium. Organ cultures often have sites of synthesis and storage of secondary metabolites in separate compartments. Elicitors, compounds triggering the formation of secondary metabolites, can be abiotic or biotic. Natural elicitors include polysaccharides such as pectin and chitosan which are also used in the immobilization and permeabilization of plant cells. Immobilization provides several advantages, such as continuous process operation, but for the development of an immobilized plant cell culture process natural or artificially induced secretion of the accumulated product into the surrounding medium is necessary (Rao and Ravishankar, 2002) Manipulation of the culture environment must be effective in increasing the product accumulation. The expression of many secondary metabolite pathways is easily altered by external factors such as nutrient levels, stress factors, light and growth regulators. Many of the

constituents of plant cell culture media are important determinants of growth and accumulation of secondary metabolites. Higher concentrations of sucrose at 5% (w/v) reduced the anthocyanin production in cell suspension cultures of *Aralia cordata*, where 3% (w/v) favoured the anthocyanin accumulation (Sakamoto *et al.*, 1995). The ratio of the ammonium/nitrate–nitrogen and overall levels of total nitrogen have been shown to markedly affect the production of secondary plant products. For example, reduced levels of NH_4^+ and increased levels of NO_3^- promoted the production of shikonin and betacyanins, whereas higher ratios of NH_4^+/NO_3^- increased the production of berberine and ubiquinone (Bohm and Rink, 1988, Nakagawa *et al.*, (1999).

2.6.2. Production of secondary metabolites from medicinal plants

Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavour and fragrance ingredients, food additives, and pesticides (Balandrin and Klocke, 1988). The search for new plant- derived chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Rao and Ravishankar, 2002). Cell suspension culture systems could be used for large scale culturing of plant cells from which secondary metabolites could be extracted. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products.In order to obtain high yields suitable for commercial exploitation, efforts have focused on isolating the biosynthetic activities of cultured cells, achieved by optimizing the cultural conditions, selecting high-producing strains, and employing precursor feeding, transformation methods, and immobilization techniques (Dicosmo and Misawa, 1995). Transgenic hairy root cultures have revolutionized the role of plant tissue culture in secondary metabolite production. They are unique in their genetic and biosynthetic stability, faster in growth, and more easily maintained. Using this methodology a wide range of chemical com- pounds have been synthesized (Shanks and Morgan, 1999). Advances in tissue culture, combined with improvement in genetic engineering, specifically

transformation technology, have opened new avenues for high volume production of pharmaceuticals, nutraceuticals, and other beneficial substances.

Large-scale plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers a controlled supply of biochemical's independent of plant availability (Sajc *et al.,* 2000) detailed the impact of specific engineering-related factors on cell suspension cultures. Current developments in tissue culture technology indicate that transcription factors are efficient new molecular tools for plant metabolic engineering to increase the production of valuable compounds (Gantet and Memelink, 2002). In vitro cell culture offers an intrinsic advantage for foreign protein synthesis in certain situations since they can be designed to produce therapeutic proteins, including monoclonal antibodies, antigenic proteins that act as immunogenes, human serum albumin, interferon, immune-contraceptive protein, ribosome unactivator trichosantin, ant hypersensitive drug angiotensin, leuenkephalin neuropeptide, and human haemoglobin (Hiatt *et al.,* 1989).

The synthesis of bioactive compounds chemically is difficult because of their complex structure and high cost. Wide variations in medicinal quality and content in phytopharmaceutical preparations have been observed. They are influenced mainly by cultivation period, season of collection. Plant tissue culture proved an important technology being used for the conservation of important plants either through organogenesis, somatic embryogenesis and genetic transformation (Sajc *et al.,* 2000). The major advantages of cell cultures includes is the synthesis of bioactive secondary metabolites independently from climatic and soil conditions. The negative biological influences that affect secondary metabolites production in the nature are eliminated (microorganisms and insects). To select cultivars with higher production of secondary metabolites with automatization of cell growth control and metabolic processes regulation, cost price can decrease and production increase (Jha *et al.,* 2003; Junaid *et al.,* 2009; Junaid *et al.,* 2010). Although the production of pharmaceuticals using plant cell cultures have been highlighted, other applications have also been suggested as a new route for the synthesis, products from plants difficult to grow, or in short supply, as a source of novel chemicals and as biotransformation systems. It is expected that the use, production of market price and structure would bring some of the other compounds to a commercial scale more rapidly and *in vitro* culture products may see

further commercialization. (Rao and Ravishankar, 2002; Junaid *et al.*,2009). Plant cell culture holds much promise as a method for producing complex secondary metabolites in vitro (Ravishankar and Venkataraman, 1993; Junaid *et al.*, 2009; Junaid *et al.*, 2010).

2.7. *Agrobacterium* mediated transformation and hairy root culture

The genus *Agrobacterium* has been divided into a number of species. However, this division has reflected, for the most part, disease symptomology and host range. Thus, *A. radiobacter* is an "avirulent" species, *A.tumefaciens* causes crown gall disease, A. Rhizogenes causes hairy root disease, and A. rubi causes cane gall disease. Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants (Birch, 1997), using *Agrobacterium*-mediated or direct transformation methods. The idea that some species cannot accept the integration of foreign DNA in its genome and lack the capacity to be transformed is unacceptable under the increasing number of species that have been transformed.

A. tumefaciens has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumour-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Nester *et al.*, 1984; Binns and Thomashaw, 1988). Virulent strains of *A.tumefaciens and A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crow gall and hairy roots, respectively. These strains contain a large mega plasmid (more than 200 kb) which plays a key role in tumour induction and for this reason it was named Ti plasmid or Ri in the case of *A. rhizogenes*. Ti plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a cis element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (vir genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced

by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The 30 kb virulence (vir) region is a regulon organized in six operons that are essential for the T-DNA transfer (virA, virB, virD, and virG) or for the increasing of transfer efficiency (virC and virE) (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995).

Agrobacterium mediated gene transfer into monocotyledonous plants was not possible until recently, when reproducible and efficient methodologies were established on rice, banana, corn, wheat and sugarcane. Reviews on plant transformation using Agro bacterium tumefaciens and the molecular mechanisms involved have been published during the last years (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995). A thorough analysis of the strategies for practical application of this methodology has been published recently (Birch, 1997). *Agrobacterium rhizogenes* infects higher plants to produce so-called "hairy roots" with altered phenotype from the wound sites. The transformed roots can be excised to establish axenic root cultures and indefinitely propagated in growth regulator free medium. The root exhibit fast, plagiotropic growth characterized by profuse lateral branching and rapid root tip elongation (Tepfer and Tempé, 1981; Chilton *et al,* 1982; Tepfer, 1984). Putatively transformed root lines can be easily screened with these morphological markers. Foreign genes can also be introduced into transformed roots by using binary vectors (Hamill *et al*, 1988). The rapid biomass accumulation in transformed root cultures is comparable, if not superior, to unorganized cell cultures and the fastest biomass doubling time is 1-day in Datura stramonium (Maldonado-Mendoza *et al,* 1993). The advantage of this transformation approach is that each primary root produced at the infection site is the result of a single transformation event a clone (Chilton *et al,* 1982). However, somaclonal variations in transformed root cultures are also known (Sevón *et al,* 1998). Transformed root cultures have been established in several species, including many medicinal plants (Tepfer, 1989).

Manipulation of the plant genome by introducing foreign genes has become a core tool in plant biology. Targets include enhancement in productivity by increasing resistance to abiotic and biotic stresses as well as fundamental studies such as identification and characterization of key regulatory genes. Plant transformation methods in use employ *Agrobacterium,* micro projectile bombardment, microinjection

and electroporation of protoplasts (Otoni *et al,* 2006). Among these, Agro bacterium-mediated plant transformation is the most extensively used method. It exploits the natural ability of Agro bacterium to transform plants to complete its own life cycle (Otoni *et al,* 2003). *A.tumefaciens* mediated transformation also leads to homologous recombination and facilitates gene knock-out. Recently, it has been shown that the host range of *A. tumefaciens* can be extended to 80 non-plant organisms, mainly fungi including yeasts but also mammalian cells, microalgae and prokaryotic cells. Although the development of methods for *Schizochytrium* transformation has advanced significantly in the past few years, it remains unknown whether *A. Tumefaciens* could mediate transformation in *Schizochytrium.*

Spontaneous and direct development of adventitious shoot buds from older regions of transformed hairy roots in hormone- free media without any callus formation is reported in a number of plant species like *Armoracia lapathifolia, Taraxacum platycarpum, Centaurium erythraea, Hypericum perforatum, T. Indica, Bacopa monnieri, Atropa belladonna, Plumbago indica, Brassica oleracea var. Botrytis, B. oleracea var. sabauda, B. oleracea var. capitata, Populus tremula, Lotus corniculatus, Ajuga reptans var. atropurpurea,* etc. These adventitious shoots when excised and cultured on hormone-free basal media regenerated into whole plants. However, culture conditions and time required for regeneration varied from plant to plant. Shoot regeneration from transformed roots can be light dependent or independent. In *A. lapathifolia* (Noda *et al,* 1991), roots maintained in dark showed induction of shoot buds on transfer to light throughout the root except the root tips, but no adventitious shoot bud formation took place in those kept in dark. Non-transformed roots rarely developed adventitious shoot buds on transfer to light. LBA 9402 transformed roots of *P. tremula* (Tzfira *et al.,* 1996) and A4 transformed roots of *B. monnieri* (Majumdar *et al.,* 2011) showed spontaneous shoot bud regeneration when cultured under 16/8 h (light/dark) photoperiod. Interestingly, LBA 9402 transformed roots of B. monnieri did not show any regeneration but spontaneously dedifferentiated into callus. In *L. corniculatus* and *P. Indica,* spontaneous shoot organogenesis is reported, when transformed roots were transferred to continuous light from dark. The hairy roots of *P. indica* did not regenerate in dark even after application of exogenous hormones to the media. Contrastingly, in *T. Indica,* 17 % of

transformed root clones are reported to regenerate shoots directly on hormone-free MS media in light independent manner.

2.7.1. *Agrobacterium*- mediated transformation in *Piper* species

Black pepper, the 'king of spices' is one of the major export earning crops. Preliminary attempts at transformation in black pepper were attempted by Sasikumar and Veluthambi (1994, 1996) and primary transformants were obtained for kanamycin resistance in the cotyledons using *Agro bacterium tumefaciens* binary vector strains LBA-4404 and EHA-105. The optimum concentration of kanamycin concentration (50 µg/ml) to completely inhibit callus formation and growth was also standardised. Sim *et al.* (1998) reported *Agro bacterium* mediated transfer with GUS marker black pepper. But regeneration of the transformed tissue has not reported. Reports are available on optimization of *Agro bacterium* mediated genetic transformation system in black pepper and related *Piper species* aimed towards development of disease resistant varieties against the most dreaded disease, foot rot, caused by Phytophthora capsici. Babu *et al.,* (2005) successfully transformed black pepper leaf tissues with osmotin gene, a pathogenesis-related (PR) protein known for inducing Phytophthora resistance. *Agro bacterium* mediated transformation was attempted using osmotin gene construct in pGV2260. Among the 70 putative transgenic regenerated, five putative transgenic showed delayed responses to infection and decreased spread of foot rot caused by Phytophthora capsici.

Asha and Rajendran (2009) reported in plant transformation in black pepper variety Panniyur 2 via pollen tube pathway using the total exogenous DNA of Piper colubrinum, a wild relative species of *Piper* resistant to Phytophthora capsici. The resulted putative transformant seeds were germinated in vitro by embryo rescue and the germinated plantlets were screened in vitro by incorporating the toxic culture filtrate of the pathogen *P. capsici* in the rooting media. The surviving putative transformant plantlets were later screened artificially for disease tolerance under ex vitro conditions. 39.21% of the putative transformants survived the screening and RAPD analysis of these plantlets showed variation in banding pattern compared to the DNA recipient parent *P.nigrum* variety Panniyur-2. Genetic transformation in black pepper showed retarded regeneration potential from mature tissues due to associated problems like high phenolic exudation and presence of endophytic fungi. A very

efficient micropropagation strategy through somatic embryogenesis developed by Nair and Gupta (2003) is promising for rapid regeneration of transformed tissues which can ease genetic manipulations of black pepper. The utility of Coat protein of Cucumber mosaic virus (CMV) gene *(Bhat et al., 2001)* in inducing virus resistance in black pepper was being studied using transgenic pathway.

Gene constructs were prepared in plant transformation vector (pBI 121) and mobilized into *Agrobacterium:* (i) Cucumber mosaic virus coat protein (CMV-CP) in sense and antisense orientation, (ii) Portion of open reading frame (ORF) III of Piper yellow mottle virus (PYMoV) in sense and antisense orientation. Putative transgenic developed in black pepper (IISR Subhakara and Panniyur 1) are under evaluation (IISR, 2011-2012). Maju and Soniya (2012) established genetic transformation system for *Piper nigrum* L. var. Panniyur-1 plants by infecting seedling derived explants with *Agrobacterium tumifaciens* strain EHA105 carrying binary plasmid pCAMBIA 1301, which contains scorable marker, β-1,3-glucuronidase and selectable marker hygromycin phospho-transferase gene (hpt) under the control of CaMV 35S promoter. Shoots were regenerated directly from the explants containing hygroysin (20 mg/ml) and analysed. PCR and GUS histochemical analysis confirmed the transformation.

2.8. Characterization of secondary metabolite through chromatographic techniques

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase.

Chromatography involves a sample (or sample extract) being dissolved in a *mobile phase* (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible *stationary phase.* A component which is quite soluble in the stationary phase will take longer to travel through it than

a component which was not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase. Techniques such as High Performance Liquid Chromatography and Gas Chromatography use *columns* - narrow tubes packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is called *elution*. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase. HPTLC is an improved method of TLC which utilizes the conventional techniques of TLC in more optimized way.

2.8.1. Analysis through Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC)

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose. This layer of adsorbent is known as the stationary phase. The phytoconstitutes of *P.nigrum and P. longum* fruits include volatile oil, other minor alkaloids such as piplartin, piperlogumine, piperidine, starch, resin and pungent alkaloid piperine (Kokate *et al,* 1994; Khare *et al,* 2006). Piperine is the main therapeutically active constituent of this plant. Because of its availability in the pure form it has been used as a characterizing compound in this study. Literature survey reveals that, various chromatographic methods such as HPTLC (Suthar *et al,* 2003), HPLC have been reported for the quantification of piperine.

Santosh *et al,* (2005) used HPLC method to quantify the piperine content in *Piper longum* and *P. nigrum* using methanol and water as mobile phase. The detection and quantification was performed at a wavelength of 345nm. Linearity of detect or response for piperine was between the concentrations 0.005% to 0.1%. The correlation coefficient obtained for the linearity was 0.998. The assay value of piperine for fruit and root of P. longum was found to be 0.879% and 0.31%. The assay value of piperine for fruit of P. nigrum was 4.5%. The recovery value of standard

piperine was 99.4%. Low value of standard deviation and coefficient of variation are indicative of high precision of the method.

2.9. Antimicrobial activity

Many focus on determining the antimicrobial activity of plant extracts found in folk medicine (Ngwendson *et al.*, 2003), essential oils (Almas *et al.*, 2001) or isolated compounds such as alkaloids (Klausmeyer *et al.*, 2004), flavonoids, sesquiterpene lactones, diterpenes (*El-Seedi et al.*, 2002), among others. Some of these compounds were isolated or obtained by bioguided isolation after previously detecting antimicrobial activity on the part of the plant. Secondary metabolites synthesized by plants serve as defence mechanism against microorganisms (Cowan, 1999). The effect of plant extract on bacteria has been studied by a large number of researchers in different parts of the world (Reddy *et al*, 2009; Maheshwari *et al*, 1986).

Antibiotics were produced by pharmacological industries in the last three decades. However, these antibiotics have failed to discourage the growth of many bacteria that have genetic ability to transmit and acquire resistance to drugs. Thus, infections with these bacteria are associated with high morbidity and mortality especially with immune compromised patients. Biological effects of these plants on prokaryotic and eukaryotic organisms have been discussed (Bakkali, 2008). Many studies all over the world have been showed that these plants and their extract have multi-antimicrobial properties. While 25 to 50 % of current pharmaceuticals are derived from plants, none is used as antimicrobials (Cowan, 1999). On the other hand, black pepper inhibits the expansion of genes encoding the nitric oxide synthase (iNOS) and the cyclooxygenase-2 (COX-2) (Mueller *et al*, 2010). The iNOS and COX-2 stimulate the production of many pro-inflammatory mediators such as (Interleukin-4 (IL-4), Interleukin-10 (IL-10), Interleukin-13 (IL-13), interferon-alpha (α-IFN)), and the transformation growth factor β-TGF (Hanada and Yoshimura, 2002; Makarov, 2000).

CHAPTER-111

MATERIAL AND METHODS

The present study was carried out at Department of Agricultural Biotechnology, College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar, Odisha.

Selection of plant: Different *Piper* species were selected for the study i.e *Piper betle, Piper longum, Piper nigrum and Piper chaba.*

Taxonomy of Plants:

Kingdom	Plantae
Subkingdom	Viridaeplantae
Division	Tracheophyta
Class	Magnoliopsida
Order	Piperales
Family	Piperaceae
Genus	*Piper* L.
Species	*Piper betle, Piper longum, Piper nigrum, Piper chaba.*

3.1. MATERIALS

3.1.1. Plant material

The different plant species of *Piper* were collected from the Medicinal garden of All India Coordinated Research Project on Medicinal and Aromatic Plants (AICRP), Orissa University of Agriculture & Technology, in the month of August - September 2013 and was identified and authenticated by Dr. G. Das, Associate Professor and officer in-charge, AICRP, OUAT.

3.1.2. Explants source

Leaf, shoot tip, root and nodal explants were collected from the field grown plants of *Piper* species i.e. *Piper longum, Piper betle and Piper nigrum* and were used for *in vitro* studies. The experiment was carried out in the laboratory of Department of Agricultural Biotechnology, College of Agriculture, Bhubaneswar.

3.1.3. Plant nutrient media

For *in vitro* studies, two basal media such as Murashige and Skoog (MS) and Woody Plant Medium (WPM) were used to carry out the different *in vitro* experiments. Woody Plant Medium (WPM) (1978) as well as Murashige and Skoog (1962) basal salts (MS) were used supplemented with different concentration of auxins and cytokinins.

3.1.4. Plant growth regulators

For standardization of *in vitro* shoot multiplication, rooting and plant regeneration, different auxins like indole-3-acetic acid (IAA), 1-naptheleneaetic acid (NAA) and 2, 4-dichlorophenoxy acetic acid (2, 4-D) and cytokinins like 6-benzylaminopurine (BAP) and kinetin (Kn) at different concentration were used. The solution was prepared as given below.

Preparation of stock solution

3.1.4.1. BAP: 40 mg of 6-benzylaminopurine initially dissolve in 500µl of 0.1 N HCl and volume makeup to 80 ml with sterile distilled water.

3.1.4.2. Kn: 40 mg of kinetin initially dissolve in 500µl of 0.1 N HCl and volume makeup to 80 ml with sterile distilled water.

3.1.4.3. 2, 4-D: 40 mg of 2, 4-D initially dissolves in 500µl of 100% ethanol and volume makeup to 80 ml with sterile distilled water.

3.1.4.4. NAA: 40 mg of 1- naphthalene acetic acid dissolve in 500µl of 100% ethanol and volume makeup to 80 ml with sterile distilled water.

3.1.4.5. IAA: 40 mg of indole-3-acetic acid dissolve in 500µl of 100% ethanol and volume makeup to 80 ml with sterile distilled water.

All the prepared solution was kept in the freeze for further use.

3.1.5. Antibiotics (source: M.P. Biomedical)

Both Carbenicillin and Cefotaxime were used for the experiment were purchased from EMERK, Biosciences, India and appropriate stocks were prepared. Stocks of antibiotic were prepared as given below:

Carbenicillin: 1gm of carbenicillin was dissolved in 50% ethanol. Filter sterilized by using syringe filter into sterile eppendorf tube aseptically and store at 4^0c. Cefataxime: 1gm of cefataxime was dissolved in double distilled water. Filter sterilized by using syringe filter into sterile eppendorf tube aseptically and store at 4 ^{0}c.

3.1.6. Antimicrobial activity

The bacterial strains *Escherichia coli, Micro-coccus and S. Epidermidis* species and fungal strains *Aspergillus Niger, A. Flavus and R.Solani* were collected from germplasm centre of Department of Microbiology, college of Basic Sciences and Humanities, OUAT, Bhubaneswar. Both pure culture of bacterial strains and fungal strains were used for antimicrobial activity.

3.1.7. Preparation of standard solution "Piperine" for quantification analysis.

Piperine was purchased from M/S Sigma, USA. The standard solution was prepared as 1mg/ml.

3.1.8. Agrobacterium strain and plasmid used

Agrobacterium rhizogenes (A4) strain harbouring root inducing plasmid (Ri-plasmid). The culture medium for *Agrobacterium* A4 strains are: Nutrient agar (PH 7.2), Incubation time: 24 hours, temperature -28^0c, Subculture-30 days

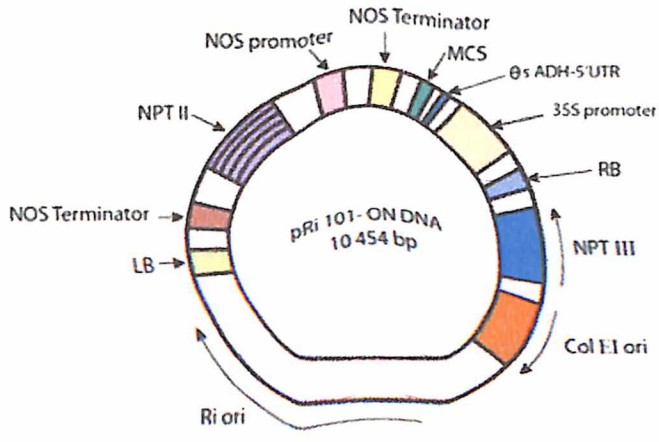


Fig. 3.1 Schematic map of ri plasmid

3.2. Sterilization

3.2.1 Sterilization of glassware and Media

Sterilization refers to any process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses, spore forms, etc.) from a working surface, equipment or biological culture medium. Sterilization can be achieved through application of heat, chemicals, high pressure or filtration.

3.2.2 Steam sterilization

A widely-used method for heat sterilization is the autoclave. Autoclaves commonly use for steam sterilization at 121^0C. To achieve sterility, a holding time of at least 15 minutes at 121^0C. Additional sterilizing time is usually required for liquids and instruments packed in layers of cloth, as they may take longer to reach the required temperature. After sterilization, autoclaved liquids kept in the media preparation room for normal temperature for further use.

3.2.3 Filter sterilization

Hormones that would be damaged by heat irradiation can be sterilized by mechanical filtration. This method is commonly used for sensitive pharmaceuticals and protein solutions in biological research. A filter with pore size 45µm will effectively remove bacteria. The filtration equipment and the filters themselves may be purchased as presterilized disposable units in sealed packaging, or must be

sterilized by the user, generally by autoclaving at a temperature that does not damage the fragile filter membranes.

3.2.4 Glassware's used

Glassware's like culture tubes (25×150 mm) conical flasks (250 ml) petriplates (80 mm) beakers (500ml) etc., were purchased from Borosil (India) and used for the experiments. All the chemicals and plant growth regulators were of analytical grade and are procured from standard chemical manufacturing companies.

3.2.5 Cleaning of glassware's

Glassware's were rinsed in water and then soaked in 0.15% chromic acid overnight. The chromic acid was drained out and the glassware's were washed with clean soap solution. The thoroughly washed glassware's are rinsed in distilled water and dried in a hot air oven. The instruments like forceps, scalpels etc., were also cleaned and dried.

3.2.6 Sterilization of glassware's

Clean glassware's were rinsed in double distilled water and dried in oven at 80^0C and sealed with aluminium foil, petriplates placed in autoclavable covers small instruments like scalpel, forceps, and blade holders wrapped in aluminium foil were autoclaved at 121^0C in 17 lbs pressure for 15 minutes. The glassware's were then transferred to sterile inoculation chambers.

3.2.7 Disinfection of laminar- flow chamber

All steps in this experiment like the sterilization of explants preparation, inoculation of the explants, sub culturing were conducted under aseptic condition in the laminar- flow cabinet. Before the laminar flow cabinet was used, the working surface of the chamber is sterilized by swabbing with 70% alcohol. The chamber was then exposed to UV light for 15 minutes. The walls of the chamber were also swabbed with 70% alcohol to ensure total sterility. Before taking the materials into cabinet, they were swabbed with 70% alcohol. In case of glassware's, the mouth of the bottles,

Composition of basal MS medium

MACRO SALT	(mg/l)
KNO_3	1900
$NH_4 NO_3$	1650
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2o$	440
$KH_2 PO_4$	170
MICRO SALTS	**(mg/l)**
$MnSO_4.\ H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025
Na_2Fe-EDTA	37.24

ADDITIVES	**(mg/l)**
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	30000

Growth regulators like 6-benzylaminopurine (BA), 3-indolebutyric acid (IBA), kinetin (Kn), adenine sulphate (Ads) were added to the basal medium either singly or in various combinations. The concentrated stock solutions of all the ingredients were prepared and stored under refrigeration. To prepare stock solution of micro salts, all the micro salts in required quantities were dissolved in one litre of distilled water and used as stock solution. Likewise stock solutions of all other ingredients are also prepared and kept under refrigeration. Similarly stock solutions of

flasks etc. are flamed before and after use. Before starting the experiment, the hands are swabbed well with alcohol.

3.3. Preparation of explants

For the surface sterilization, the explants first were washed thoroughly in running tap water for 30 minutes. After that they were again washed with liquid detergent (Labolene, Qualigen, India) and Tween 20 (Himedia Laboratories, India) for 10 minutes with vigorous shaking. After washing with detergent, the explants were again washed with running tap water to remove any traces of detergent for 30 minutes and kept in 1% w/v solution of Bavistin (BASF India Limited) for 15 min. Further, the explants were taken out and dipped in 70% ethyl alcohol for 30 seconds. After alcohol dip, explants were surface sterilized with freshly prepared 0.1% (w/v) aqueous solution of mercuric chloride for 5 minutes. Subsequently, the explants were thoroughly washed with sterile distilled water for 2 - 3 times to remove any traces of mercuric chloride.

3.4. Preparation of media

The basal medium used for the culture is Murashige and Skoog medium (MS, 1962) with sucrose 3% (Analytical grade, Himedia, India) and 0.8% agar (Bacteriological grade, Himedia, India).

Table 3.1 Amount of stock solutions added to the media

S no	Stock solution	Strength	Amount to be added (ml)
1.	Macronutrients	20X	50
2.	Micronutrients	1000X	1
3.	Iron source	200X	5
4.	Organic supplements	1000X	1

Agar (8g/l) and Myoinositol (100mg/l) were added separately

growth hormones were also prepared. Cytokinins were dissolved in few drops of acidic solutions (0.1N HCl) and Auxins were dissolved in few drops of basic solutions (0.1N KOH), after dissolving final volume is made up with the help of distilled water and kept at 4^0c.

The medium was prepared by adding required quantities of all the ingredients in the conical flask. After adding all the ingredients in required amounts, the final volume is made up with the help of distilled water. pH of the medium was adjusted to 5.8 by using 0.1N KOH or 0.1N HCl. After adjusting the pH, agar (Himedia Labs Limited, India) was added to the medium at the rate of 0.8% (w/v) for solidification of the medium. For preparing liquid medium (wherever used) agar was not added to the medium. After pouring media (25 ml culture tube & 100 ml flask) were tightly capped and labelled properly. After that media was autoclaved at 121°C for 20 min at 17psi.

3.5. Standardization of regeneration protocol

3.5.1. Culture conditions

All the *In vitro* culture experiment was carried out aseptically in a laminar airflow chamber and the culture were incubated in the culture room maintained at 25 ± 2^0C, under 3000 lux intensity with fluorescent tubes over 16 h photoperiod.

3.5.2. Inoculation of explants

The sterilized explants (leaf, stem and root) were inoculated in culture tubes, flask and bottles aseptically. For inoculation explants were transferred to large sterile glass flask with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed to make them in suitable sizes. Trimming and leaves were removed with sterile scalpel blade. After cutting explants into suitable size (1-2cm), explants are transferred to culture bottles containing MS medium with various concentration of auxins and cytokinins. After vertically inoculating the explants in the culture vessel the mouth of tubes, flask and bottle was quickly flamed and tightly capped to avoid entry of external air. After proper labelling, clearly mentioning media code, date of inoculation etc. the bottles was transferred to growth room.

3.5.3. Callus induction

The different explants were cultured on MS medium supplemented with different growth regulators and incubated in culture room for callus induction. Observations were recorded on days to callus initiation, number of explants responding, type of callus, and colour of callus, visual callus quality and percentage of callus induction.

Response of explants to callus initiation was assessed by calculating number of explants responded for callus initiation and expressed in percentage.

$$\text{Percent callus induction} = \frac{\text{No. of explants with callus initiation}}{\text{Total no. of explants cultured}} \times 100$$

3.5.4. Shoot bud proliferation

For shoot proliferation, BA (0.25 – 4.0 mg/ml) and IAA (0.25-2.0 mg/ml) at different concentrations in combination with adenine sulphate (100 mg/ml) and agar (0.8%) were used. After 4 weeks of culture period the newly growing shoots were separated under aseptic conditions with help of sterile scalpel blade and sterile forceps and inoculated into new tubes/bottles containing fresh solid MS basal medium with different set of growth hormones as mentioned earlier. Two shoots per culture bottle were used and 2-3 replicates per treatment were also used. Data were recorded every 15 days of culture and only shoots greater than 0.5cm was considered for taking data. Every possible care has been taken to prevent any further contamination.

3.5.5. Rooting of microshoots

Newly grown shoots measuring about 1 - 2cm in length were excised individually from the parent culture and transferred to different rooting media. Three types of rooting medias were used one MS basal media without hormone and other MS basal media with hormone IBA or NAA. Both liquid as well as solid mediums were used for the experiment. Data were recorded after 15 days interval.

3.5.6. Acclimatization

After 4 weeks of culture on rooting media, the plantlets were shifted to plastic pots for their hardening prior to final transfer to soil to natural conditions. For hardening of plants, plants with newly formed roots were taken out from the culture

bottles with the help of forceps with utmost care to prevent any damage to newly formed roots and washed in tap water to remove the agar gel medium. After removing media, plants were dipped in 1% w/v solution of bavistine to prevent any fungal infection to newly developed plants. After bavistine treatment the plantlets were carefully planted in plastic pots containing 1:1 mixture of soil and farmyard manure. After planting the plants were thoroughly watered and kept under playhouse with 80% humidity and 31°C temperature for ten days. There after the plants were shifted to shade house with less humidity level and indirect sunlight. In shade house, watering was made in everyday two times to prevent wilting.

3.6. Optimization for efficient *Agrobacterium rhizogenes* mediated transformation method

3.6.1. Preparation of explants for the co-cultivation

Young leaves were excised from *in-vitro* grown shoots cut into small segments (0.5 x 0.5 cm) and friable callus was used as explants for transformation.

3.6.2. Bacterial culture preparation

Agrobacterium rhizogenes A4 strain was used for hairy root induction in plants. The bacteria were maintained on nutrient agar medium. The bacteria were streak on fresh plates and incubated in 28°c. After 24 h, the single colony was transferred to the broth medium and cultured for 48h in the dark on a rotary shaker at 90 rpm. Bacterial count was measured by OD at 620nm. The OD must be 0.5 - 0.6 i.e. (10^6 cells/ml). The bacterial suspension was centrifuged at 6000 rpm for 10 min and pellet was collected for co-cultivation.

3.6.3. Co-cultivation with the explants/calli

The prepared explants were co-cultivated with Agrobacterium. The pellet was suspended in Liquid Plant Growth Medium (LPGM) along with 100µl Acetosyringone. The explants were injured with sterile needle and co-cultivation wrapped with aluminium foils and incubated at 25°c for 30 min at 90 rpm. After 30 minute, the infected explants were taken out and blotted dry on sterile filter paper and

transferred to petridish on blotting paper moistened with LPGM (liquid plant growth medium) along with acetosyringone for incubation for a period of 48-72 hrs at 25^0C.

3.6.4. Explants washing

After 2 days of incubation, the infected explants were washed with sterile distilled water for three times followed by washing with Carbenicillin / cefotaxime (0, 100,200, 300, 400 and 500 mg/l) then the washed explants were blotted on sterile filter paper. The individual explants were transferred onto basal MS medium placed in the dark at 25 ± 2^0C.

3.6.5. Establishment of hairy root culture

After about 4 weeks, explants develop roots at infected sites; they were then subculture again in MS basal medium containing 200 mg/l carbenicillin. Before transferring to solid media, the explants were rinsed with MS liquid medium containing carbenicillin / cefotaxime (0, 100, 200, 300, 400 and 500 mg/l). They were subcultured at every 14 days interval. Hairy root was excised and transferred to MS liquid medium and kept in rotary shaker at 90 rpm for 30 days. The cultures were harvested and secondary metabolites were determined.

3.7. HPTLC analysis

3.7.1. Material

Prepared TLC plates were purchased from EMerck Bioscience (India). The standard piperine was purchased from M/S Sigma, USA.

3.7.2. Preparation of extract of callus, leaf, fruit and root

The callus, leaf and root samples were air dried and were macerated in mortal and pestle to obtained a fine powder. The powdered plant material were loaded in the soxlet apparatus and then fitted into a round flask containing methanol. The solvent was boiled gently (60-80) over a heating mantle using adjustable rheostat. The extraction was continued until complete extraction was done (8-10 hrs). After extraction, collect the extract and allow the methanol to evaporate. A dry mass of

extract is weighed and make a stock of 1mg/ml concentration. This was used for HPTLC analysis for presence of standard piperine.

3.7.3. HPTLC analysis

3.7.3.1. Calibration of standard graph

Stock solution (1mg/ml) of standard compound piperine was prepared individually in methanol and different concentration was spotted onto TLC plates in order to prepare the calibration graph.

3.7.3.2. Chromatographic analysis of the samples

Aluminium plate pre-coated with silica gel 60 F254 TLC plates (10x10cm) (EMerck, India) was used as a stationary phase. A Linomat IV (Camag, Muttenz, Switzerland) automatic TLC applicator was used to apply samples and standards onto the TLC plates under a flow of nitrogen gas. The application parameters were identical for all analysis performed and the delivery speed of the syringe was 10s/µl. The extract and standard solution were applied 1.5cm away from lower edge of the plate with the help of micro capillary tube. The solvent system used to identify piperine was Toluene: Ethyl acetate (7:3 v/v) under laboratory condition. The loaded plates were then placed vertically in the chamber previously saturated with solvent system for 30 min. After the solvent moved about 90% of length, the plates were taken out and dried at room temperature.

Developed plates were dried in stream air and immersed in 5% concentrated ethanolic sulphuric acid. After drying, the plates were heated at 110^0c for 15-20 min. To develop the colour of the spots. For quantitative determination, spots corresponding to standards were scanned using a Camag TLC Scanner 3 at 254 nm and 343 nm wavelength with a slit size of 6 x 0.4mm.

The Rf values were calculated using formula

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

3.8. Plant extracts preparation

3.8.1. Material:

The leaf, root, fruit, transformed and non-transformed calli extracts were prepared by extraction with appropriate solvent using Soxhlet apparatus.

3.8.2. Crude plant extracts preparation

100 mg/ml of the various extract were prepared in methanol. From this stock, different dilution was prepared.

3.9. Phytochemical analysis of extracts

The following procedures were adopted for analysing the presence of various chemical constituents in the fractions.

Test for Steroids – The salkowski test (Ganguly *et al.*, 1975) was adopted to analyse the steroid content in various species of Piper. Chloroform (2 ml) and 2 ml of concentrated sulphuric acid were added to the 2 ml of test solution, shaken and allowed to stand. Change in the colour of lower chloroform layer to red and acid layer to greenish yellow fluorescence indicates the presence of steroids.

Test for Triterpenoids - The salkowski test (Muller, 1967) was adopted to analyse the Triterpenoids content in various species of Piper. Concentrated sulphuric acid (2 ml) was added to 2 ml of test solution. The solution was shaken and allowed to stand. The colour of lower layer changes to yellow indicating the presence of triterpenoids.

Test for Glycosides - The Keller-Kiliani test was adopted to analyse the Glycosides content in various species of Piper. Glacial acetic acid (3 - 5 drops), one drop of 5% $FeCl_3$ and conc. sulphuric acid were added to the test tube containing 2 ml of total solution. Appearance of reddish-brown colour at the junction of two layers and bluish green in the upper layer indicates the presence of glycosides.

Tests for Saponin -The Foam Test (Tadhani and Subhash, 2006) were adopted to analyse the saponin content in various plant species of Piper. Powdered extract (10-20 mg) was shaken vigorously with water (1ml). Development of persistent foam which is stable at least for 15 minutes indicates the presence of saponin.

Tests for Carbohydrates - The Molisch's test was adopted to analyse the carbohydrates content in various plant species of Piper. 3 ml of Molisch's reagent was

added to the 3 ml of test solution, shaken for few minutes. Then 2 ml of concentrated sulphuric acid was added slowly from the sides of the test tube. The development of a purple ring at the junction of two liquids indicates the presence of carbohydrates.

Tests for Alkaloids -The Dragendorff's test (Concon et al., 1979) was adopted to analyse the alkaloids content in various plant species of Piper. 3 ml of the test solution was mixed with Dragendorff's reagent (potassium bismuth iodide). Appearance of reddish brown precipitate indicates the presence of alkaloids.

Tests for Flavonoids- The Ferric-chloride test (Voesgen et al., 1980) was adopted to analyse the flavonoids content in various plant species of Piper. Test solution with few drops of ferric chloride solution shows intense green colour indicating the presence of flavonoids.

Tests for Tannins - The Ferric-chloride test (Trease and Evans, 1983) was adopted to analyse the tannins content in various plant species of Piper. 3 ml of test solution treated with few drops of ferric chloride solution. Development of dark colour indicates the presence of tannins.

Test for Proteins The Biuret test (Ferdinand Rose, 1833) was adopted to analyse the protein content in various plant species of *piper.* 3 ml of the test solution was treated with 4% sodium hydroxide (3-5 drops) and 1% copper sulphate solution (3-5 drops). The appearance of blue colour indicates the presence of proteins.

Plant constituents	**Test/Reagent**
Alkaloid	Dragendorff's
Steroids	Salkowski test
Triterpenoids	Salkowski test
Glycosides	Keller-Kiliani
Saponin	Foam
Carbohydrates	Molish's
Flavonoids	Ferric-chloride
Tannins	Ferric-chloride
Proteins	Biuret

3.10. Growth and maintenance of test microorganism for antimicrobial studies

Bacterial cultures like *Escherichia coli, Micrococcus* and *S. epidermis* and fungal culture like *Aspergillus Niger, A. Flavus, Fusarium, pencellium* and *R. solani* were obtained from the Department of Microbiology, College of Basic Sciences and Humanities, Orissa University of Agriculture and Technology, India and were used for antimicrobial test. The bacterial culture was maintained on nutrient broth (NB) at 37^0 C and fungus was maintained on Potato Dextrose Agar (PDA) at 28^0 C.

3.10.1. Antibacterial susceptibility test

The leaf, fruit, root and callus extracts were tested on the test bacteria using the paper disc diffusion method. The nutrient agar plates were seeded with the 24 hr broth culture (10^5 cfu /ml) of the bacteria was aseptically introduced and was poured in the pre-sterilised petridishes and was allowed to solidify. The sterile paper discs (5mm) were soaked in the leaf extract of different concentration viz. 20 mg/ml, 40mg/ml, 60mg/ml for 2 hours. The paper discs containing the extracts were placed at different area on the surface of each plate. The plate was incubated at 37^0c for 24 h.

3.10.2. Antifungal susceptibility testing

The leaf, fruit, root and callus extracts were tested on antifungal testing by using potato dextrose agar plates. The PDA plates were seeded with fungal spores and were poured in the pre-sterilized petridishes and were allowed to solidify. The sterile paper disc (5mm) were soaked in the leaf extract of different concentration viz. 20 mg/ml, 40mg/ml, 60mg/ml for 2 hours. The paper disc containing the extract was placed at different areas on the surface of each plate. The plates were incubated at 28^0 C for 24hr. A disc soaked in methanol was used as control. Antimicrobial activity of the extract against the test bacteria and fungus was indicated by growth – free "zone of inhibition" near the respective disc. Two replications per test.

3.11. Statistical analysis

As all the studies were done in laboratory under controlled condition with maintaining temperature, light intensity and photoperiod. All the experiment is completely randomized design (CBD). For accurate statistical analysis, each experiment was repeated. All the data were analyzed using ANOVA test, used for comparisons among means.

CHAPTER-1V

RESULTS AND DISCUSSION

Medicinal plants are gaining great interest in pharmaceutical industries for the production of high valued secondary compounds (Rout *et al.*, 2000: Das and Rout, 2002). There has been significant rise in number of various studies on *Piper* species around the globe. *Piper* has recently attracted the attention of researchers because it has a wide range of medicinal and pharmacological application. In traditional medicine mature spike of female plant thick stem and roots is extensively used in the treatment of bronchial disease, worms, amobiasis and aphrodisiac agent. It is widely used in pharmaceutical and food industry. Piper plants are important sources for research on and development of new anticancer agents.

In vitro plant development is considerable to be completely dependent upon the species. Single cells of the tissue can regenerate into whole plant (totipotency) through shoot tip, leaf, roots and axillary buds or stem segment. Both intrinsic and extrinsic factors that influence the morphogenic response of explanted tissue under culture condition. These factors were either physical or physiological in nature depending upon the type of explants used, media composition and culture environment.

The culture media contains inorganic and organic salts (macronutrients, micronutrients and vitamins) as well as energy source (sucrose), growth regulators and agar-agar are used as nutrient source to plant. In most of the cases, Murashige and Skoog (1962) basal medium was used for plant growth and development. Growth regulators like auxins and cytokinins were used for shoot proliferation, adventitious shoot development, root induction and callus proliferation. Shoots produced through *in vitro* are generally easy to induction of root by manipulating the growth regulators, nutrient media and culture condition. The resulting rooted shoots are transfer to greenhouse for acclimatization and subsequently transferred to field condition. *In vitro* culture systems are also been adapted for mass multiplication of various plant species including medicinal and aromatic plant for propagation, conservation and utilization of secondary metabolites.

4.1. *In vitro* regeneration.

The standardization of protocol on an efficient plant regeneration is utmost important for successful commercialization of plant and also crop improvement through genetic transformation. The present investigation deals with the induction of shoot multiplication either from apical shoots or axiliary buds by manipulating growth regulators and physiological condition. Plant growth regulators concentrations in the medium and additional media amendments also play a determining role in morphogenesis (Narayanaswamy, 1977).

Table. 4.1. Effect of different concentrations of cytokinins on shoot proliferation from shoot tip explants of *Piper longum* after 8 weeks of culture.

MS+ growth regulator mg/l			% of response (Mean± SE)*	No. of multiple shoots/culture (Mean± SE)*
BAP	Kn	Ads		
0	0	0	0.0	0.0
0.25	0.5	100	13.6±0.6 a	1.5±0.2 a
0.50	0.5	100	16.8±0.3 b	1.6±0.3 a
1.0	0.5	0	23.3±0.4 c	1.4±0.4 a
1.5	0.5	100	23.4±0.5 c	1.6±0.5 a
2.0	1.0	0	56.6±0.6 i	1.4±0.4 a
1.5	1.0	100	43.3±0.5 f	1.4±0.6 a
2.0	1.5	100	46.6±0.6 g	2.0±0.7 b
0.0	1.5	0	34.3±0.7 d	2.4±0.6 c
0.50	1.5	100	33.3±0.8 d	2.3±0.4 c
1.0	1.5	100	36.6±0.6 e	2.4±0.6 c
1.5	1.5	100	46.8±0.5 g	2.2±0.5 b
2.0	**1.0**	**100**	**66.2±0.4 k**	**2.8±0.5 c**
1.5	1.0	100	63.4±0.3 i	2.6±0.6 c
2.0	1.0	100	43.3±0.5 f	2.5±0.8 c
0	2.0	0	36.6±0.7 e	1.8±0.4 b
3.0	-	100	56.5±0.6 i +	1.6±0.5 a+
3.5	-	100	46.6±0.5 g +	1.8±0.6 b+
3.5	2.0	100	53.3±0.4 h+	1.4±0.5 a+
4.0	2.0	100	56.8±0.6 i+	1.2±0.4 a+

*15 replicates/treatment; repeated thrice;
+ - Callusing at the basal end
Mean followed by different letters are significantly different at the 5% level.

4.1.1. Effect of cytokinins on shoot multiplication.

Three cytokinins (BAP, Kn and Ads) have been tested for shoot multiplication from apical and axillary shoots of *Piper longum*, *Piper betle* and *Piper nigrum*. Among the three cytokinins, 6-benzylaminopurine and kinetin favoured maximum rate of shoot proliferation (Tables 4.1, 4.2 & 4.3). Without cytokinin, the shoots did not show any positive response. The medium supplemented with BAP, Kinetin and adenine sulphate showed the maximum rate of shoot growth and multiplication. Among the different concentrations of nutrient tried, the full strength MS medium supplemented with BAP, Kinetin and adenine sulphate showed significant response as compared with other treatment. The explants initially exposed to tap water for longer period and subsequently transferred to culture medium showed the positive impact on shoot growth. The higher concentrations of BAP (2.0 mg/l) along with 1.0 mg/l kinetin and 100 mg/l adenine sulphate showed higher response in case of *Piper longum* (Table 4.1, Fig. 4.1A). However, the medium having 2.0 mg/l BAP + 0.5 Kn and 2.0 mg/l Kn + 1.5 mg/l BAP + 100 mg/l adenine sulfate favoured shoot multiplication in *Piper betle* and *Piper nigrum* respectively (Tables 4.2 and 4.3). Low concentration of BAP was found to be ineffective for shoot induction. The shoot of *Piper longum* showed maximum percentage of shoot multiplication (66.2%) on MS medium supplemented with BAP 2.0 mg/l along with 1.0 mg/l kinetin and 100mg/ml of adenine sulphate. Increase the concentration of from 0.5 to 4.0 mg/l BAP showed increase in the percentage of response but the number of multiple shoots per culture was reduced. The average number of shoots varied from 1.2 - 2.8 per culture. In *Piper betle*, the percentage of multiple shoot formation was 40.4% in MS medium supplemented with 2.0 mg/l BAP along with 0.5 mg/l Kn. The average number of shoots varied from 1.0 - 2.3 shoots per culture (Table 4.2). In case of *Piper nigrum*, the maximum percentage of multiple shoot formation was 36.4 % on MS medium supplemented with 2.0 mg/l Kn along with 1.5 mg/l BAP and 100mg/ml of adenine sulphate. The average number of multiple shoots per culture was ranged from 1.0 - 2.4 (Table 4.3). The combinations of cytokinins favour the shoot proliferation and elongation. It indicates that cytokinin/auxin ratio being the principal players in the induction of shoot multiplication from explants. Similar observations

indicating the effect of cytokinin and auxin on shoot multiplication were previously reported in *Clerodendrum colebrookianum* (Mao *et al.* 1995), *Plumbago* (Rout *et al.* 1999), *Lawsonia inermis* (Rout *et al.* 2001), *Ocimum gratissimum* (Gopi et al. 2006). The present findings suggest a high frequency of shoot production from organogenic calli could be obtained by manipulating the growth regulators and culture condition. There were differences between treatments both in the percentage of cultures with response and in the mean number of shootbuds per culture. Many authors reported that cytokinin is required in optimal quantity for shoot proliferation in many genotypes but an inclusion of a low concentration of auxin along with cytokinin increases the rate of shoot bud proliferation (Sharma *et al.* 1993; Sharma and Singh 1997; Shasany et al. 1998; Rout *et al.* 2000; Rout, 2005). However, the molecular mechanisms through which auxin-cytokinin crosstalk act in concert to exert the shoot meristem induction are still poorly understood. A lower concentration of BA (< 3.0 mg/l) in the culture medium inhibited the growth of the shoot buds. The number of shoot buds per culture varied from 1.8 – 28.6 in case of leaf and 2.4 to 16.2 in case of stem in different treatments. The rate of shoot bud regeneration increased as the number of subcultures increased. Similar observations were reported for *Gentiana kurroo* (Sharma et al. 1993) and *Plumbago* species (Rout *et al.* 1999).

4.1.2. Effect of cytokinins and auxins in shoot multiplication

The combination of cytokinins and auxins also favoured positive impact on shoot proliferation and multiplication. In the present investigation, one best cytokinin and two auxins were tested for shoot proliferation and multiplication. Among the two auxins tested, BAP along with IAA favoured the maximum rate of shoot multiplication. The medium supplemented with BAP and NAA did not show any positive response. In medium having 1.5 mg/l BAP along with 0.5 mg/l IAA showed the highest rate of shoot growth and proliferation in case of *Piper longum* (Table 4.4). Increase the concentrations of either BAP or IAA, the cultures did not showed any more positive response. In case of *Piper longum*, the maximum percentage (83.3%) of shoot proliferation was observed in MS medium supplemented with 1.5 mg/l BAP, 0.5 mg/l IAA and 3% sucrose within 8 weeks of culture (Table 4.4). The average number of multiple shoots per culture was varied from 1.0 - 3.4 in different treatments (Fig. 4.1B) . In case of *Piper betle,* the maximum percentage of response

on shoot multiple was 40% on MS medium supplemented with BAP 1.5 mg/l along with IAA 1.5 mg/l and 0.2 mg/l NAA (Table 4.5). The average number of shoots per culture varied from 1.1 - 1.8 shoots per culture. In case of *Piper nigrum,* the maximum percentage of shoot proliferation was 34.2 % on MS medium supplemented with 2.0 mg/l BAP along with 2.0 mg/l IAA and 0.2 mg/l NAA (Table 4.6). The average number of shoots varied from 1.0 -1.4 shoots per culture (Fig.4.2A). The manipulation of cytokinin and auxin helps in shoot multiplication of different medicinal plant species was reported by various authors (Aminduddin *et al.*, 1993; Johri *et al.*, 1996, Mathews and Rao, 1984; Philip *et al.*, 1992 (Bhat *et al.*, 1995; Anand and Rao, 2000; Bhat *et al.*, 1992; Philip *et al.* 1992; Bhat *et al.* 1995; Joseph *et al.* 1996; Philip *et al.*, 2000; Soniya and Das, 2002; Nair and Gupta 2006; Thomas *et al.*, 2008).

Table 4.2. Effect of different concentrations of cytokinins on shoot proliferation from shoot tip explants of *Piper betle* after 8 weeks of culture.

MS+ growth regulator mg/l			% of response (Mean ± SE)*	No. of multiple shoots/culture (Mean ± S.E)*
BAP	Kn	Ads		
0	0	0	0.0	0.0
0.25	0.5	100	No response	No response
0.50	0.5	100	No response	No response
1.0	0.5	100	13.4±0.4 a	1.2±0.4 a
1.5	0.5	100	13.8±0.5 a	1.2±0.5 a
2.0	1.0	0	28.6±0.6 b,c	1.3±0.4 a
1.5	1.0	100	23.3±0.5 b	1.3±0.6 a
2.0	1.5	100	36.2±0.6 d	2.2±0.7 b
0	1.5	0	13.8±0.7 a	1.2±0.6 a
0.50	1.5	100	16.6±0.8 a	1.3±0.4 a
1.0	1.5	100	16.8±0.6 a	1.3±0.6 a
1.5	1.5	100	26.6±0.5 b	1.3±0.5 a
2.0	**0.5**	**0**	**40.4±0.4 d**	**2.3±0.5 b**
1.5	2.0	100	34.5±0.3 d	1.8±0.6 a
0	2.0	0	32.3±0.5 d	1.8±0.8 +a
2.5	-	100	26.6±0.7 b	1.3±0.4 a
3.0	-	100	30.8±0.6 +c	1.8±0.5 +a
3.5	-	100	32.3±0.5 +c,d	2.0±0.6 +b
3.5	2.0	100	33.6±0.4 +d	2.0±0.5 +b
4.0	2.0	100	25.6±0.6 +b	1.3±0.4 +a

*15 replicates/treatment; repeated thrice.

+ - Callusing at the basal end

Mean followed by different letters are significantly different at the 5% level.

4.1.4. Effect of auxins on callus proliferation

Callus is a mass of unorganised cells obtained from plant tissues and used for different biological research and crop improvement program. The development of callus is greatly influenced by the growth regulators and physiological condition. Generally, higher concentration of auxin in the culture medium helps to proliferate the growth of callus. The culture condition like temperature and photoperiod has great potential for callus production from various explants like stem, leaf and root. In the present study, three auxins (2, 4-D, IAA and NAA) were used for induction of callus from leaf and stem explants of *Piper* species.

Table 4.3. Effect of different concentrations of cytokinins on shoot proliferation from shoot tip explants of *Piper nigrum* after 8 weeks of culture.

MS+ growth regulator mg/l			% of response (Mean ± SE)*	No. of Multiple Shoots/culture (Mean ± S.E)*
BAP	Kn	Ads		
0	0	0	0.0	0.0
0.25	0.5	100	NR	NR
0.50	0.5	100	NR	NR
1.0	0.5	100	NR	NR
1.5	0.5	100	12.8±0.4 a	+
2.0	1.0	100	20.6±0.6 d	1.0±0.4 a
1.5	1.0	100	14.3±0.5 b	1.0±0.1 a
2.0	1.5	100	26.6±0.6 e	2.0±0.4 b
0	1.5	0	13.3±0.7 a,b	1.0±0.6 a
0.50	1.5	100	16.3±0.8 c	1.0±0.4 a
1.0	1.5	100	20.4±0.6 d	1.0±0.1 a
1.5	1.5	100	26.2±0.5 e	1.0±0.5 a
2.0	0.5	100	34.6±0.4 +h	1.0±0.5 a+
1.5	**2.0**	**100**	**36.4±0.3 + i**	**2.4±0.2 b+**
2.0	2.0	100	30.3±0.5 +g	2.1±0.2 b+
0	2.0	0	30.6±0.7 +g	1.0±0.4 a+
3.0	-	100	20.0±0.6 d	1.6±0.5 b
3.5	-	100	+	+
3.5	2.0	100	+	+
4.0	2.0	100	+	+

*15 replicates/treatment; repeated thrice, NR – no response, + - Callusing at the basal end Mean followed by different letters are significantly different at the 5% level.

The result showed that the green friable callus was developed from leaf as well as stem after 4 - 5 weeks of culture on medium supplemented with 2, 4-D + IAA or NAA. The callus further proliferated in subsequent subculture, the maximum proliferation of callus was observed from leaf and stem explants in *Piper betle* in the medium containing 2.5 mg/l 2, 4-D within 8 weeks of culture (Table 4.7). The callus proliferation became declined if the medium having either lower concentrations of auxin alone or without auxin. The maximum proliferation of callus from stem and leaf explants were 73.3% and 83.4% respectively on medium containing 2.5 mg/l 2, 4-D (Table 4.7; Fig. 4.3 A & B). The combination of growth regulators like 2, 4-D, NAA and IAA did not show any positive impact on callus proliferation. In case of *Piper longum,* the highest callus proliferation was obtained in medium having 2.0 mg/l 2, 4-D and 2.0 mg/l IAA within 8 weeks of culture (Fig. 4.4A & B). The maximum percentage of response on callus proliferation from leaf and stem explants were 58.3% and 54.0 % respectively (Table 4.8). On the basis of comparative analysis, it was observed that the medium having 2, 4-D + IAA showed higher callus proliferation than the 2, 4-D + NAA or NAA + IAA. In case of *Piper nigrum*, the maximum proliferation of callus from leaf and stem plants were observed in medium having 2.0 mg/l 2, 4-D and 2.0 mg/l IAA (Table 4.9).

4.1.5. Effect of cytokinins and auxins on callus proliferation and plant regeneration

The proliferation of callus and plant regeneration was depended on the type of growth medium, culture conditions and source of explant. In most of the cases, the combination of auxin and cytokinin favoured the rate of callus growth and regeneration of shoots from the callus. The present study indicated that the combination of auxin and cytokinin in the growth medium favoured higher percentage of callus proliferation by using both stem and leaf explants. The maximum percentage of callus proliferation from leaf and stem explant of *Piper betle* on MS medium supplemented with 2.0 mg/l 2,4 D, 1.5 mg/l BAP and 1.0 mg/l NAA within 8 weeks of culture. The callus was green and friable in nature. The percentage of organogenenic response of callus derived from leaf and stem were 63.3 % and 38.6% respectively (Table 4.10; Fig.4.3 C, D). The calluses were again subculture into fresh medium with same composition for shoot bud regeneration. There was no shoot bud

regeneration observed in any of the medium tested in this study. However, the callus proliferation and shoot bud regeneration was achieved in leaf derived callus of *Piper longum* on MS medium supplemented with 1.0 – 1.5 mg/l 2, 4-D and 1.5 mg/l BAP (Fig. 4.4 C, D). The stem derived callus did not produce positive response with regard to shoot bud regeneration even the culture kept for longer period or subculture twice in same medium (Table 4.11). The subculturing of callus was made in every 4 weeks interval. Shoot bud regeneration did not achieved in callus derived from stem and leaf explants of *Piper nigrum*. The callus was compact in nature but the organogenic shoot primordial like appearance observed during the study period. The source of explant was important in determining the morphogenetic and regenerative potential, which were significantly influenced by the physiological conditions of the donor plant (Debergh and Maene, 1981; Read, 1988).

Table. 4.4. Effect of different concentrations of auxins and cytokinins on shoot proliferation from shoot tip explants of *Piper longum* after 8 weeks of culture.

MS+ growth regulator mg/l			% of response (Mean ± SE)*	No. of multiple Shoots/culture. (Mean ± S.E)*
BAP	IAA	NAA		
0	0	0	0.0	0.0
0.25	0.5	-	23.4±0.6 a	1.0±0.3 a
0.50	0.5	-	46.6±0.3 e	1.8±0.6 a
1.0	0.5	-	63.3±0.4 h	2.8±0.4 b
1.5	**0.5**	**-**	**83.3±0.5 j**	**3.4±0.5 c**
2.0	1.0	-	76.6±0.6 i	3.2±0.4 c
1.5	1.0	-	53.6±0.5 f	2.4±0.3 b
2.0	1.5	-	56.2±0.6 g	2.6±0.2 b
0.25	1.5	0.2	33.2±0.7 b	1.6±0.6 a
0.50	1.5	0.2	34.3±0.8 b	1.6±0.4 a
1.0	1.5	0.2	56.4±0.6 g	2.6±0.6 b
1.5	1.5	0.2	62.6±0.5 h	3.0±0.5 b
2.0	0.5	0.2	56.4±0.4 g	3.0±0.5 b
1.5	2.0	0.2	62.3±0.3 h	2.8±0.6 b
2.0	2.0	0.2	43.3±0.5 d	1.6±0.8 a
2.5	-	0.2	36.6±0.7 c	1.3±0.4 a
3.0	-	0.5	56.6±0.6 g+	2.6±0.5 b+
3.5	-	1.0	46.8±0.5 e+	1.8±0.6 +a
3.5	2.0	0.5	53.3±0.4 f+	2.4±0.5 +b
4.0	2.0	0.5	56.6±0.6 g+	2.6±0.4 +b

*15 replicates/treatment; repeated thrice

+ - Callusing at the basal end

Mean followed by different letters are significantly different at the 5% level.

Maintaining the donor plants in clean and controlled environmental conditions delivers healthy and sterile explants (Sagare *et al.*, 2001). The physiological age of explants and the explant type and size are the other factors which influenced formation of organs *in vitro* (Rout et al., 2000). Gunay and Rao (1978) concluded that Kn was inefficient in inducing differentiation and reported only callus production.

Table 4.5. Effect of different concentrations of auxins and cytokinins on shoot proliferation from shoot tip explants of *Piper betle* after 8 weeks of culture.

MS+ growth regulator mg/l			% of response (Mean ± SE)*	No. of multiple shoots/culture (Mean ± SE)*
BAP	IAA	NAA		
0	0	0	0.0	0.0
0.25	0.5	-	NR	NR
0.50	0.5	-	NR	NR
1.0	0.5	-	16.6±0.4 b	1.2±0.4 a
1.5	0.5	-	18.2±0.5 b	1.2±0.5 a
2.0	1.0	-	26.2±0.6 d	1.3±0.4 a
1.5	1.0	-	22.3±0.5 c	1.3±0.6 a
2.0	1.5	-	36.4±0.6 g	1.8±0.2 b
0.25	1.5	0.2	12.6±0.7 a	1.1±0.6 a
0.50	1.5	0.2	14.6±0.8 a	1.1±0.4 a
1.0	1.5	0.2	26.6±0.6 d	1.3±0.4 a
1.5	**1.5**	**0.2**	**40.0±0.5 h**	**1.8±0.5 b**
2.0	0.5	0.2	30.0±0.4 e	1.3±0.5 a
1.5	2.0	0.2	32.3±0.3 f	1.6±0.6 b
2.0	2.0	0.2	34.4±0.5 g	1.6±0.2 b
2.5	-	0.2	26.6±0.7 d	1.3±0.4 a
3.0	-	0.5	30.0±0.6 e	1.3±0.5 a
3.5	-	1.0	32.3±0.5 f+	1.6±0.6 +b
3.5	2.0	0.5	30.2±0.4 e+	1.6±0.5 +b
4.0	2.0	0.5	24.6±0.6 d+	1.3±0.4 +a

*15 replicates/treatment; repeated thrice, NR - No response

\+ - Callusing at the basal end

Mean followed by different letters are significantly different at the 5% level.

Maintaining the donor plants in clean and controlled environmental conditions delivers healthy and sterile explants (Sagare *et al.*, 2001). The physiological age of explants and the explant type and size are the other factors which influenced formation of organs *in vitro* (Rout et al., 2000). Gunay and Rao (1978) concluded that Kn was inefficient in inducing differentiation and reported only callus production.

Table 4.5. Effect of different concentrations of auxins and cytokinins on shoot proliferation from shoot tip explants of *Piper betle* after 8 weeks of culture.

MS+ growth regulator mg/l			% of response (Mean ± SE)*	No. of multiple shoots/culture (Mean ± SE)*
BAP	IAA	NAA		
0	0	0	0.0	0.0
0.25	0.5	-	NR	NR
0.50	0.5	-	NR	NR
1.0	0.5	-	16.6±0.4 b	1.2±0.4 a
1.5	0.5	-	18.2±0.5 b	1.2±0.5 a
2.0	1.0	-	26.2±0.6 d	1.3±0.4 a
1.5	1.0	-	22.3±0.5 c	1.3±0.6 a
2.0	1.5	-	36.4±0.6 g	1.8±0.2 b
0.25	1.5	0.2	12.6±0.7 a	1.1±0.6 a
0.50	1.5	0.2	14.6±0.8 a	1.1±0.4 a
1.0	1.5	0.2	26.6±0.6 d	1.3±0.4 a
1.5	**1.5**	**0.2**	**40.0±0.5 h**	**1.8±0.5 b**
2.0	0.5	0.2	30.0±0.4 e	1.3±0.5 a
1.5	2.0	0.2	32.3±0.3 f	1.6±0.6 b
2.0	2.0	0.2	34.4±0.5 g	1.6±0.2 b
2.5	-	0.2	26.6±0.7 d	1.3±0.4 a
3.0	-	0.5	30.0±0.6 e	1.3±0.5 a
3.5	-	1.0	32.3±0.5 f+	1.6±0.6 +b
3.5	2.0	0.5	30.2±0.4 e+	1.6±0.5 +b
4.0	2.0	0.5	24.6±0.6 d+	1.3±0.4 +a

*15 replicates/treatment; repeated thrice, NR - No response
+ - Callusing at the basal end
Mean followed by different letters are significantly different at the 5% level.

Table. 4.6. Effect of different concentrations of auxins and cytokinins on shoot proliferation from shoot tip explants of *Piper nigrum* after 8 weeks of culture.

MS+ growth regulator mg/l			% of response (Mean ± SE)*	No. of multiple shoots/culture (Mean ± SE)*
BAP	IAA	NAA		
0	0	0	0.0	0.0
0.25	0.5	-	NR	NR
0.50	0.5	-	NR	NR
1.0	0.5	-	NR	NR
1.5	0.5	-	NR	NR
2.0	1.0	-	18.6±0.6 c	1.0±0.4 a
1.5	1.0	-	16.3±0.5 b	1.0±0.1 a
2.0	1.5	-	20.6±0.6 d	1.2±0.7 a
0.25	1.5	0.2	12.3±0.7 a	1.0±0.6 a
0.50	1.5	0.2	18.3±0.8 c	1.0±0.4 a
1.0	1.5	0.2	20.6±0.6 d	1.1±0.6 a
1.5	1.5	0.2	22.6±0.5+ e	1.2±0.5 a
2.0	0.5	0.2	24.0±0.4 f	1.4±0.5 a
1.5	2.0	0.2	26.6±0.3+ g	1.1±0.6 a
2.0	**2.0**	**0.2**	**34.2±0.5+ h**	**1.4±0.8 a**
2.5	-	0.2	26.6±0.7+ g	1.0±0.4 a
3.0	-	0.5	28.0±0.6+ g	1.3±0.5 a
3.5	-	1.0	18.8±0.6+ c	1.2±0.3 a
3.5	2.0	-	18.6±0.6 +c	1.0±0.2 a
4.0	2.0	-	16.0±0.6 +b	1.2±0.4 a

*15 replicates/treatment; repeated thrice, NR - No response,
+ - Callusing at the basal end.
Mean followed by different letters are significantly different at the 5% level.

Table. 4.7. Effect of different concentrations of auxins on callus proliferation from leaf and stem explants of *Piper betle* after 8 weeks of culture (subculture was made every 4 weeks interval).

MS+ growth regulator mg/l			% of response (Mean ± SE)*		Colour and texture of callus
			Leaf	Stem	
2,4-D	IAA	NAA			
0	0	0	0.0	0.0	-
0.25	0.5	-	23.3±0.6 a	NR	Green friable
0.50	0.5	-	36.4±0.3 c	NR	Green friable
1.0	0.5	-	43.2±0.4 d	NR	Green friable
1.5	0.5	-	53.3±0.5 f	48.2±0.4 e	Green friable
2.0	1.0	-	66.8±0.6 i	36.4±0.6 b	Green friable
1.5	1.0	-	62.3±0.5 g	43.3±0.5 d	Green friable
2.0	1.5	-	68.6±0.6 j	46.6±0.6 e	Green friable
0.25	1.5	0.2	33.2±0.7 b	33.3±0.7 a	Green friable
0.50	1.5	0.2	36.6±0.8 c	41.6±0.8 c	Green friable
1.0	1.5	0.2	46.2±0.6 e	36.4±0.6 b	Green friable
1.5	1.5	0.2	54.6±0.5 f	46.6±0.5 e	Green friable
2.0	0.5	0.2	66.6±0.4 i	62.6±0.7 h	Green friable
1.5	2.0	-	52.3±0.3 f	55.2±0.3 g	Green friable
2.0	-	-	73.3±0.5 k	63.3±0.5 h	Green friable
2.5	-	-	**83.4±0.7 m**	**73.3±0.7 i**	**Green friable**
3.0	-	0.5	76.6±0.6 l	56.4±0.6 g	Green friable
3.5	-	1.0	56.6±0.5 g	50.2±0.6 f	Green friable
3.5	2.0	0.5	53.3±0.4 f	42.2±0.6 c	Green friable
4.0	2.0	0.5	54.6±0.6 f	48.4±0.6 e	Green friable

*15 replicates/treatment; repeated thrice; NR- No response

+ - Callusing at the basal end

Mean followed by different letters are significantly different at the 5% level.

Table. 4.8. Effect of different concentrations of auxins on callus proliferation from leaf and stem explants of *Piper longum* after 8 weeks of culture (subculture wasmade every 4 weeks interval).

MS+ growth regulator mg/l			% of response (Mean ± SE)*		Colour and texture of callus
2,4-D	IAA	NAA	Leaf	Stem	
0	0	0	0.0	0.0	-
0.25	0.5	-	NR	NR	Green friable
0.50	0.5	-	NR	NR	Green friable
1.0	0.5	-	13.3±0.2 a	NR	Green friable
1.5	0.5	-	24.3±0.4 c	26.2±0.6 b	Green friable
2.0	1.0	-	32.6±0.6 d	28.6±0.1 b	Green friable
1.5	1.0	-	24.3±0.2 c	23.3±0.4 a	Green friable
2.0	1.5	-	36.6±0.1 e	30.4±0.2 c	Green friable
0.25	1.5	0.2	20.0±0.2 b	23.3±0.8 a	Green friable
0.50	1.5	0.2	26.6±0.3 b	22.4±0.6 a	Green friable
1.0	1.5	0.2	46.6±0.8 h	36.6±0.9 d	Green friable
1.5	1.5	0.2	56.6±0.4 j	43.3±0.7 e	Green friable
2.0	0.5	0.2	56.8±0.1 j	48.0±0.4 f	Green friable
1.5	2.0	-	43.3±0.1 g	42.3±0.1 e	Green friable
2.0	**2.0**	**-**	**58.3±0.4 k**	**54.0±0.8 g**	**Green friable**
2.5	-	-	46.3±0.7 g	35.3±0.3 d	Green friable
3.0	-	0.5	48.6±0.3 i	34.6±0.2 d	Green friable
3.5	-	1.0	36.0±0.6 e	35.8±0.3 d	Green friable
3.5	2.0	0.5	40.3±0.5 f	34.3±0.3 d	Green friable
4.0	2.0	0.5	36.6±0.2 e	32.3±0.3 c	Green friable

*15 replicates/treatment; repeated thrice, NR- No response
+ - Callusing at the basal end
Mean followed by different letters are significantly different at the 5% level.

Table 4.9. Effect of different concentrations of auxins on callus proliferation from leaf and stem explants of *Piper nigrum* after 8 weeks of culture (subculture was made every 4 weeks interval).

MS+ growth regulator mg/l			% of response (Mean ± SE)*		Colour and texture of callus
			Leaf	Stem	
2,4-D	IAA	NAA			
0	0	0	0.0	0.0	-
0.25	0.5	-	NR	NR	Green friable
0.50	0.5	-	NR	NR	Green friable
1.0	0.5	-	NR	NR	Green friable
1.5	0.5	-	26.3±0.6 a	24.3±0.6 a	Green friable
2.0	1.0	-	36.4±0.6 d	28.0±0.7 c	Green friable
1.5	1.0	-	28.3±0.5 b	26.3±0.8 b	Green friable
2.0	1.5	-	38.6±0.6 e	34.3±0.4 d	Green friable
0.25	1.5	0.2	26.4±0.7 a	28.6±0.3 c	Green friable
0.50	1.5	0.2	30.6±0.8 c	28.8±0.6 c	Green friable
1.0	1.5	0.2	36.6±0.6 d	38.3±0.5 f	Green friable
1.5	1.5	0.2	40.2±0.4 f	42.6±0.5 g	Green friable
2.0	0.5	0.2	44.3±0.4 g	46.2±0.4 i	Green friable
1.5	2.0	-	53.3±0.3 i	48.6±0.8 j	Green friable
2.0	**2.0**	**-**	**62.6±0.6 k**	**53.3±0.5 k**	**Green friable**
2.5	-	-	43.3±0.4 g	46.4±0.7 i	Green friable
3.0	-	0.5	52.6±0.5 i	53.4±0.6 k	Green friable
3.5	-	1.0	56.4±0.6 j	44.6±0.5 h	White Green
3.5	2.0	0.5	46.6±0.3 h	38.4±0.5 f	White green
4.0	2.0	0.5	42.8±0.5 g	36.4±0.4 e	White green

*15 replicates/treatment; repeated thrice, NR- No response.

+ - Callusing at the basal end

Mean followed by different letters are significantly different at the 5% level.

Table 4.10. Effect of different concentrations of cytokinins and auxins on callus proliferation and plant regeneration from leaf and stem explants of *Piper betle* after 8 weeks of culture (subculture was made every 4 weeks interval).

MS+ growth regulator mg/l			Organogenic response (%) (Mean ± SE)*		No. of shoot buds/culture (Mean ± SE)*	
			Leaf	Stem		
2, 4-D	BAP	NAA			Leaf	Stem
0	0	0	0.0	0.0	0.0	0.0
0.25	0.5	0.5	0.0	0.0	0.0	0.0
0.50	0.5	0.5	0.0	0.0	-	-
1.0	0.5	0.5	26.3±0.4 a	23.3±0.6 a	-	-
1.5	0.5	0.5	28.2±0.5 b	24.6±0.4 b	-	-
2.0	0.5	0.5	36.6±0.6 d	28.6±0.5 c	-	-
1.5	1.0	1.0	26.6±0.5 a	23.3±0.4 a	-	-
2.0	1.5	1.0	48.0±0.6 e	35.0±0.4 d	-	-
0.25	1.5	1.0	28.3±0.7 b	23.6±0.3 a	-	-
0.50	1.5	1.0	33.2±0.8 c	28.4±0.5 c	-	-
1.0	1.5	1.0	46.6±0.6 e	36.6±0.7 d	-	-
1.5	1.5	1.0	56.6±0.5 g	43.0±0.6 e	-	-
2.0	**0.5**	**1.5**	**63.3±0.4 i**	**48.6±0.3 g**	-	-
1.5	2.0	1.5	46.4±0.3 e	43.3±0.5 e	-	-
2.0	2.0	1.5	53.3±0.5 f	44.3±0.8 e	-	-
2.5	-	1.5	42.6±0.7 d	42.3±0.7 e	-	-
3.0	-	1.5	56.8±0.8 g	46.2±0.4 f	-	-
3.5	-	2.0	60.0±1.0 h	43.3±0.9 e	-	-
3.5	2.0	2.0	53.3±0.4 f	52.3±0.6 h	-	-
4.0	2.0	2.0	56.6±0.6 g	52.3±0.8 h	-	-

*15 replicates/treatment; repeated thrice.

+ - Callusing at the basal end

Mean followed by different letters are significantly different at the 5% level.

Table 4.11. Effect of different concentrations of cytokinins and auxins on callus proliferation and plant regeneration from leaf and stem explants of *Piper longum* after 8 weeks of culture (subculture was made every 4 weeks interval)

MS+ growth regulator mg/l			Organogenic response (%) (Mean ± SE)*		No. of shoot buds /culture (Mean ± SE)*	
2, 4-D	BAP	NAA	Leaf	Stem	Leaf	Stem
0	0	0	0.0	0.0	0.0	0.0
0.25	0.5	0.5	0.0	0.0	-	-
0.50	0.5	0.5	0.0	0.0	-	-
1.0	0.5	0.5	0.0	0.0	-	-
1.5	0.5	0.5	22.3±0.5 a	20.4±0.6 a	-	-
2.0	1.0	0.5	36.6±0.8 e	28.2±0.4 d	-	-
1.5	1.0	1.0	30.4±0.5 c	32.3±0.5 e	-	-
2.0	1.5	1.0	42.6±0.3 g	40.0±0.7 f	-	-
0.25	1.5	1.0	23.3±0.8 a	23.8±0.5 b	-	-
0.50	1.5	1.0	26.6±0.6 b	28.4±0.4 d	-	-
1.0	**1.5**	**0**	**46.6±0.6 h**	**23.3±0.6 b**	**2.4 ± 0.5 a**	-
1.5	1.5	0	36.3±0.5 e	28.0±0.4 d	2.2 ± 0.4 a	-
2.0	0.5	1.5	30.0±0.4 c	26.6±0.7 c	-	-
1.5	2.0	1.5	33.3±0.3 d	26.0±0.3 c	-	-
2.0	2.0	1.5	38.6±0.5 f	27.8±0.9 d	-	-
2.5	-	1.5	42.2±0.7 g	38.6±0.5 f	-	-
3.0	-	1.5	36.6±0.6 e	33.4±0.9 e	-	-
3.5	-	2.0	36.3±0.7 e	32.4±0.6 e	-	-
3.5	2.0	2.0	43.6±0.8 g	40.6±0.5 f	-	-
4.0	2.0	2.0	52.4±0.6 i	43.3±0.7 g	-	-

*15 replicates/treatment; repeated thrice.

+ - Callusing at the basal end

Mean followed by different letters are significantly different at the 5% level.

Table. 4.12. Effect of different concentrations of cytokinins and auxins on callus proliferation and plant regeneration from leaf and stem explants of *Piper nigrum* after 8 weeks of culture (subculture was made every 4 weeks interval)

MS+ growth regulator mg/l			Organogenic response (%) (Mean ± SE)*		No. of shoot buds/culture (Mean ± SE)*	
			Leaf	Stem		
2, 4-D	BAP	NAA			Leaf	Stem
0	0	0	0.0	0.0	0.0	0.0
0.25	0.5	0.5	0.0	0.0	0.0	0.0
0.50	0.5	0.5	0.0	0.0	_	_
1.0	0.5	0.5	0.0	0.0	_	_
1.5	0.5	0.5	20.6±0.5 a	18.4±0.7 a	_	_
2.0	1.0	0.5	26.2±0.6 c	23.4±0.5 b	_	_
1.5	1.0	1.0	32.6±0.5 d	30.3±0.6 c	_	_
2.0	1.5	1.0	43.3±0.8 g	36.6±0.9 g	_	_
0.25	1.5	1.0	23.8±0.7 b	28.0±0.6 d	_	_
0.50	1.5	1.0	26.6±0.8 c	25.2±0.7 c	_	_
1.0	1.5	1.0	30.8±0.6 d	32.6±0.4 f	_	_
1.5	1.5	1.0	36.8±0.5 f	32.0±0.8 f	_	_
2.0	0.5	1.5	43.3±0.9 g	36.8±0.6 g	_	_
1.5	**2.0**	**1.5**	**53.3±0.8 i**	**48.3±0.5 j**	_	_
2.0	2.0	1.5	44.0±0.7 h	43.3±0.8 i	_	_
2.5	-	1.5	42.3±0.5 g	38.3±0.5 h	_	_
3.0	-	1.5	37.4±0.8 f	36.6±0.8 g	_	_
3.5	-	2.0	34.8±0.5 e	32.3±0.6 f	_	_
3.5	2.0	2.0	42.8±0.9 g	40.6±0.8 h	_	_
4.0	2.0	2.0	45.3±0.7 h	42.8±0.4 i	_	_

*15 replicates/treatment; repeated thrice.

+ - Callusing at the basal end

Mean followed by different letters are significantly different at the 5% level.

Fig. 4.3. Callus induction from leaf explants of *Piper betle* on MS medium supplemented with 2.5 mg/l 2,4-D after 4 weeks (A) and after 8 weeks (B) of culture. Callus proliferation on MS medium supplemented with 2.0 mg/l 2,4-D, 0.5 mg/l BAP and 1.5 mg/l NAA after 8 weeks of culture.

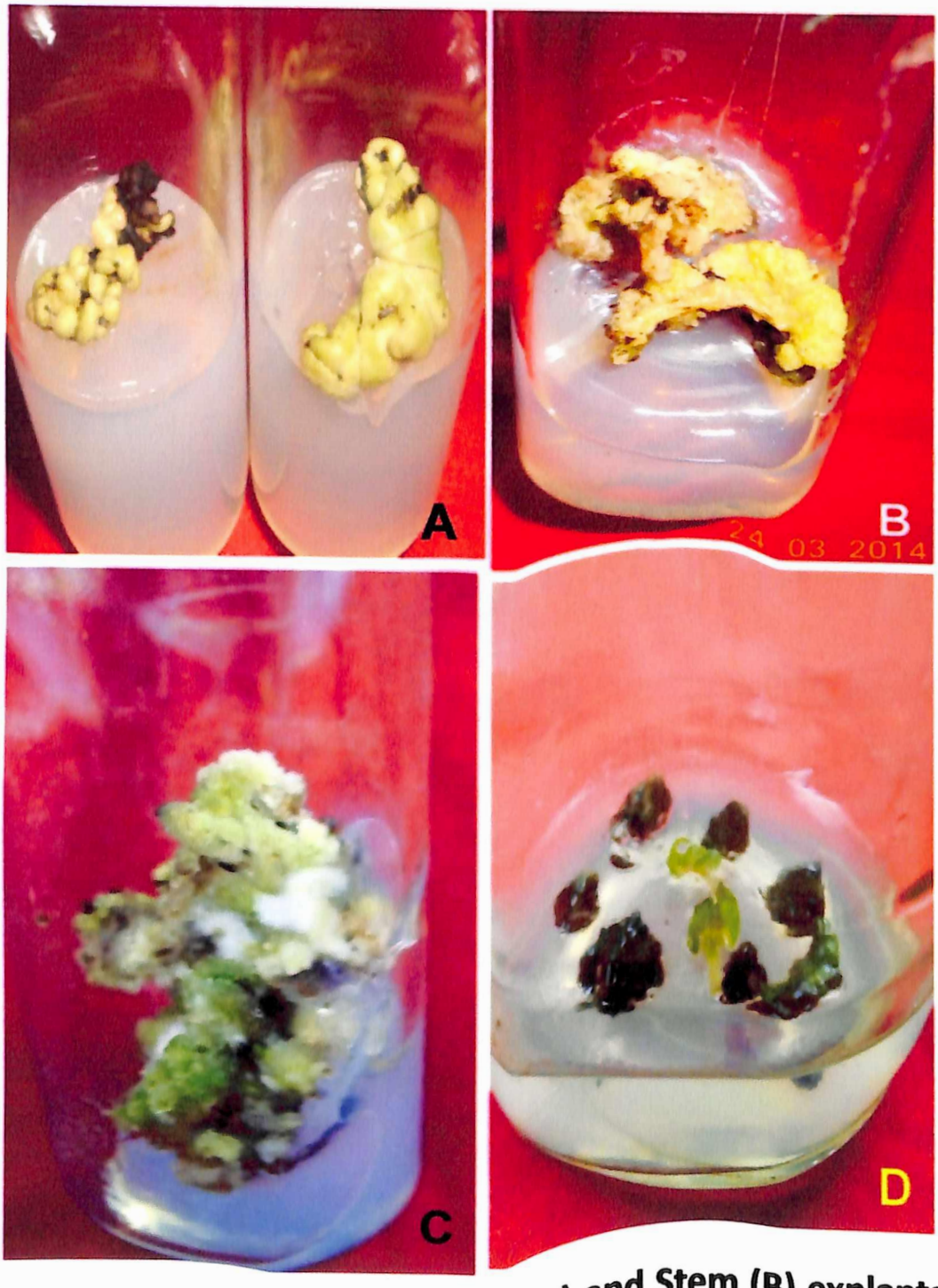


Fig. 4.4 . Callus induction from leaf (A) and Stem (B) explants of *Piper longum* on MS medium supplemented with 2.0 mg/l 2,4-D, 2.0 mg/l IAA after 8 weeks of culture. Shoot bud initiation from callus after 8 weeks (C,D) on MS medium supplemented with 1.0 mg/l 2,4-D and 1.5 mg/l BAP and 3% sucrose.

4.1.6. Effect of auxins on root induction

The process of *in vitro* root initiation, development and elongation normally require medium supplemented with auxin (Azad *et al.*, 2003). The healthy microshoots were separated from the clump and transferred to different medium having various concentrations of auxins with 2% (w/v) sucrose for induction of rooting. No rooting was observed in basal full strength or half strength MS media without growth regulators. The maximum percentage of rooting was observed in half strength MS media supplemented with 0.25 mg/l IBA and 2% sucrose. The medium supplemented with NAA also favoured the induction of rooting but the efficiency was more in IBA containing medium. The higher percentage of rooting was 73.3% in case of *Piper longum* (Table 4.13, Fig. 4.1C) and 33.3% in case of *Piper nigrum* (Table. 4.14; 4.2C). Abbasi *et al* (2010) reported that the micro shoots obtained from *in vitro* either shoot multiplication or adventitious shoot regeneration were rooted in ½ and ¼ MS basal medium under light (16 h photoperiod) and dark culture conditions. 100% rooting response was observed under dark incubation.

Table. 4.13. Effect of different concentrations of auxins on root induction from microshoots of *Piper longum* after 4 weeks of culture.

½ MS+ growth regulator (mg/l) + 2% (w/v) sucrose		% of response (Mean ± SE)*	No. of roots/ microshoots	Days to rooting
NAA	**IBA**			
0	0	0.0	-	-
0.25	0	23.3±0.6 a	2.2±0.5 a	28
0.50	0	26.6±0.5 b	2.3±0.4 a	26
1.0	0	43.3±0.4 d	3.4±0.3 a	25+
0	**0.25**	**73.3±0.6 g**	**4.5±0.6 b**	**20**
0	0.50	56.6±0.7 f	4.2±0.5 b	22
0	1.0	43.3±0.6 c	3.4±0.6 a	25+
0.25	0.25	46.6±0.8 e	3.8±0.7 a,b	26+

*12 replicates/treatment; repeated thrice.
+ - Callusing at the basal end
Mean followed by different letters are significantly different at the 5% level.

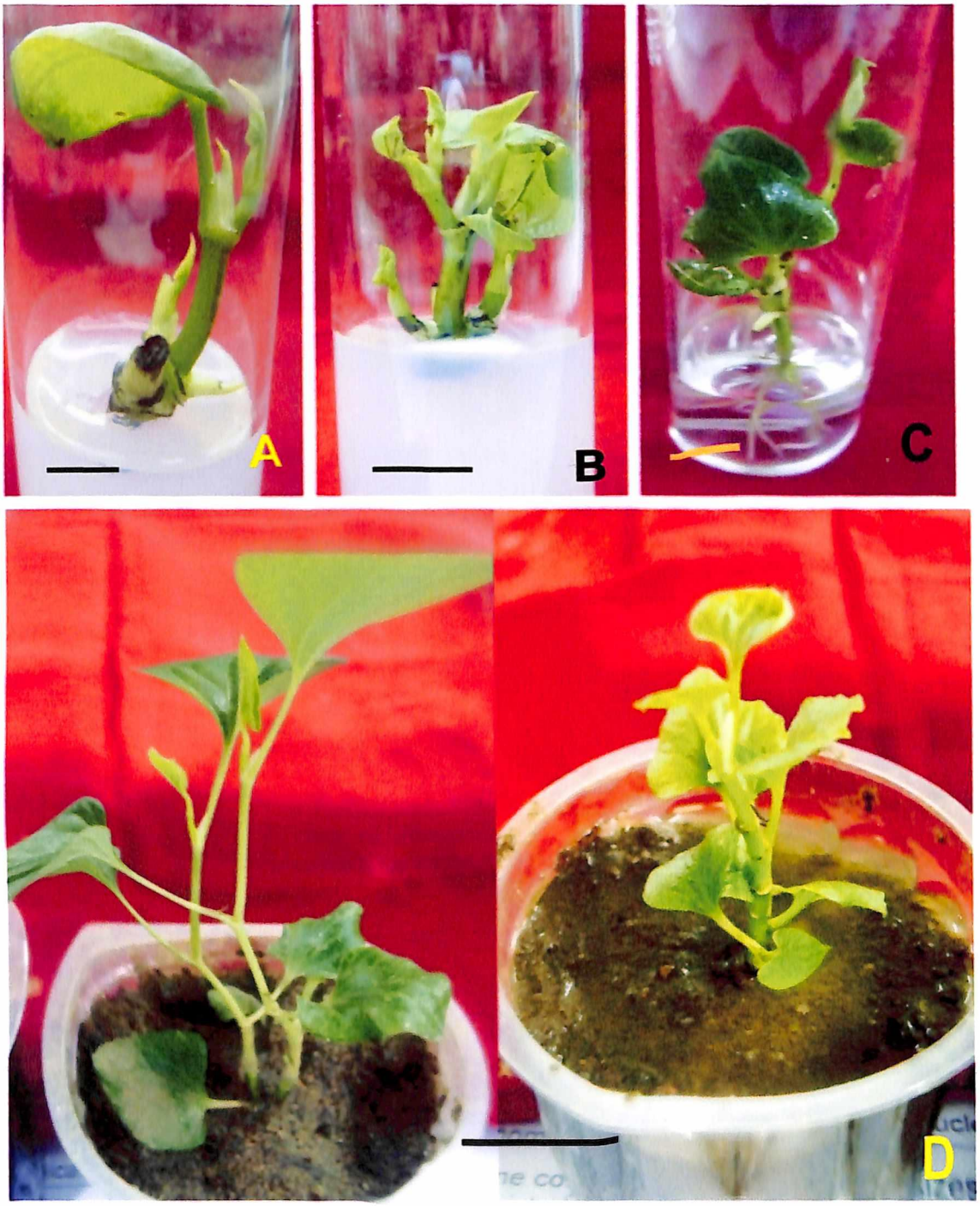


Fig. .4.1 *In vitro* Shoot multiplication of *Piper longum*.

A. Shoot proliferation on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l Kn + 100 mg/l Ads + 3% sucrose after 4 weeks of culture (Bar= 10 mm), **B.** Induction of multiple shoots on MS + 1.5 mg/l BAP + 0.5 mg/l IAA after 4 weeks of culture (Bar= 10 mm), **C-** Induction of rooting from microshoot on ½ MS + 0.25 mg/l IBA + 2% sucrose (Bar = 20 mm), **D.** *In vitro* raised plantlets established in polyglass with soil manure after one month of transfer (Bar= 100 mm).

4.1.7. Acclimatization of *in vitro* raised plantlets.

The rooted plants were transferred to plastic pots containing mixture of sand: soil: FYM (1:1:1) and kept in green house with 85% humidity for acclimatization. About 90% of the plantlets were established in the green house within 1-2 weeks of transfer. The plant grew well and attained 6 – 8 inches within 4 weeks of transfer (Fig. 4.1D & Fig. 4.2D). The prevailing conditions (humidity and temperature) of transplanting season greatly influenced the initial survival of potted plantlets (Azad et al., 2003). The plantlets were transplanted to soil and acclimatized in the growth chamber under high humidity conditions. The rooted plants were transferred to field nursery for hardening (Anand and Rao, 2000).

Table 4.14. Effect of different concentrations of auxins on root induction from microshoots of *Piper nigrum* after 4 weeks.

½ MS+ growth regulator (mg/l) + 2% sucrose		% of response (Mean±SE)*	No. of roots/ microshoots	Days to rooting
NAA	**IBA**			
0	0	0.0	-	-
0.25	0	13.3±0.6 a	2.1±0.5 a	28
0.50	0	23.3±0.5 b	2.2±0.4 a	24
1.0	**0**	**33.3±0.4 d**	**3.4±0.3 b**	**22**
0	0.25	23.3±0.6 b	2.2±0.6 a	23
0	0.50	26.6±0.7 c	2.4±0.5 a	25+
0	1.0	23.3±0.6 b	2.2±0.6 a	28+
0.25	0.25	26.6±0.8 c	2.4±0.7 a	24+

*12 replicates/treatment; repeated thrice.
+ - Callusing at the basal end
Mean followed by different letters are significantly different at the 5% level.

4.2. Development of transform calli of *Piper betle*.

The friable calli derived from leaf explants of *Piper betle* was co-cultivated with *Agrobacterium rhizogenesis* (A4 strain) for induction of roots to synthesize the higher amount of secondary metabolite (Fig. 4.5 A & B). The transformed calli were subsequently transfer to different antibiotic solution to stop the infection. To standardize lethal dose of carbencillin and cefotaxime, the putative transformed tissues were cultured on MS medium without sucrose and growth regulators and

Fig. 4.2.*In vitro* Shoot multiplication of *Piper nigrum.*

A. Shoot proliferation on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l IAA, 3% sucrose after 4 weeks of culture (Bar= 20 mm), **B & C .** Induction of rooting from microshoot on ½ MS + 0.25 mg/l IBA + 2% sucrose (Bar = 20 mm), **D.** *In vitro* raised plantlets established in polyglass with soil manure after one month of transfer (Bar= 70 mm).

inclusion of different concentration of carbencilin or cefotaxime (0, 100, 200, 300, 400 and 500 mg/l). The results showed that the agrobacterium growth was not inhibited in medium having 100 and 200 mg/l carbencilin or cefotaxime. The treatment with 300 mg/l carbenicilin or cefotaxime appears to have a slightly toxic effect over the calli causing loss of chlorophyll, but the agrobacterium infection was not able to inhibit. The medium having 500 mg/l carbenicillin or cefotaxime had a deleterious effect. The growth of agrobacterium was fully control over infection in transformed calli. Therefore, 500 mg/l carbenicilin was used as a selection agent (Table 4.15). The co-cultivated callus was grew on this concentration of carbenicilin which considered as a putative transformants.

Table 4.15. Detection of lethal concentration of Carbenicillin and Cefotaxmine in selection medium (MS + 2,4-D 2.5 mg/l).

Carbenicilin (mg/l)	Percentage of calli of survival	Cefotaxmine (mg/l)	Percentage of calli survival	Appearance of the calli
0	98	0	100	Green colour
100	60	100	75	Dark green
200	46.65	200	48.28	Light green
300	36.65	300	38.62	Pale green
400	13.33	400	13.33	Yellowish green
500	0	500	0	White

*20 replicates/treatment; repeated thrice.

4.2.1. Co-cultivation period and infection time

The co-cultivation period and infection time are crucial for survival of transformed calli. A separate experiment was conducted to find out the infection time and co-cultivation period to achieve the good transformation study. The friable calli were immersed with *Agrobacterium* (A4 strain) suspension under shaking condition for 10, 15, 20 and 30 minutes for 48 h, 72h and 96h. The transformed calli were transferred to MS medium supplemented 500 mg/l cefotaxime or carbenicillin for different periods and times. The survival percentage of calli without bacterial growth was maximum after 48 hours of co-cultivation on medium having 500 mg/l cefotaxime for 30 minutes incubation period. However, about 53.3% of calli were survived without bacterial growth after 48hours of co-cultivation on medium having 500 mg/l carbenicillin for 20 min. incubation (Table 4.16). The factors like explant types, *agrobacterium* strain, growth medium, growth hormone and pH were showed

great influences on hairy root development. The time of exposure of *A. Rhizogenes* and co-cultivation period also played an important role in induction of hairy roots. Infection time of 20 - 30 min and co-cultivation period of 2 days were found to be optimum for induction of transformed root cultures. (Dupre *et al.*, 2000; Giri *et al.*, 2001, Kumar *et al.*, 2006; Azlan *et al.*, 2002; John *et al.*, 2009, Falasca *et al.*, 2000; Danesh *et al.*, 2006).

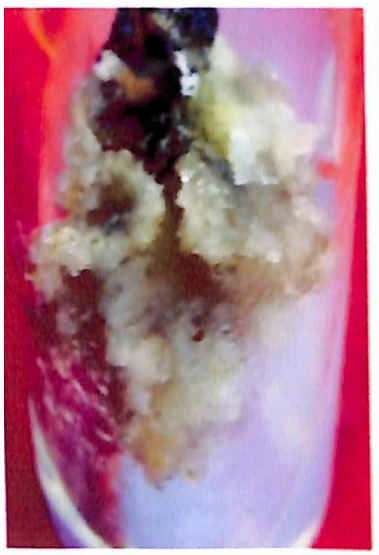
Table 4.16. Effect of infection time and co-cultivation period for transformation

Infection time (min)	Co-cultivation periods (hours)	Response after 20 days			
		Piper betle			
		After 2nd washing of cefotaxime		After 2nd washing of carbenicillin	
		Alive without bacterial growth	% of Survival	Alive	% of survival
10	48	4.33	43.3	4.67	46.7
	72	4.33	43.3	4.67	46.7
	96	3.33	33.3	3.67	36.7
15	48	4.33	43.3	4.33	43.3
	72	3.33	33.3	3.33	33.3
	96	3.33	33.3	3.67	36.7
20	48	4.67	46.7	5.33	53.3
	72	4.33	43.3	4.67	46.7
	96	3.67	36.7	4.67	46.7
30	48	5.00	50.0	4.33	43.3
	72	5.33	53.3	3.33	33.3
	96	4.33	43.3	3.67	36.7

10 calli /treatment; repeated twice

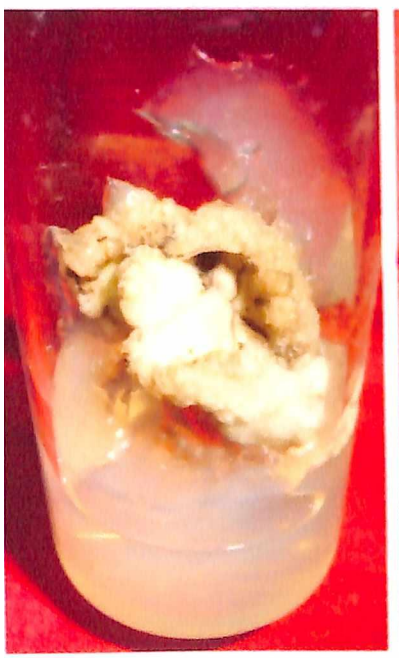
4.3. Screening of piperine content through TLC and HPTLC analysis.

The piperine content was examined in different species of Piper through TLC and HPTLC analysis. The piperine content varied from species to species and also differs in the source of explant. The root having higher content of piperine (Table 4.17). Two peaks were observed during the chromatography analysis i.e. piperine -1 & piperine-2 as reported earlier (Hamrapurkar *et al.*, 2011; Reshmi *et al.*, 2010). The piperine content was maximum in P.chaba fruit as compared with other three species. However, the piperine-2 content was more in P.nigrum fruit (Table 4.17; Figs 4.9-4.11). On the basis of TLC and HPTLC analysis, it was observed that there were two compounds such as Piperine-1 and Piperine-2 obtained from the leaf, root, transformed and non-transformed callus extract of *Piper betle* as compared to standard (Fig.4.9). The TLC analysis showed that the Rf value of Piperine -1 and



A

Fig.4.5A.
Transformed calli developed from leaf explants of *Piper betle* co-cultivation with A4 strain after 8 weeks of culture on MS medium supplemented with 2.5 mg/l 2,4-D and 3% sucrose.



B

Fig.4.5B.
Transformed calli developed from leaf explants of *Piper betle* co-cultivation with A4 strain after 8 weeks of culture on MS medium supplemented with 2.5 mg/l NAA and 3% sucrose.

Piperine -2 ranged from 0.39 - 0.40 and 0.42 – 0.46 respectively (Table 4.18). On the basis of HPTLC analysis, it was observed that the transformed calli having higher content of piperine (0.56%) with the unit of 10187.4 AU as compared with root, leaf and non-transformed calli extracts. (Figs. 4.10 & 4.11). The piperine-2 content was maximum in transformed calli extract as compared with non-transformed calli, leaf and stem extract. Pino et al. (2003) observed that the major components of the essential oil obtained from the aerial parts of *P. nigrum* were gluulol, a-pinene, ß-caryophyllene and a-terpinene. Piperine was the first amide to be isolated from piper species. Piperine the major active principle of black pepper, is closely related in structure to the known natural carcinogens-safrole, estragole and methylengenol which are also widely distributed in spices and plant oils.

Table 4.17. Comparison of piperine content in leaf, fruit and root of *Piper longum, Piper nigrum, Piper chaba and Piper betle.* The data represent the mean value of two independent experiment.

Sample (mg/ml) (5µl used in each case)	Piperine 1			Piperine 2		
	Rf	Area unit	Content(%)	Rf	Area unit	Content (%)
Standard piperine	0.42 (0.37-0.45)	27923.9	1.0	0.48 (0.45-0.56)	26133.00	1.0
P. longum leaf	0.39 (0.33-0.40)	11558.8	0.41±30	0.46 (0.40-0.51)	30056.40	1.15±10
P. longum fruit	0.43 (0.34-0.45)	22651	0.82±24	0.49 (0.45-0.58)	30052.30	1.14±90
P. longum root	*	*	*	0.50 (0.22-0.59)	102265.10	3.91±13
P. nigrum leaf	*	*	*	0.53 (0.47-0.54)	1504.80	0.05±72
P. nigrum fruit	*	*	*	0.51 (0.23-0.63)	116432.80	4.45±50
P. nigrum root	0.45 (0.22-0.48)	73451.9	2.63±10	0.48 (0.48-0.59)	37297.90	1.42±72
P. chaba leaf	0.44 (0.40-0.47)	7201.00	0.25±78	049 (0.47-53)	4126.7	0.15±79
P. chaba fruit	0.50 (0.14-0.52)	106854.0	3.82±66	0.54 (0.52-0.62)	30262.4	1.15±79
P. betle leaf	*	*	*	0.50 (0.41-0.54)	8582.50	0.32±84
P. betle root	*	*	*	0.50 (0.39-0.51)	6551.6	0.25±06

*- Not present

4.4. Phytochemical analysis of different *Piper* species and transformed and non-transformed calli of *Piper betle*.

Nine phytochemicals such as alkaloid, steroids, triterpenoid, glycosides, saponin, carbohydrates, flavonoids, tannins and proteins were quantified on the basis

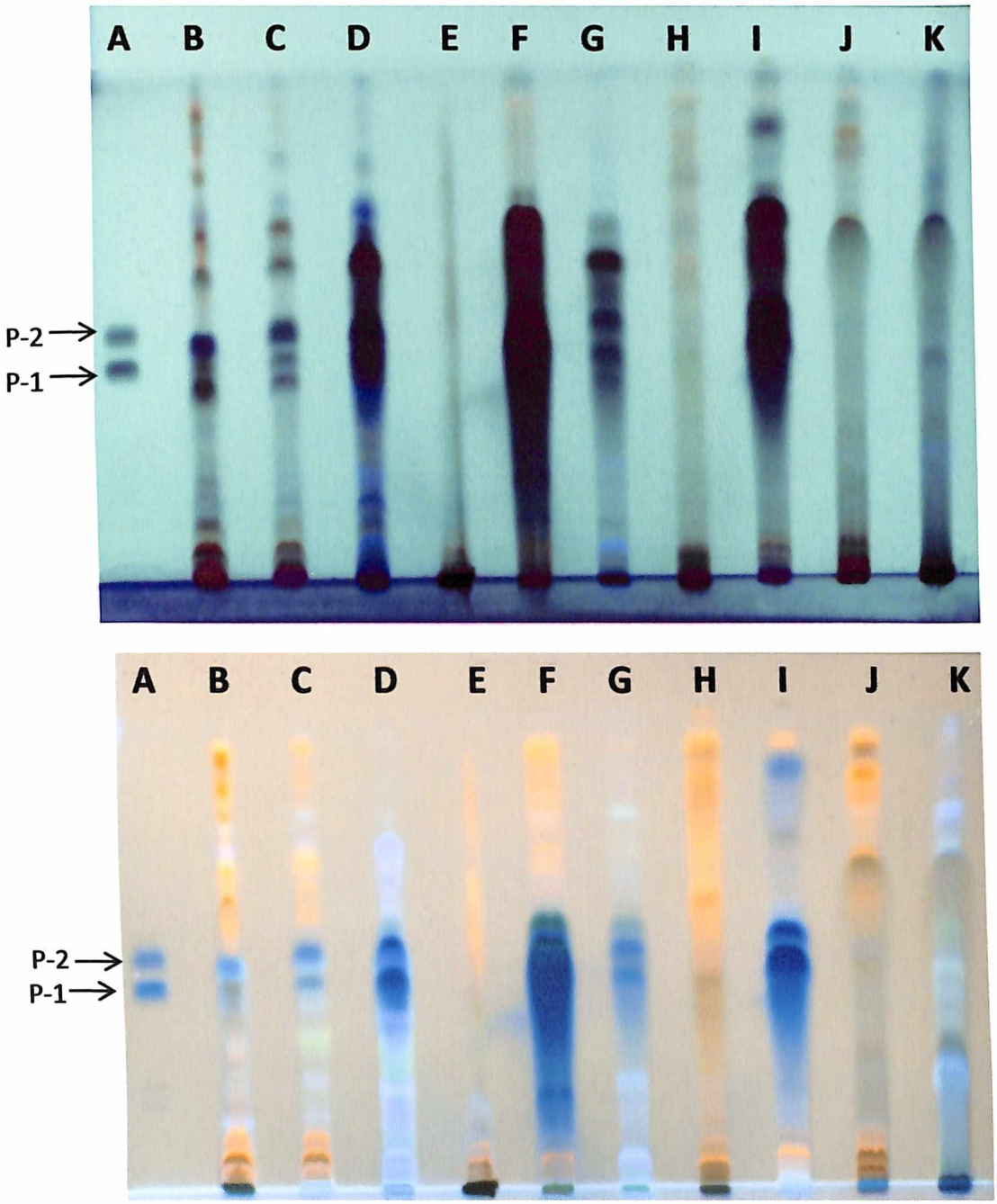


Fig. 4.6 TLC analysis of piperine content (P-1 & P-2) in leaf, root and fruit of different *Piper* species

A- standard piperine, B- *P.longum* (Leaf extract), C- P.longum (Fruit extract), D- *P.longum* (root), E- *P.nigrum* (Leaf, F- *P.nigrum* (Fruit), G- *P.nigrum* (root), H- *P.chaba* (leaf), I- *P.chaba* (Fruit), J- *P.betle* (leaf), K- P.*betle* (root).

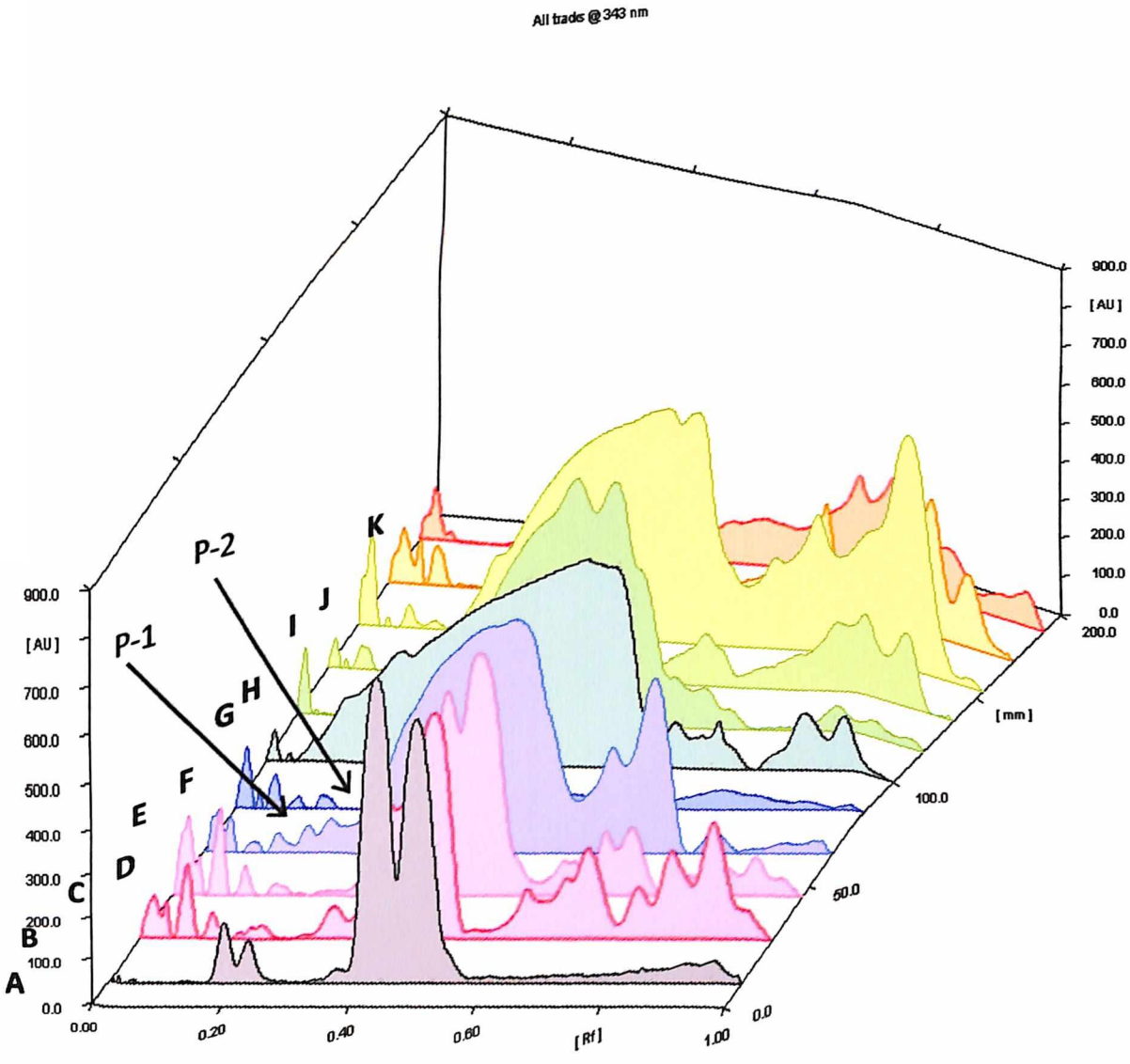


Fig. 4.7. Graphical representation of piperine content (P-1 & P-2) in leaf, root and fruit of different *Piper* species

A- standard piperine, B- *P.longum* (Leaf extract), C- *P.longum* (Fruit extract), D- *P.longum* (root), E- P.*nigrum* (Leaf, F- P.nigrum (Fruit), G- P.nigrum (root), H- *P.chaba* (leaf), I- P.*chaba* (Fruit), J- *P.betle* (leaf), K- *P.betle* (root).

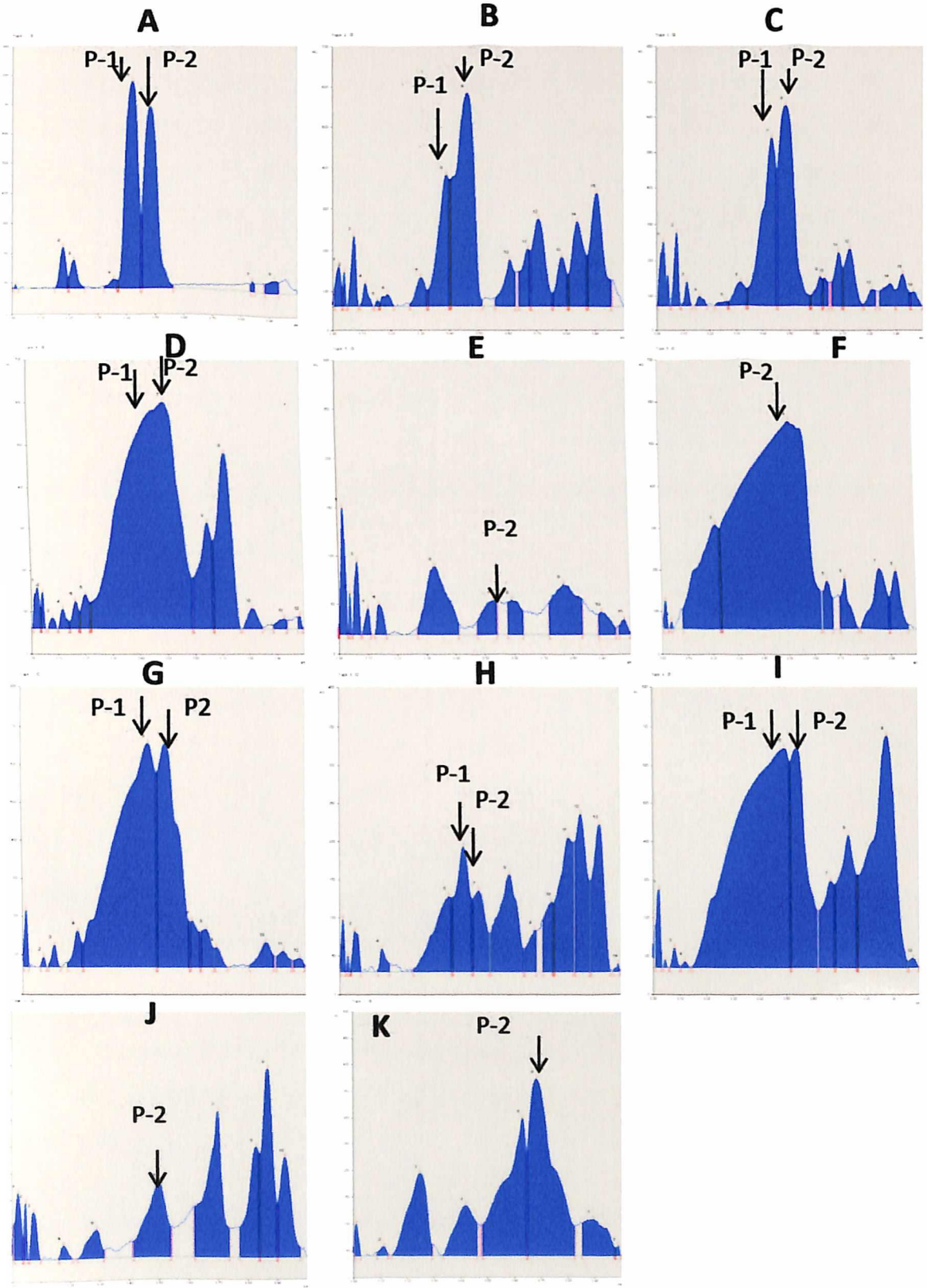


Fig. 4.8. HPTLC analysis of piperine content (P-1 & P-2) in leaf (B), fruit (C), root (D) of *P.longum*, in leaf (E), fruit (F), root (G) of *P.nigrum*, in leaf (H), fruit (I), of *P.chaba* and leaf (J) and root (K) of *P.betle* compare with standard (A)

of various tests (Gangully *et al.*, 1975; Muller *et al.*, 1967; Tadhani & Subhash, 2006; Concon *et al.*, 1979; Trease and Evans, 1983). The present study is to analyse the phytochemical constituent in different species of *Piper*, transformed and non-transformed calli of *Piper betle*. The result showed that the alkaloid and steroid content were present in all the *Piper* species as well as transform and non-transformed calli of *P.betle* (Table 4.19). The saponin was absent in all the species of *Piper* including transformed and non-transformed calli. The protein was present in leaf and fruits of *P.longum* and *Piper nigrum* and it absent in *P.betle* including transformed and non-transformed calli. The flavonoid content was high in leaf and root of *P.nigrum* and transformed calli of *P.betle*.

Table 4.18. Comparison of piperine content in leaf, root, transformed and non-transformed calli of *Piper betle*. The data represent the mean value of two independent experiment

Sample (mg/ml) (5µl used in each case)	Piperine 1			Piperine 2		
	Rf	Area unit	Content (%)	Rf	Area unit	Content(%)
Standard piperine	0.40 (0.35-0.43)	18175.5	1.0	0.46 (0.43-0.53)	14097.7	1.0
P.betle leaf	0.39 (0.35-0.43)	1999.6	0.11±10	0.46 (0.43-0.49)	1339.8	0.09±50
P.betle root	0.36 (0.31-0.40)	4519.4	0.24±86	0.42 (0.40-0.47)	2975.7	0.21±11
Transformed callus	0.41 (0.34-0.43)	10187.4	0.56±05	0.47 (0.43-0.53)	19162.7	1.35±92
Untransformed callus	0.4 (0.34-0.42)	9284.1	0.51±07	0.45 (0.42-0.52)	14010.3	0.99±38

4.5. Antimicrobial study of the extract derived from leaf, root, transformed and non -transformed callus of *piper species.*

The antimicrobial activity was tested as per the zone of inhibition formed by the tissue extract against bacteria and fungus. Leaf, root, transformed and non-transform calli extracts of *Piper betle* were tested against the pathogenic bacteria namely *Escherichia coli, Micrococcus luteus, Streptomyces epidermidis* and fungal culture of *Aspergillus niger, Aspergillus flavus* and *Rhizoctonia solani* at different concentrations (0, 20, 40, 60 mg/ml). The plant extracts showed antibacterial activity against all bacteria tested in the present study. The zone of inhibition was maximum in all the bacterial strains in the concentrations of 60 mg/ml of root extract of *P.betle* i.e 22 mm in case of *Micrococcus*, 18 mm in case of *S.epiderdimis* and 14 mm in case

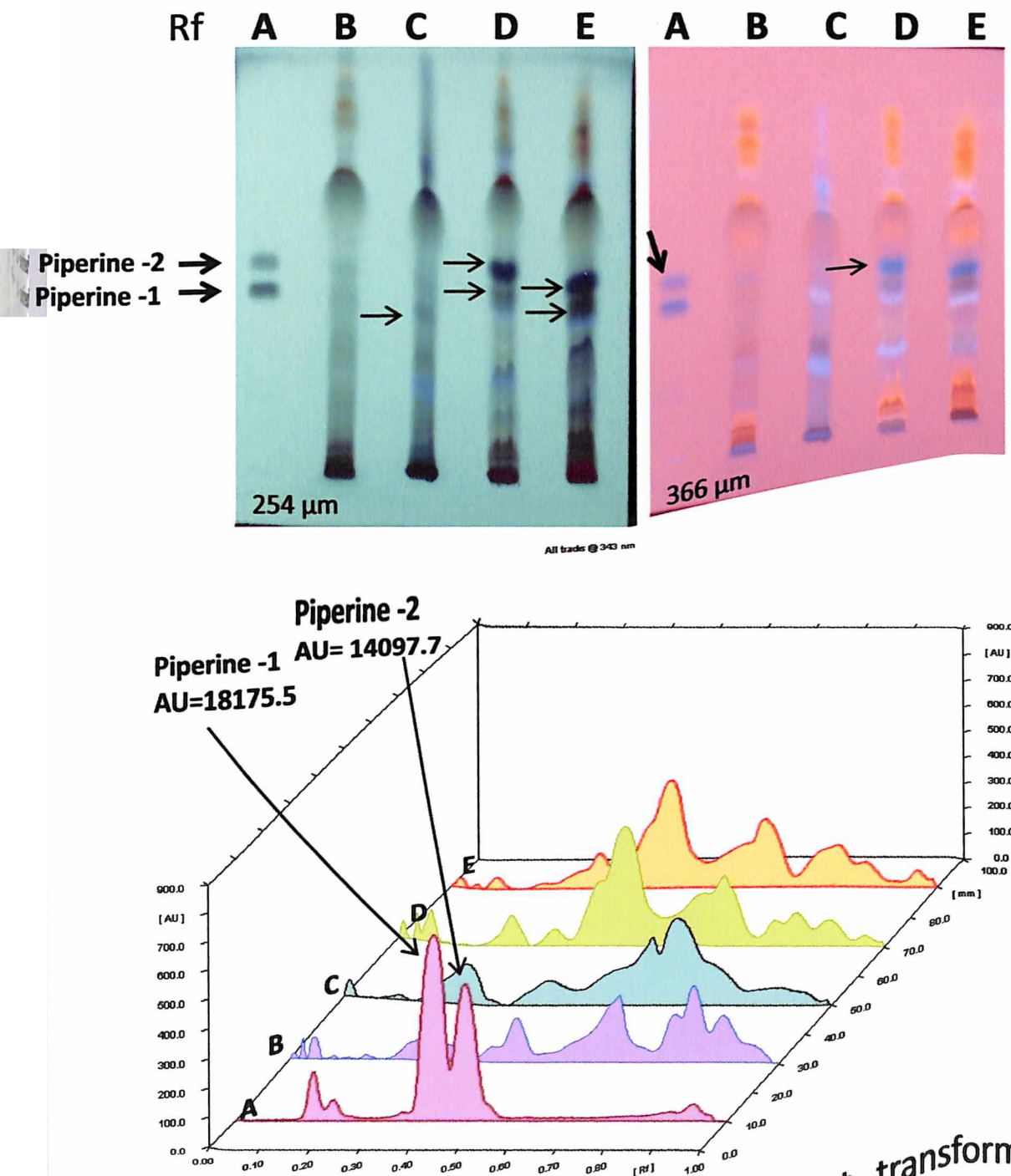


Fig. 4.9. Analysis of piperine content in leaf, root, transformed callus and non-transformed callus of Piper betle through TLC and HPTLC analysis. A- Standard of piperine 1 (AU= 18175.5) & piperine 2 (AU=14097.7), B- leaf extract sample, C- root extract sample, D- Transformed callu, E- Non-transformed callus

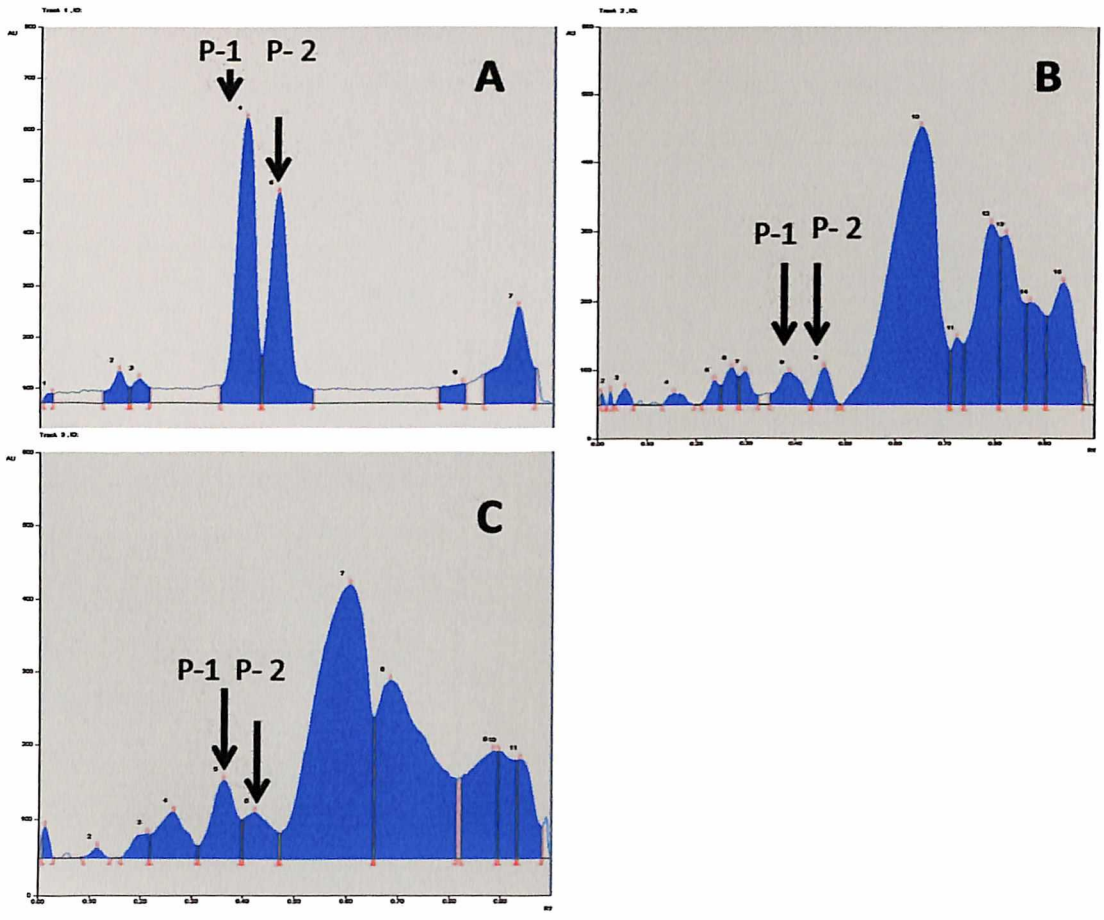


Fig. 4.10. HPTLC analysis of piperine content (P-1 & P-2) in leaf extract (B), root extract (C) of *Piper betle* as compare with standard (A)

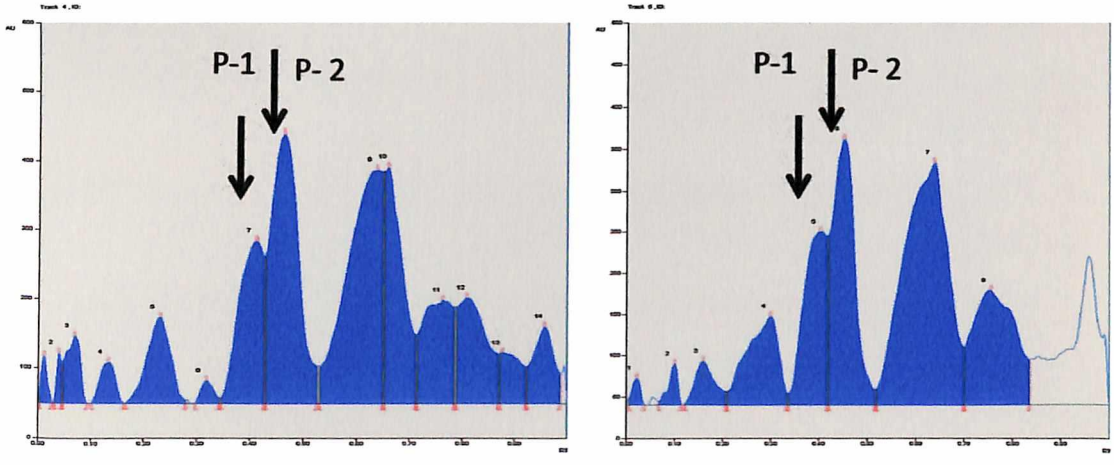


Fig. 4.11. HPTLC analysis of piperine content (P-1 & P-2) in transformed callus (D) and non-transformed callus (E) of *Piper betle* as compare with standard (A)

of *E.coli* (Fig. 4.12). However, the extract derived from transformed calli has also showed significant zone of inhibition i.e 19mm, 17 mm and 13 mm against *Micrococcus*, *S.epiderdimis* and *E.coli* respectively (Table 4.20). Bacteria are prokaryotes with thin cell wall and relatively simple genetic system, which enhance easy penetration of bioactive substances, leading to manipulation of genetic system as a result of bioactive interruption. Gram-positive bacteria possess a permeable cell wall that usually does not restrict the penetration of antimicrobials as does the gram-negative bacteria. The structural composition and arrangement of the cell wall of gram-negative bacteria is such that does not easily permit the penetration of the bioactive compounds. Grampositive bacteria are of a single layer, whereas the gramnegative cell wall as multi-layered structure (Lambert, 2002; Russell, 2002; Cetin and Gurler, 1989). In addition microorganisms show variable sensitivity to chemical substances related to different resistance levels between strains (Ogundare *et al.*, 2006). It may be also noted that some compounds do not shows antimicrobial activity, this may be because the active compound. Lack of activity can thus only be proven by using large doses. Alternatively, if the active principles are present in high enough quantities, there could be other constituents exerting anatagonistic effects or negating the positive effects of the bioactive agents (Jager *et al.*, 1996; Taylor *et al.*, 2001). The zone of inhibition was observed in different fungal strains such as *A. Flavus* and *R. Solani*. There was no zone of inhibition occurred in case of *Aspergillus Niger*. (Fig. 4.12). The zone of inhibition was maximum in *A.flavus* and *R.solani* against 60 mg/ml of the root extract and transformed callus of *P.betle* (Table 4.20). These results show that organic extracts particularly alcoholic exhibited better antibacterial principles either polar or non-polar are effectively extracted only through the organic solvent medium (Essawi and Srour, 2000). The antibacterial activity of plant extracts was not likely to be due to any one main active constituent but to the combined action of additional other compounds (Bai, 1990: Essawi and Srour, 2000). The aqueous extracts appear to have less antibacterial activity than any of the organic extracts. This is interesting in that the traditional method of treating a bacterial infection was by administering a decoction of the plant or apart there by boiling it in water, whereas according to our results an organic solvent is better, hence, this may be more beneficial.

Table.4.19. Phytochemical analysis of leaf (L), fruits (F), root (R), transformed (T) and non-transformed (NT) calli of *Piper betle* and other *Piper* species.
The data represent the mean value of two independent experiment.

Plant constituents	*Piper longum*			*Piper nigrum*			*Piper betle*			
	L	F	R	L	F	R	L	R	NT	T
Alkaloid	++	++	+	++	+++	++	+	++	+	++
Steroids	++	++	+	++	+++	++	+	++	+	++
Triterpenoid	++	+	++	++	+	+	++	+	++	++
Glycosides	++	++	-	-	++	-	+	+	+	++
Saponin	_	_	_	_	_	_	_	_	-	-
Carbohydrates	++	++	+	++	++	++	++	++	++	++
Flavonoids	+	++	++	+	+++	+++	+	+	++	+++
Tannins	+++	+	++	+++	++	+	+++	+++	+++	+++
Proteins	++	++	-	++	+	-	-	-	-	-

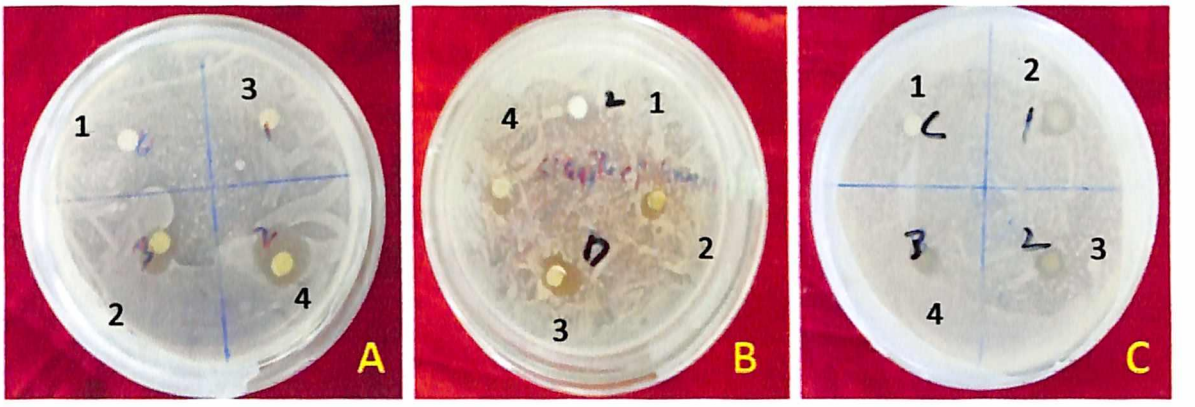
+ - Low, ++ - Medium, +++ - High

Table. 4.20. Antimicrobial activity of different explant extract, transformed and untransformed calli of *Piper betle.* The data represent the mean value of two independent experiment.

Plant species	Different plant extract used	Concentration (mg/ml)	Zone of inhibition		
			Bacterial Strains		
			Micrococcus	*S. epiderdimis*	*E.coli*
Piper betle	Leaf	0	0	0	0
		20	7mm	10mm	8mm
		40	16mm	13mm	9mm
		60	17mm	15mm	10mm
	Root	20	9mm	12mm	11mm
		40	20mm	16mm	13mm
		60	22mm	18mm	14mm
	Transformed Callus	20	8mm	11mm	9mm
		40	18mm	14mm	12mm
		60	19mm	17mm	13mm
	Untransformed callus	20	7mm	10mm	9mm
		40	17mm	14mm	10mm
		60	17mm	16mm	12mm

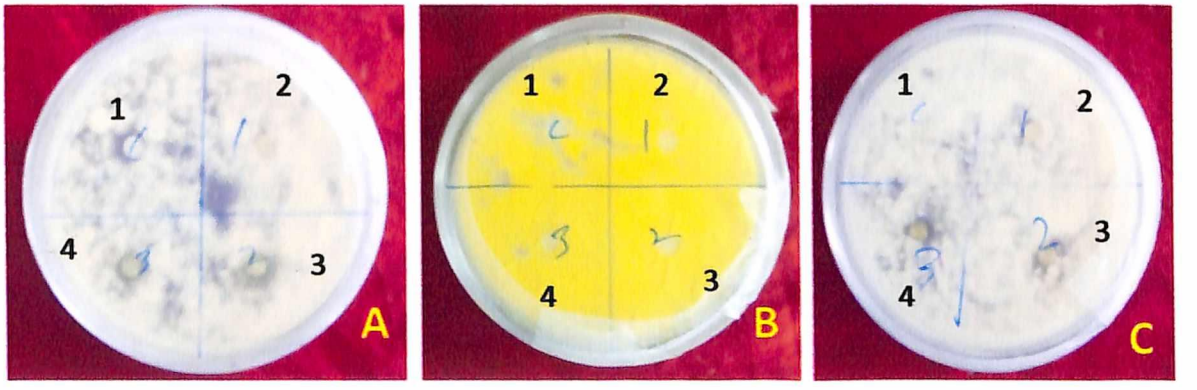
Plant species	Different plant extract used	Concentration (mg/ml)	Zone of inhibition		
			Fungal strains		
			A. flavus	*A. niger*	*R. solani*
Piper betle	Leaf	0	0	0	0
		20	6mm	NA	7mm
		40	9mm	NA	8mm
		60	11mm	NA	10mm
	Root	20	8mm	NA	9mm
		40	11mm	NA	10mm
		60	13mm	NA	12mm
	Transformed Callus	20	8mm	NA	8mm
		40	12mm	NA	8mm
		60	14mm	NA	11mm
	Untransformed callus	20	6mm	NA	8mm
		40	7mm	NA	8mm
		60	11mm	NA	11mm

Fig. 4.12A. Antibacterial activity (1- Control Methanol (1mg/ml), 2- 20mg/ml extract, 3- 40 mg/ml extract, 4- 60 mg/ml extract)



Effect of leaf extract of *P.chaba* on *Microccous* (A), fruit extract of P. *nigrum* on *Streptomyces epiderdimis* (B), leaf extract of *P.betle* on *E.coli* (C).

Fig. 4.12B.Antifungal activity (1- Control Methanol (1mg/ml), 2- 20mg/ml extract, 3- 40 mg/ml extract, 4- 60 mg/ml extract)



Effect of leaf extract of *P.chaba* on *Aspergillus flavus* (A), leaf extract of P. *longum* on *Aspergillus nizer* (B), leaf extract of *P.betle* on *Rhizoctonia solani (C).*

CHAPTER-V

SUMMARY AND CONCLUSION

Medicinal plants are gaining great interest in pharmaceutical industries for the production of high valued secondary compounds (Rout *et al.*, 2000: Das and Rout, 2002). There has been significant rise in number of various studies on piper species around the globe. Piper has recently attracted the attention of researchers because it has a wide range of medicinal and pharmacological application. The genus *Piper* (Piperaceae) is largely distributed in tropical and subtropical regions of the world. The genus has more than 1000 species but the most well known species are *P.nigrum*, *P.longum* and *P.betle*. In *P.nigrum*, abot 51 cultivars have been reported from the tropical and sub-tropical regions of the India. The fruits of *P.nigrum* are also used to produce white pepper and green pepper and valued due to presence of piperine. It has high amount of secondary compounds such as unsaturated amides, flavonids, lignans, long and short chain esters, terpenes, steroids and alkaloids and also having insecticidal activity. On the view of above fact, the present study is to standardize the in vitro protocol on plant regeneration of different Piper species and comparative analysis of secondary metabolite content in different explants as well as transformed and non-transformed calli of *Piper betle*.

Three cytokinins (BAP, Kn and Ads) have been tested for shoot multiplication from apical and axillary shoots of *Piper longum*, *Piper betle* and *Piper nigrum*. Among the three cytokinins, 6-benzylaminopurine and kinetin favoured maximum rate of shoot proliferation. Without cytokinin, the shoots did not show any positive response. The medium supplemented with BAP, Kinetin and adenine sulphate showed the maximum rate of shoot growth and multiplication. Among the different concentrations of nutrient tried, the full strength MS medium supplemented with BAP, Kinetin and adinine sulphate showed significant response as compared with other treatment. The medium having 2.0 mg/l BAP + 0.5 kinetin and 2.0 mg/l Kn + 1.5 mg/l BAP + 100 mg/l adenine sulfate favoured shoot multiplication in *Piper betle* and *Piper nigrum* respectively The shoot of *Piper longum* showed maximum percentage of shoot multiplication (66.2%) on MS medium supplemented with 2.0 mg/l BAP along with 1.0 mg/l Kinetin and 100 mg/ml of adenine sulphate. Increase

the concentration of BAP from 0.5 to 4.0 mg/ml showed increase in the percentage of response but the number of multiple shoots per culture was reduced. The average number of shoots varied from 1.2 - 2.8 per culture. In *Piper betle*, the percentage of multiple shoot formation was 40.4% in MS medium supplemented with 2.0 mg/l BAP along with 0.5 mg/l Kn. The average number of shoots varied from 1.0-2.3 shoots per culture. In case of *Piper nigrum,* the maximum percentage of multiple shoot formation was 36.4 % on MS medium supplemented with 2.0 mg/l Kn along with 1.5 mg/l BAP and 100mg/ml of adenine sulphate. The average number of multiple shoots per culture was ranged from 1.0 - 2.4. The combination of cytokinins and auxins also favoured positive impact on shoot proliferation and multiplication. Among the two auxins tested, BAP along with IAA favoured the maximum rate of shoot multiplication. In medium having 1.5 mg/l BAP along with 0.5 mg/l IAA showed the highest rate of shoot growth and proliferation in case of *Piper longum.* In case of *Piper longum*, the maximum percentage (83.3%) of shoot proliferation was observed in MS medium supplemented with 1.5 mg/l BAP, 0.5 mg/l IAA and 3% sucrose within 8 weeks of culture. The average number of multiple shoots per culture was varied from 1.0 - 3.4 in different treatment. In case of *Piper betle,* the maximum percentage of response on shoot multiple was 40% on MS medium supplemented with BAP 1.5 mg/l along with IAA 1.5 mg/l and 0.2 mg/l NAA. The average number of shoots per culture varied from 1.1 - 1.8 shoots per culture. In case of *Piper nigrum,* the maximum percentage of shoot proliferation was 34.2 % on MS medium supplemented with 2.0 mg/l BAP along with 2.0 mg/l IAA and 0.2 mg/l NAA. The average number of shoots varied from 1.0 -1.4 shoots per culture.

The development of callus is greatly influenced by the growth regulators and physiological condition. The result showed that the green friable callus was developed from leaf as well as stem after 4 - 5 weeks of culture on medium supplemented with 2, 4-D + IAA or NAA. The maximum proliferation of callus was observed from leaf and stem explants in *Piper betle* in the medium containing 2.5 mg/l 2, 4-D within 8 weeks of culture. The callus proliferation became declined if the medium having either lower concentrations of auxin alone or without auxin. The maximum proliferation of callus from stem and leaf explants were 73.3% and 83.4% respectively on medium containing 2.5 mg/l 2, 4-D. On the basis of comparative analysis, it was observed that the medium having 2, 4-D + IAA showed

higher callus proliferation than the 2, 4-D + NAA or NAA + IAA. In case of *Piper nigrum*, the maximum proliferation of callus from leaf and stem plants were observed in medium having 2.0 mg/l 2, 4-D and 2.0 mg/l IAA. Shoot bud regeneration did not achieved in callus derived from stem and leaf explants of *Piper nigrum*. The callus was compact in nature but the organogenic shoot primordial like appearance observed during the study period.

The friable calli derived from leaf explants of *Piper betle* was co-cultivated with *Agrobacterium rhizogenesis* (A4 strain) for induction of roots to synthesize the higher amount of secondary metabolite. The transformed calli were subsequently transferred to different antibiotic solution to stop the bacterial infection. To standardize lethal dose of carbencillin and cefotaxime, the putative transformed tissues were cultured on MS medium with different concentration of carbencilin / cefotaxime (0, 100, 200, 300, 400 and 500 mg/l). The results showed that the agrobacterium growth was not inhibited in medium having 100 and 200 mg/l carbencilin or cefotaxine. The medium having 500 mg/l carbenicillin / cefotaxime had a deleterious effect. The growth of agrobacterium was fully control over infection in transformed calli. Therefore, 500 mg/l carbenicilin was used as a selection agent. Infection time of 20 - 30 min and co-cultivation period of 2 days were found to be optimum for induction of transformed root cultures.

The healthy microshoots were separated from the clump and transferred to different medium having various concentrations of auxins with 2% sucrose for induction of rooting. The maximum percentage of rooting was observed in half strength MS media supplemented with 0.25 mg/l IBA and 2% sucrose. The higher percentage of rooting was 73.3% in case of *Piper longum* and 33.3% in case of *Piper nigrum*. The rooted plants were transferred to plastic pots containing soil mixture and kept in green house with 85% humidity for acclimatization. About 90% of the plantlets were established in the green house within 1-2 weeks of transfer.

The piperine content was varied in different species of Piper. The resultindicate that the roots having higher content of piperine than leaf and non-transformed calli. On the basis of TLC and HPTLC analysis, it was observed that there were two compounds such as Piperine-1 and Piperine-2 obtained from the leaf, root, transformed and non-transformed callus extract of *Piper betle* as compared to

standard. The TLC analysis showed that the Rf value of Piperine -1 and Piperine -2 ranged from 0.39 - 0.40 and 0.42 – 0.46 respectively. On the basis of HPTLC analysis, it was observed that the transformed calli having higher content of piperine (0.56%) with the unit of 10187.4 AU as compared with root, leaf and non-transformed calli extracts. The piperine -2 content was maximum in transformed calli extract as compared with non-transformed calli, leaf and stem extract.

The phytochemical analysis showed that the alkaloid and steroid content were present in all the *Piper* species as well as transform and non-transformed calli of *P.betle.* The saponin was absent in all the species of *Piper* including transformed and non-transformed calli. The protein was present in leaf and fruits of *P.longum* and *Piper nigrum* and it absent in *P.betle* including transformed and non-transformed calli. The flavonoid content was high in leaf and root of *P.nigrum* and transformed calli of *P.betle.*

The antimicrobial activity was tested as per the zone of inhibition formed by the tissue extract against bacteria and fungus. Leaf, root, transformed and non-transform calli extracts of *Piper betle* were tested against the pathogenic bacteria namely *Escherichia coli, Micrococcus luteus, Streptomyces epidermidis* and fungal culture of *Aspergillus niger, Aspergillus flavus* and *Rhizoctonia solani* at different concentrations (0, 20, 40, 60 mg/ml). The zone of inhibition was maximum in all the bacterial strains in the concentrations of 60 mg/ml of root extract of *P.betle* i.e 22 mm in case of *Micrococcus*, 18 mm in case of *S.epiderdimis* and 14 mm in case of *E.coli.* However, the extract derived from transformed calli has also showed significant zone of inhibition i.e 19mm, 17 mm and 13 mm against *Micrococcus, S.epiderdimis* and *E.coli* respectively. The zone of inhibition was observed in different fungal strains such as *A. Flavus* and *R. Solani.* There was no zone of inhibition occurred in case of *Aspergillus Niger.* The zone of inhibition was maximum in *A.flavus* and *R.solani* against 60 mg/ml of the root extract and transformed callus of *P.betle.* The antibacterial activity of plant extracts was not likely to be due to any one main active constituent but to the combined action of additional other compounds. The aqueous extracts appear to have less antibacterial activity than any of the organic extracts. This is interesting in that the traditional method of treating a bacterial infection was by administering a decoction of the plant or apart there by boiling it in

water, whereas according to our results an organic solvent is better, hence, this may be more beneficial.

In conclusion, the outline of protocol offers a potential system for improvement, conservation and micro-propagation of *Piper species* from leaves, stem and internodal explants. Further research and conservation of all plant species including medicinal plants is needed to preserve nature's natural drugs. Advances in plant tissue culture will enable rapid multiplication and sustainable use of medicinal plants for future generations.

CHAPTER- VI

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APPENDICES

Appendix I.

Preparation of LPGM (Liquid Plant Growth Medium)
LPGM was prepared same as MS medium except sucrose and agar

Appendix - II

Yeast extract mannitol agar (YEMA)

Chemical	Quantity
Yeast extract	0.1g
Mannitol	1.0g
KH_2PO_4	0.05g
$MgSo47H_2O$	0.02g
NaCl	0.01g
Agar	1.5g

By adding all chemical in 100ml of D/W media was autoclaved and used for next purpose.

Appendix –III

Nutrient agar (100ml)

Chemical	Quantity
Beef extract	0.1g
Yeast extract	0.2g
Peptone	0.5g
NaCl	0.5g
Agar	1.5g

By adding all chemical in 100ml of D/W media was autoclaved and used for next purpose.

Appendix –IV

Potato dextrose agar

Chemical	Quantity
Potato infusion	0.2g
Dextrose	0.02g
Agar	0.02g

Dextrose Agar (100ml)
By adding all chemical in 100ml of D/W media was autoclaved and used for next purpose.

Appendix –V

LPGM (Liquid Plant Growth Medium)
Liquid MS Medium + 2 mg/l BAP + 200Mm Acetosyringone

Appendix -VI

1. **Mobile Phase for HPTLC**
   Toluene: Ethyl acetate (7:3)
   Toluene - 70ml
   Ethyl acetate - 30ml

2. **Spraying reagent for HPTLC**
   Ethanol : Sulphuric acid
   Ethanol -95ml
   H_2SO_4 - 5ml

Appendix VII: Equipments used

- Autoclave (Arch Tech)
- B.O.D. incubator (Remi)
- Deep freeze -20 0C (Blue star)
- Electronic balances (Sartorios)
- Hot air oven (Wiswo)
- Laminar flower (Clear)
- Microwave ovens (Samsung)
- Magnetic stirrer (Remi)
- Microscope (Zeiss)
- PH meter (EU-Tech)
- Refrigerated Centrifuges (Remi)
- Refrigerator (Whirlpool)
- UV transilluminator (UVI Tech)
- Vortex mixer (Geni)
- Ice maker (orumsem)
- Incubator shaker (Pelican)
- Mini centrifuge (Biofuge)
- Spectrophotometer (BL-190)
- Water bath (GFL)
- Water purification system (Borosil)
- Lyophilizer (Christ)
- Rotary shaker (Remi)