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DETECTION AND QUANTIFICATION OF PIPERINE FROM *IN VITRO* CULTURES OF BLACK PEPPER (*Piper nigrum* L.)

By

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(2009-11-103)

THESIS

Submitted in partial fulfillment of the
requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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2011

DECLARATION

I hereby declare that the thesis entitled **"Detection and quantification of piperine from *in vitro* cultures of black pepper (*Piper nigrum* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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Certified that the thesis entitled **"Detection and quantification of piperine from *in vitro* cultures of black pepper (*Piper nigrum* L.)"** is a record of research work done independently by **Mr. Achintya Kumar Dolui** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

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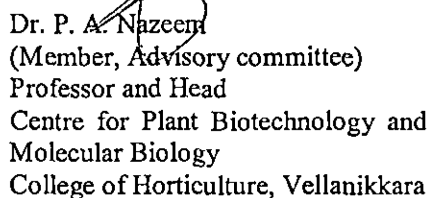

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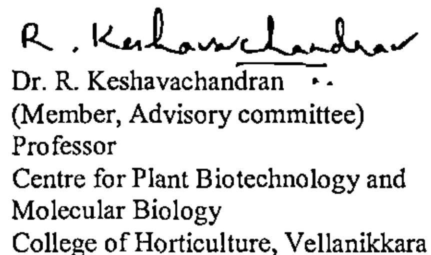

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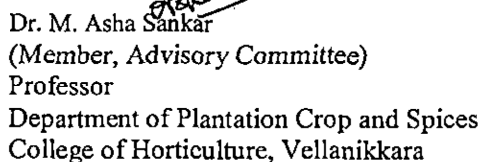

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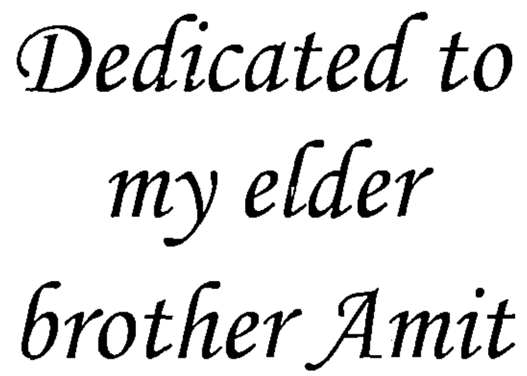
Dedicated to
my elder
brother Amit

ACKNOWLEDGEMENT

First and foremost I bow my head before my baba and ma who enabled me to successfully complete the thesis work.

With immense pleasure I avail this opportunity to express my deep sense of gratitude and indebtedness to my major advisor Dr. Sujatha. R., Associate Professor, Centre for Plant Biotechnology and Molecular Biology, College of Horticulture and Chairperson of my Advisory Committee for her valuable guidance, constructive suggestions, unfailing patience, kind considerations, friendly approach, timely help at various stages of my work.

I would like to express my extreme indebtedness to Dr. P. A. Nazeem., Professor & Head, Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara and member of my Advisory Committee for her meticulous help, well timed support and critical scrutiny of the manuscript which has helped a lot for the improvement and preparation of the thesis. I owe a deep sense of gratitude to her.

I sincerely thank Dr. R. Keshavachandran, Professor, Centre for Plant Biotechnology and Molecular Biology, College of Horticulture and member of my Advisory Committee for his expert advice and constructive criticisms rendered throughout the study period.

I express my heartiest gratitude to Dr. M. Asha Sankar, Professor, Department of Plantation Crop and Spices, College of Horticulture and member of my Advisory Committee for her ever willing help, valuable guidance and creative suggestions during the period of my work.

I am obliged to Dr. S. Krishnan, Associate Professor & Head, Department of Agricultural Statistics, College of Horticulture, who helped me in doing the statistical analysis.

I am obliged to Dr. D. Girija, Professor & Head, Department of Agricultural Microbiology, Dr. P.A. Valsala, Professor and Dr. P.S. Abida, Assistant Professor, Centre for Plant Biotechnology and Molecular Biology, whose moral support have helped to complete this venture successfully.

I thank Mr. Srikumar, Farm Officer, Centre for Plant Biotechnology and Molecular Biology, for his help during the conduct of this research work.

I express my deep sense of gratitude to Vidya chechi, Biji chechi, Laxmi chechi, Priya chechi, Aswini chechi, Dale chechi, Shamini chechi, Soorya chechi and Arun who helped me in several ways for the completion of this venture.

I take this opportunity to thank my roommates Chinmoy, Ashok, Danish and batch mates Lubina, Sahna, Rakesh, Manjunath, Somya, Adarsha, Sarveshwar, Pradeep, Elaverson Kannan, and Liju for their prompt help and cooperation for the entire period of study.

I am in dearth of words to thank my friends Bishnu da, Dwaipayan, Prabir and Manju chechi, who stood by me during all the hard times.

I wish to express my thanks to my loving juniors Amar, Ashok, Renju, Meera, Reshmi, Pawan, Narender, Pramod, Rohit, and Alex anna from College of Horticulture, KAU.

I wish to express my sincere thanks to my friends Harish, Varun, Nitin, Sreenath, Divya, Saju cheta and Jusna chechi of CPBMB for their whole hearted cooperation.

Words cannot express the help that I relished from my lovely parents and my younger brother Apurba for the heartfelt help and support rendered throughout the study period.

My deepest gratitude goes to my family, Jatha, Kaka, Kakima, Shikha didi, Asim da, Amiyo, Jharna, Rita, Sadhna, Sourish, Dipu, Hiru, Anannya , for their unflagging love and support throughout my life.

I wish to express my thanks to Department of Biotechnology for the financial support in the form of JNU fellowship for the M Sc. programme.

A note of thanks to one and all who worked for the successful completion of this endeavour.

Achintya Kumar Dolui

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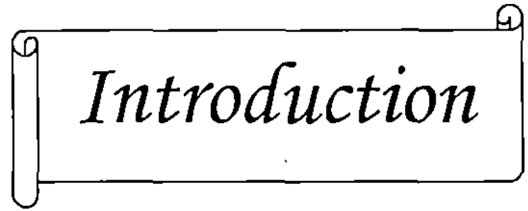
ABBREVIATIONS

ABA	Abscisic acid
BA	6-benzylaminopurine
°C	Degree Celsius
Cm	Centimeter
Dw	Dry weight
g	Gram
GA	Gibberelic acid
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KAU	Kerala Agricultural University
Kin	Kinetin
L	Litre
LS	Linsmair and Skoog
LAF	Laminar Air Flow
μl	Micro liter
M	Molar
mg	Milligram
ml	Milliliter
μM	Micromolar
min	minutes
mM	Millimolar
Mm	Milli mitre
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
%	Percent

PDA	Photo Diode Array
pH	Hydrogen ion concentration
ppm	Parts per million
SE	Standard Error
SH	Schenk and Hildeberandth
UV	Ultra violet
V/V	Volume/Volume
W/V	Weight/Volume
2, 4-D	2, 4-dichlorophenoxyacetic acid


Introduction

1. INTRODUCTION

Since early days of mankind, plants with secondary metabolites have been used by humans to treat infections, health disorders and illness (Wyk and Wink, 2004). Many plants are major sources of useful secondary metabolites which are used in pharmaceutical, agrochemical, flavor and aroma industries. Biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives for production of desirable medicinal compounds from plants (Rao and Ravishankar, 2002).

Plant tissue culture has been applied for the production of secondary metabolites on a commercial scale since late 1950s, when atropine from the roots of *Atropa belladonna* was synthesized and accumulated in roots and in callus (West-jr and Mika, 1957). The advantage of this method is that it removes all seasonal constraints and eliminates the geographic barriers for production of secondary metabolites (Karuppusamy, 2009).

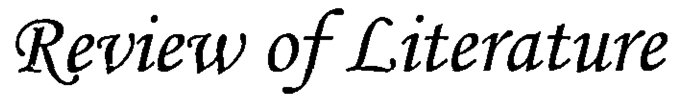
Among the plants investigated to date, one showing enormous potential for secondary metabolite is the pepper family otherwise known as Piperaceae (Dodson *et al.*, 2000). Among the spices, *Piper nigrum* (black pepper) popularly known as the 'King of spices' is a monoecious or dioceous climbing vine native to southern India and SriLanka (Reshmi *et al.*, 2010). Pepper contributes to about 35 per cent of the world's spice trade. Around 416 different chemicals are reported in black pepper among which the alkaloid piperine is the major pungent principle, which accounts for 90 to 95 per cent of the total pungency of the spice. The piperine content in the different cultivars of pepper is reported to vary from 2.0 to 7.4 per cent (Ravindran *et al.*, 2000).

Majeed *et al.* (1999) reported that piperine is widely used in various herbal cough syrups for its potent anti-tussive and bronchodilator properties. It is used as an anti-inflammatory, anti malarial and anti leukemial agent.

Recent medical studies have shown that it is useful in increasing the absorption of certain vitamins, selenium and beta-carotene apart from increasing the body's natural thermogenic activity (Reshmi *et al.*, 2010). It has antimicrobial (Dorman and Deans, 2000), antimutagenic (El *et al.*, 2003), antioxidant and radical scavenging properity (Gulcin, 2005) and inhalation of black pepper oil increase the reflexive swallowing movement (Vijayakumar *et al*, 2004).

The crop is also amenable to *in vitro* manipulation as repoted by Sujatha *et al.* (2003). Though protocol for the callus culture in black pepper has been standardized (Nazeem *et. al.*, 1992), the possibility of exploitation of *in vitro* cultures for secondary metabolite production in this crop has not been looked into.

Keeping in view of the significance piperine has attained, the present study envisages exploring the possibility of enhancement of piperine production under *in vitro* conditions. The objectives of this work are to standardize the protocol for *in vitro* synthesis of piperine in black pepper and to detect and quantify the *in vitro* synthesized piperine.


Review of Literature

2. REVIEW OF LITERATURE

The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. However, it is not the only commercial significance that drives the research initiatives. The deliberate stimulation of defined chemical products within carefully regulated *in vitro* cultures provides an excellent material for in-depth investigation of biochemical and metabolic pathways, under highly controlled micro environmental regimes.

Secondary products in plant cell culture can be generated on a continuous year-round basis; there are no seasonal constraints. Production is reliable, predictable, and independent of ambient weather. At least in some cases, the yield per gram fresh weight may exceed that which is found in nature (Karuppusamy, 2009).

2.1 Brief description of black pepper

2.1.1 Botanical description

Black pepper is a climbing perennial shrub. Branches are stout, trailing and rooting at the node. The stout climbing stem is highly flexible with leathery blackish green leaves. Leaves are entire, variable in length and breadth, 12.5 to 17.5 cm by 5.0 to 12.5 cm. Flowers are minute in spikes, dioecious, often female,

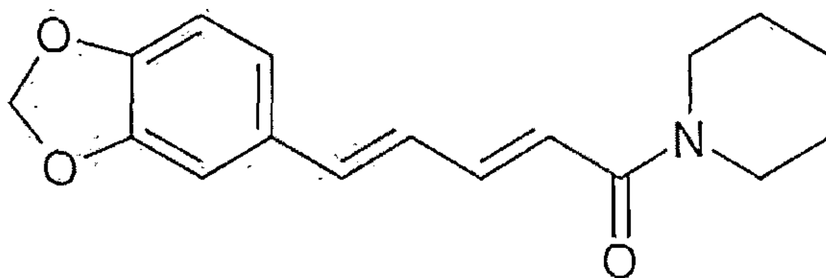
bears 2 anthers and the male, pistillode. Fruiting spikes are variable in length. Fruits are globose and bright red when ripe, seeds usually globose. Fruits botanically described as drupe.

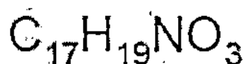
2.1.2 Active principle in Black Pepper

Black pepper is valued for its distinct biting quality attributed to piperine and its isomers (Govindarajan, 1977). Piperine (1-peperoylpiperidine), the primary pungent alkaloid in black pepper corns derived from the fruit bodies of *Piper nigrum*, is commonly ingested in many diets throughout the world. In addition to its common utility as a gustatory enhancer, it has found many rather diverse applications ranging from brandy flavouring to its use as an insecticide (Budavari *et al.*, 1989).

Commercial products of piperine are available in market by the name Bioperine®. It also enters into anticonvulsant, antidepressant and muscle relaxing drug formulations. Piperine is the trans stereoisomer of 1-piperoylpiperidine. It is also known as (E, E)-1- piperoylpiperidine and (E, E)-1- [5-(1, 3- benzodioxol-5-yl)-1-oxo-2, 4-pentdienyl] piperidine.

Piperine.





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2.2 Accumulation of secondary metabolites in *in vitro* cultures

Fujita (1988) for the first time reported the *in vitro* production of secondary metabolite on a commercial scale from *Lithospermum erythrorhizon,* which yielded 15 to 20 per cent shikonine in comparison to one to two percent by the field grown plants. The present status of secondary metabolite production by *in vitro* techniques are given in Table 1.

2.3 Establishment of callus culture

2.3.1 Selection of explants

Different explants like leaves, internodes, stem segments, seeds are used as the source for establishing *in vitro* cultures.

Regeneration from callus cultures of *Centella sp.* using stem and leaf explant from green house grown mother plant (Patra *et al.*, 1998). Saba *et al.*(1999) reported use of nodal segments in *Ammi majuss* L. Tiwari *et al.* (2000a) reported use of nodal segments for clonal propagation of *Centella asiatica.* Morphogenetic potential of node, internode and leaf explants of Brahmi (*Bacopa monniera* L. Wettst.) was investigated to develop reliable protocols for shoot regeneration and somatic embryogenesis (Tiwari *et al.*, 2000b). Shoot tip, nodal and internodal segments were reported in *Phyllanthus amarus* (Ghanti *et al.*, 2004).

Callus cultures were established from nodal and leaf explants. Leaf explants showed better callus initiation than nodal explants from *Vanilla planifolia* (Janarthanam and Seshadri, 2008).

Table 1. ***In vitro*** **production of plant secondary metabolites**

Metabolite	**Species**	**Yield g/l**	**Dry weight (%)**	**Reference**
Berberine	*Coptis japonica*	7.0	12	Fujita and Tabata, 1987
Rosmarinic acid	*Coleus blumei*	5.6	20	Ulbrich *et al.*, 1985
Anthocyanins	*Salvia officianlis*	6.4		Hippolyte *et al.*, 1992
Shikonin	*Lithospermum erythrorhizon*	3.5	12	Fujita, 1988
Anthraquinones	*Morinda citrifolia*	2.5	18	Zenk *et al.*, 1975
Raucaffricine	*Rauwolfia serpentina*	1.6	3.0	Schubel *et al.*, 1985
Paclitaxel	*Taxus* species	0.1 – 0.3		Yukimune *et al.*, 1996
Jatrorrhizine	*Berberis wilsoniae*	3.0	12	Breuling *et al.*, 1985

2.3.2 Sterilization treatment

Surface sterilization is the most important step before inoculation of explants. Different steps have been employed for treatment of explants. Different sterilization treatment was followed by Mathur and Kumar (1998) in which leaves and stem explants of *Bacopa monnieri* were shaken for 10 minutes in Tween 20 and Savlon for 10 min, rinsed in running water for 30 min, treated with 0.1 per cent mercuric chloride for 3 to 4 min and washed several times with sterile water for reduction of microbial loads.

Shrivastava and Rajani (1999) have described sterilization treatment of *Bacopa*, which includes use of 0.1 per cent mercuric chloride for 2 min followed by rinsing thoroughly with sterile distilled water.

Tiwari *et al.* (2000a) suggested that for contamination free micropropagation of *Centella asiatica*, plants were washed thoroughly for 30 min under running tap water followed by removal of leaves, which was followed by soaking in the mixture of 1 per cent cetrimide solution containing 150 mg l^{-1} bavistin and 50 mg l^{-1} Trimethoprim for 25 min. The explants were finally treated with 0.1 per cent mercuric chloride for 3-4 min followed by rinsing in sterile distilled water for 4-5 times.

Guava seeds were taken from mature fruits thoroughly washed and surface sterilized with 0.05 per cent mercuric chloride ($HgCl_2$) for 5 min followed by 4 rinsing with sterile distilled water and was suitable for *in vitro* cultures free from contamination (Zamir *et al.*, 2004). Singh *et al.* (2009) reported that *Rauvolfia serpentina* leaves were treated with bavistin solution for 4-5 min., and then rinsed thoroughly with sterile distilled water. The leaves were subjected to 0.1 per cent mercuric chloride ($HgCl_2$) for 30 second, washed with distilled water and then

placed in 70 per cent ethanol for 1 min. and again washed with distilled water for culture initiation. Chordia *et al.* (2010) reported that shoot explants surface sterilized with 10 per cent sodium hypochlorite for 10 min and rinsed with sterile double distilled water 4 - 5 times was suitable for contamination free callus culture of *Vitex leucoxylon L.*

2.4 Medium for callus establishment and proliferation

Several media formulations are commonly used for the majority of cell and tissue culture work. These media formulations include those described by MS (Murashige and Skoog, 1962), LS (Linsmair and Skoog 1965), B_5 by Gamborg *et al.* (1968), SH (Schenk and Hildeberandth, 1972) and McCown (Llyod and McCown, 1980) media.

Medium standardization has been an important parameter in plant cell culture technology since the composition of the culture media is known to influence the biomass yield as well as secondary metabolite production. Medium composition, culture conditions and exogenous phytohormone combinations together influence the metabolite accumulation in the cell (Seabrook, 1980). Callus cultures of *Phyllanthus acidus* (Skeels) were established on MS media for production of phyllanthusol A as the intact plant does (Duangporn and Siripong, 2009). Murashige Skoog (MS) Medium is one of the most widely used medium for rapid growth of callus (Shilpa *et al.*, 2010).

The internodal stem bits from node tip of *Gymnema sylvestre* were used to initiate callus on full strength MS medium (Murashige and Skoog, 1962) supplemented with 4 mg l^{-1} 2, 4-D (Rani *at el.*, 2010). Raziuddin *et al.* (2010) tested two basal media, LS and MS supplemented with 3 per cent sucrose and 0.8 per cent agar for the induction of embryogenic calli of *Triticum aestivum* L. Rajmohan *et al.* (2010) supplementd MS medium with copper sulphate (15 mg l^{-1}) for overcoming the the inheritance of systemic bacteria in *in vitro* cultures of

black pepper, as it is having algicidal, fungicidal, bactericidal and herbicidal properties.

2.5 Strategies to improve secondary metabolite production

Manipulation of physical aspects and nutritional elements in a culture is perhaps the most fundamental approach for optimization of culture productivity, as reported by various investigators. A number of chemical and physical factors affecting production of secondary metabolites, such as media components; phytohormones, pH, etc. have been extensively studied (Lee and Shuler, 2000). Rosmarinic acid by *Coleus bluemei* (Ulbrich *et al.,* 1985), ubiquinone-10 from *Nicotiana tabacum* (Fontanel and Tabata, 1987), berberin by *Coptis japonica* (Matsubara *et al.,* 1989), shikonin from *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991), ginsenosides from *Panax ginseng* (Choi *et al.,* 1994), are a few examples of compounds which accumulated in much higher levels in cultured cells than in the intact plants.

2.5.1 Modifying sucrose concentration

Zenk *et al.* (1975) reported that 5 per cent sucrose concentration was best for the anthraquinone yield in cell suspension cultures of *Morinda citrifolia*. For indole alkaloid production in cell cultures of *Catharanthus roseus*, 8 per cent sucrose was found to be optimal in the tested concentration of 4 to 12 per cent (Knobloch and Berlin, 1980). Suzuki *et al.* (1984) reported that 5 per cent sucrose was best with respect to optimal anthraquinone production in *Rubia cordifolia* cell suspension culture. It was found that maximum alkaloid yield was obtained with a medium with 8 percent sucrose (Winjsma *et al.,* 1986). Kim *et al.* (1995) suggested the role of sucrose in culture medium for biomass accumulation and taxol production. Ellis *et al.* (1996) were able to stimulate taxol production in *Taxus sp.* by increasing sucrose concentration to 8 per cent. Sucrose concentration in the culture medium affected the morphological parameters such as growth,

primary metabolism and yield of secondary products in the plants (Jacob and Malpathak, 2004).

When high concentration (30-45 g l^{-1}) of sucrose was applied, optimal bromelain production was obtained in *in vitro* culture of *Ananas comosus* (Ngampanya and Phongtongpasuk, 2006). High chlorophyll content was observed in plants grown on Murashige and Skoog basal medium supplemented with 131.85 mM sucrose (Anirudhan and Nair, 2009). Among the various concentrations of sucrose (1 -8%) tested, four percent sucrose was found suitable for withanolide A production (Nagella and Murthy, 2010).

2.5.2 Manipulation of nitrate and phosphate concentration

An overall level of total nitrogen has been found to affect production of secondary metabolites. Apart from nitrogen source, phosphates were also found to play an important role in increasing the secondary metabolite production. It has been observed that added phosphates in the culture medium resulted in prolonged cell growth in *Catharanthus roseus* cultures (Mac-Carthy *et al.*, 1980). Increased phosphate also stimulated the production of digitoxin in *Digitalis purpurea* (Hagimori *et al.*, 1982).

It has also been observed that reduced levels of total nitrogen improved the production of anthraquinones in *Morinda citrifolia* and anthocyanins in *Vitis* species (Yamakawa *et al.*, 1983). When the ratio of ammonium to nitrate nitrogen was 1:1, the total anthraquinone yield was about 100 per cent higher than that of the control culture in *Rubia cordifolia* cell suspension culture (Suzuki *et al.*, 1984).

For example, reduced levels of ammonia and increased nitrate levels were found to promote the production of shikonin and betacyanins, whereas higher ammonia to nitrate ratio was found to increase the production of berberine and

ubiquinone (Bohm and Rink, 1988). Similar results were obtained in a separate study for the production of betacyanins in callus cultures of *Beta vulgaris* (Bohm and Rink, 1988).

With respect to anthocyanin biosynthesis, cultured cells of *Vitis sp.* showed that maximum anthocyanin was accumulated when it was grown in a medium containing ammonium and nitrate in 1:1 ratio. Production of anthraquinone in *Morinda citrifolia* rosmaric acid in *Anchusa officinalis* and betacyanin in *Phytolaca americana* was improved when phosphate concentration was increased (Mizukami *et al.*, 1991).

2.5.3 Plant growth regulators

In a plant cell tissue culture study, growth regulator concentration plays a crucial role in secondary metabolite accumulation (Di-Cosmo and Towers, 1984). The concentration of auxin and cytokinin individually or in combination significantly alters both the growth and the secondary metabolite accumulation in cultured cells (Mantell and Smith, 1984). In cases where secondary metabolite production is inhibited, elimination of 2, 4-D or replacement of 2, 4-D with other auxins such as Naphthalene Acetic Acid (NAA) or Indole Acetic Acid (IAA) has been found to enhance the production of nicotine in suspensions of *Nicotiana tabacum* and shikonin in suspensions *of Lithospermum erythrorhizon* (Sahai and Shuler, 1984).

Cytokinins have been observed to have different effects depending on the type of plant species and metabolite. Kinetin has been found to stimulate the production of anthocyanin in *Haplopappus gracilis* but inhibited the formation of anthocyanins in Populus cell cultures (Seitz and Hinderer, 1988). Apart from auxins and cytokinins, other plant growth regulators such as Gibberellic acid and abscisic acid have been reported to suppress production of anthocyanins in a number of cultures (Bohm and Rink, 1988).

The growth regulator 2, 4-dichlorophenoxyacetic acid (2, 4-D) was reported to play an important role in callus initiation. There are reports where cultures grown in the presence of 2, 4-D have shown secondary metabolite induction for eg. carotenoid and anthocyanin production in callus cultures of *Daucus carota* (Rajendran *et al.*, 1992) and *Oxalis linearis* (Meyer and Staden,1995), respectively.

Umamaheswari and Lalitha (2007) cultured callus of *Capsicum annum* L. on MS Media with the various combinations of GA, IAA, NAA, 2, 4-D and kinetine. Of all combinations of growth hormones tried, MS medium with 2.0 mg l^{-1} 2, 4-D and 0.5 mgl^{-1} Kinetin was producing significant (1.6 mg g-1 of fresh weight of the callus) amount of capsaicin.

2.5.4 Elicitation of *in vitro* products creating condition of stress

Mannitol, a sugar which also creates an osmotic stress or shock in the culture medium would enhance the production of alkaloids to even 4-fold (Jian *et al.*, 2000) in *Catharanthus roseus* cell cultures. High osmotic pressure conditions generated by non-metabolic sugar (mannitol and sorbitol) enhanced paclitaxel production by about two-fold in suspension cell cultures of *Taxus chinensis* (Kim *et al.*, 2010). Addition of Methyl Jasmonate (MJ) inhibited the cell growth and promoted secondary metabolite production with cell suspension cultures of *Panax ginseng* C.A. Meyer (Kim *et al.*, 2004).

The highest values of total alkaloids (per cent) percentage of Vinblastin and Vincristine as relative to Vinblastine and Vincristine of intact plant were recorded with leaf derived calli cultures from MS medium when supplemented with 8 per cent of mannitol (Taha *et al.*, 2009). The highest value of total alkaloids (Vinblastin and Vincristine) were recorded with leaf derived calli cultures from MS media which were supplemented with 8 per cent of mannitol (Taha *et al.*, 2009).

2.5.5 Precursor feeding for secondary metabolite production

Favourable influence of the precursor phenylalanine on the expression of alkaloid in leaf and stem calli of Sida cordifolia was evident at 50 mg l^{-1} and 100 mg l^{-1} (Sankar, 1998).

It has been noticed that addition of precursors or related compounds (which intermediate at the beginning of a secondary metabolite biosynthetic route) to the culture media sometimes stimulate secondary metabolite production (Mulabagal and Tsay, 2004). Production of triterpenes in leaf derived callus and cell suspension cultures of *Centella asiatica* was enhanced by the feeding of amino acids. In the callus culture, manifold increase of asiaticoside accumulation was reported with the addition of Lucine (Kiong *et al.*, 2005). The effect of coniferyl alcohol as a precursor of flavonolignan biosynthesis on silymarin components production in *Silybum marianum* suspension culture was reported (Tumova *et al.*, 2006).

Feeding the shoot cultures with unlabelled isoleucine at a concentration of 2 mM induced a 3-7-fold increase in the production of adhyperforin. Addition of 3 mM threonine, a precursor of isoleucine, stimulated a 2-fold increase in the accumulation of adhyperforin (Karppinen *et al.,* 2007).To stimulate the production of paclitaxel, phenylisoserine was added to the cell suspension cultures of *Taxus cuspidate.* The level of production was enhanced 3.4 fold compared to that of the control when the phenylisoserine (0.5 mg l^{-1}) was added (Tachinbana *et al.*, 2007).

2.6 Biochemical estimation of alkaloid

2.6.1 Extraction of alkaloids

Two major extracting phases are suggested in literature for the extraction of alkaloids, such as methanol and ethyl acetate, however acetic acid (Renaudin,

1989), ethanol (Uniyal *et al.*, 2001), phosphoric acid (Blom *et al.*, 1991), and acetone for secologanin (Contin *et al.*, 1998) were also used.

High performance liquid chromatography (HPLC) method is described for detecting ergot (alkaloid) in ground or pelleted forages and grains. Samples were extracted with alkaline chloroform, filtered, and applied to silica gel/organic binder cleanup columns (George *et al.*, 1993). Gymnemic acid was quantified directly in callus. Ten gram of callus was weighed, dried in lyophilizer and extracted with 60 per cent methanol for HPLC analysis (Villareal *et al.*, 1997).

For HPLC analysis of ramiflorine A and andramiflorine B alkaloid, the crude extract was obtained using methanol as solvent (Bindoi *et al.*, 2004). One gram each of the air-dried leaves of the cultivated parent plants, *in vitro* regenerated leaves and callus of the leaves of *Hyoscyamus muticus* L. was accurately weighed and extracted using chloroform as solvent. Each chloroform extract was filtered through 0.2 μm PTFE membrane filter before injection into HPLC column (Ibrahim *et al.*, 2009).

The content of vincristine in field-grown plants and in vitro cultures were estimated using vincristine (Sigma–Aldrich) as standard. One gram each of various harvested *in vitro* cultured sample and field grown *Catharanthus roseus* plant were dried separately at 45.8C for a week and pulverized in a mortar and pestle. Dried material was shaken in 20 ml methanol for 24 h, evaporated up to 2 ml and then 20 ml of 0.5N H_2SO4 (Hi-media Lab., India) was added (Aslam *et al.*, 2009).

2.6.2 Separation of alkaloids

HPLC can be efficiently coupled to different detectors such as Photo Diode Array (PDA), fluorescence, ESI-MS (Electrospray Ionization Mass Spectrometry), NMR (nuclear magnetic resonance) and CD (circular dichroism),

(Bringmann *et al.,* 2002) for precise identification and/or quantification of alkaloids.

For estimation of the alkaloid, vasicine, from *Adhatoda zeylanica,* high performance liquid chromatography (HPLC) is used an isocratic mode with methanol and water as mobile phase (Shimadzu-LC-6A and a recorder CR3A). All chromatograms were obtained using a 25-cm ODS, C18 column with a particle size of 5mm, with a flow rate of 0.7 ml per min and a run time of 12 min. The peak absorbance was recorded at 298 nm. Chromatograms of standard vasicine and extracts of callus samples were obtained and the relative amounts of the alkaloid were calculated using vasicine as the reference (Jayapaul *et al.,* 2005).

For HPLC analysis of the vincristine alkaloid, A Merck LiChro CART C18 column (125 mm X mm X5 mm) and the solvent systems (acetonitrile and methanol; 3:1) were used at a constant flow rate of 1.0 ml min^{-1}. A Photo Diode Array detector was employed for detection of peaks, set at wavelength of 220 nm and bandwidth of 5 nm. The alkaloid was quantified by using regression equation of calibration curve (Aslam *et al.*, 2009).

The chromatographic conditions for the analysis of camptothecin were as follows: mobile phase: acetonitrile: water (60: 40), column: ODS (Octadecyl silane) C18, size 250 X 4.6 mm (Supelco), Detector: SPD-M 10 A, photo diode array detector, wave length: 254 nm, flow rate: 1.0 ml min^{-1}, injection volume: 25 μl, retention time: 6.4 min (Singh *et al.*, 2010).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled 'Detection and quantification of piperine from *in vitro* cultures of black pepper (*Piper nigrum* L.)' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, during the period 2009-2011. The materials used and the methodology adopted are presented in this chapter.

3.1 Materials

3.1.1 Laboratory chemicals, glassware and equipments

All the chemicals, used as ingredients and additives in the MS medium and for High Performance Liquid Chromatography analysis were procured from Merck India Ltd., Sisco Research Laboratories Pvt. Ltd., British Drug House and Sigma Ltd. Glassware used was of borosilicate (Borosil), which was cleaned by soaking in solution of potassium dichromate in sulfuric acid for half an hour. Later, the glass containers were washed with jets of tap water to remove traces of potassium dichromate solution followed by rinsing twice with double distilled water. These were then dried in hot air oven at more than 100°C for 24 hours and later stored in cupboards, free of dust till further use. Culture inoculation was carried out in laminar air flow chamber (Kirloskar Pvt. Ltd). Piperine was estimated by HPLC system (Shimadzu, Japan).

3.1.2. Plant materials

Explants for culture initiation were taken from variety Panniyur 2 and Panniyur 5 (leaf and nodal cuttings only) grown in pots kept in net house at CPBMB and were regularly sprayed with plant protection chemicals against pest and diseases.

Initially bush pepper plants of the variety Panniyur 2 was selected for the study which is reported to have high piperine content (6.6%) and all the explants were tried from this variety. Since incidence of quick wilt was noticed in the net house, the leaf explants inoculated were lost due to fungal contamination during the culture initiation stage. Hence Panniyur 5, another high yielding variety growing in a separate net house was used for taking the leaf and nodal explants but, since it was raised from runners, fruits were not available for inoculation. However, berries were available from the variety of Panniyur 2 at a later stage and were used for culture initiation from immature berries, epicarp and seed.

3.2 Methods

The experiment is divided into the following sections:

1. *In vitro* culture initiation
2. Callus proliferation in modified media for *in vitro* piperine synthesis
3. Estimation of piperine content in the calli using High Performance Liquid Chromatography

3.2.1 *In vitro* culture initiation

3.2.1.1 Collection, pretreatment and surface sterilization of explants

Different types of explants such as green shoots with axillary buds, tender pale green leaves (2nd or 3rd from tip of the shoot), immature berries (4-5months old) and ripened berries(yellow-reddish in color) were collected from potted plants of the pepper varities Panniyur 2 and Panniyur 5. All the explants were washed thoroughly with a detergent solution (Pril) to remove any soil particles and extraneous matters present on the surface followed by washing under running tap water. These were then wiped with clean swab of cotton soaked in 70 per cent alcohol and dipped in 0.1% bavistin for 10 min followed by washing with aquaguard water. For extraction of seeds from ripened berries, the outer epicarp

was removed and the seeds thus obtained were washed thoroughly in tap water followed by aquaguard water to remove any adhering tissue.

Further sterilization for all the explants was done in the Laminar Air Flow chamber (LAF). All the explants were treated with 0.1 per cent mercuric chloride for 5 min, 7 min and 10 min. This was followed by rinsing with sterile distilled water thrice and kept open on a sterile plate with blotting paper to remove the traces of water.

3.2.1.2 Preparation of media

The callus initiation and establishment medium consisting of half strength MS (Murashige and Skoog, 1962) supplemented with IAA (1 mg l^{-1}) and BA (1mg l^{-1}) (Nazeem *et al.*, 1993) was used for the present study. In order to reduce the systemic bacterial contamination, 15.0 mg l^{-1} copper sulphate was also added into the medium (Rajmoham *et al.*, 2010). ½ MS supplemented with 2, 4-D (3 mg l^{-1}) was also tried for callus initiation.

Stock solutions of major and minor elements and growth regulators were prepared and stored in refrigerated condition. A clean steel vessel rinsed with distilled water was used to prepare the media. Required volume of each stock solution of MS medium was pipetted into the vessel containing approximately 200 ml distilled water, stirred thoroughly and required quantity of sucrose, inositol, copper sulphate and growth regulators were added. The desired volume was made up by adding distilled water and pH was adjusted to 5.7-5.8 using 1N NaOH. The medium was heated to melt the agar, dispensed into test tubes (around 15 ml per tubes) when warm and autoclaved (15 Pounds per square inch at 121°C for 15 to 20 min). The composition of MS medium and components of ½ MS basal medium are provided as Appendix I.

3.2.1.3 Inoculation of explants and culture conditions

Inoculation was carried out under strict aseptic condition inside the LAF. The working floor of the chamber was thoroughly wiped with 70 per cent alcohol to remove any traces of dust or microbes. Sterilized forceps, surgical blades, scalpels and blotting papers were kept inside the chamber and were sterilized with UV light for 30 min before inoculation. The leaf explants were cut into small segments, each containing a portion of the midrib or vein and 2-3 pieces were inoculated into each test tube. For inoculating the epicarp, the individual berry was cut into two equal halves and the inner epicarp was scooped out after removing the endosperm. For immature berries and seeds, 2-3 berries or seeds were inoculated into each test tube (Plate 1).

The cultures were incubated in a culture room with 3000 lux fluorescent light for a period of 16 hours followed by 8 hours of dark period daily. The temperature was maintained at 26±2°C.

3.2.1.4 Sub culturing of the calli

Sub culturing was done every 25 to 30 days interval into fresh medium with the same composition. To avoid the effect of light on phenol exudation in the culture initiation medium, the cultures were kept initially in the dark culture room for 3 days and then shifted to light. The following observations were recorded for the cultures.

Observations recorded

(a) Number of days for callus initiation: The time interval between the day of inoculation to the day when first visible signs of callus growth was seen, was counted as number of days for callus initiation

a. Nodal cutting

b. Tender leaf

c. Immature berry

d. Epicarp of berry

e. Seed

Plate 1: Explants used for culture initiation

(b) Percentage of cultures initiating calli: Number of cultures which showed indications of callusing were counted and were expressed as percentage of total number of inoculated test tubes.

(c) Callus growth score: After 60 days of inoculation, calli were scored according to the spread in the medium by visual assessment as given in Table 2.

(d) Callus index: Callus index (CI) was calculated as CI = P X G (Labade, 2009) Where, P= percentage of cultures initiating calli and G= Growth score.

Since the growth score ranged from 0 to 4, the percentile value of callus index ranged between 0 to 400. Hence the maximum obtainable value of the callus index was 400. The treatment index near 400 was considered as the best, while those with values near 0 were considered to have poor callusing ability.

(e) Number of shoots from callus: Counted, the number of shoots, if there is any regeneration from the callus.

(f) Length of shoots: Length (cm) will be measured for the regenerated shoots, if any.

(g) Fresh weight of cultures (g): After 60days in the medium, 10 calli with callus score as 4 were selected and weight of the callus was recorded in grams.

(h) Dry weight of cultures (g): After taking the fresh weight, the calli were kept in a hot air oven at 65^0C till consistent weights were recorded.

(i) Dry matter content (%): Calculated as

$$\frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

Table 2. Growth score of *in vitro* grown calli in *Piper nigrum*

Extent of callus growth 60 days after initiation	**Growth score**
No callus initiation	0
Callus covering about ¼ th surface of medium	1
Callus covering about ½ nd surface of medium	2
Callus covering about ¾ th surface of medium	3
Callus covering the entire surface of medium	4

3.2.2 Callus proliferation in modified media for *in vitro* piperine synthesis

The leaf calli proliferating in the culture initiation medium viz., ½ MS+ IAA (1.0mgl^{-1})+BA(1.0mgl^{-1})+copper sulphate(15.0mgl^{-1})+ sucrose (3%) were sub cultured into media with the following modifications.

3.2.2.1 Modification of sucrose content in the culture initiation medium

Composition of the culture initiation medium was modified by increasing the concentration of sucrose to 5, 6, 7 and 8 per cent. The full grown calli were sub cultured into these four media along with the control medium with 3 per cent sucrose.

3.2.2.2 Modification of composition of MS macronutrient stock

Effect of reducing the nitrate nitrogen and phosphate concentration of the macronutrient stock solution of the MS medium was tried along with two concentrations of sucrose (3% and 7%) with respect to piperine. The modified macronutrient stock solution was prepared by reducing the nitrate and phosphate content to half of the quantity of standard MS medium. As the basal medium used was ½ MS, the final concentration for nitrate and phosphate were reduced to one fourth of the original concentration (Appendix II).

3.2.2.3 Addition of mannitol in the culture initiation medium

Mannitol at 4 per cent concentration was tried along with 3 and 7 per cent sucrose in the culture initiation medium as 7 per cent sucrose had a favourable effect on piperine production.

3.2.2.4 Modification of growth regulators in the culture initiation medium

Instead of IAA as the auxin in the culture initiation medium, the sub culturing medium was prepared with 2, 4-D as the auxin in three different

concentrations (0.5, 1.0 and 3.0 mg l^{-1}) along with 1.0 mg l^{-1} BA for sub culturing the leaf derived calli from the variey of Panniyur 5. Berry derived calli from the variey of Panniyur 2 were sub cultured into medium with 3.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BA and 3.0 mg l^{-1} 2, 4-D alone (without any cytokinin).

3.2.2.5 Precursor feeding

L- Lysine, identified as the amino acid precursor of the alkaloid piperine under *in vivo* condition, was incorporated at three different concentrations viz- 5, 50 and 100 mg l^{-1} in which (epicarp) derived calli from the variey of Panniyur 2 and leaf derived calli from the variey of Panniyur 5 were sub cultured.

3.2.3 Estimation of piperine content in the calli

Total piperine content was estimated by HPLC method from calli grown on culture initiation medium (as control) and the calli grown in various modified media tried for enhancing *in vitro* production of piperine.

3.2.3.1 Preparation of callus extract for HPLC analysis

The fresh clean callus (1.0 g) was ground using pestle and mortar with 5 ml of methanol. Then the extract was transferred to 10 ml centrifuge tube and kept in a water bath at 65°C for 1 hour. The crude extract was centrifuged at 5000 rpm for 10 min (Rotek). After centrifugation, 25μl of the supernatant was injected into the HPLC.

3.2.3.2 HPLC analysis

The protocol standardized by Sujatha (2010) was followed for the HPLC analysis. Gradient elution method was followed using the HPLC system 'Shimadzu Prominence' with SPD-M20A diode array detector. The stainless steel

column (250 mmX4.60mm internal dia.) used was C18 (5μm particle diameter) (Phenomenex, USA). Separation is achieved using a mixture of 0.1% *o*-phosphoric acid (A) and 90% acetonitrile in *o*-phosphoric acid (B).

To prepare solvent A, 1.75 g of liquid Ortho-phosphoric acid was made up to 1000 ml using Millipore water in a volumetric flask. The solution was filtered through cellulose filter using vacuum filtration unit. The pore size of the cellulose filter paper is 0.2 μm. Then, the bottle containing the solution was kept in an ultrasonic bath for 15 min. For preparing solvent B, 100 ml of solvent A was taken into a separate bottle and 900 ml of acetonitrile was added and mixed thoroughly. The mixture was then sonicated for 10 min. as described above.

The HPLC programme used was as follows.

Total time for running = 40 min
First 20 min: A: B = 60:40
Next 5 min : A: B = 0:100
Last 25-40 : A: B = 60:40
Flow rate: 1.0 ml /min

Pure piperine (Merck, India) was used for identifying the retention time and for calculating the constant for estimating piperine from the peak area obtained. Using the standardized procedure (Sujatha, 2010) retention time of known quantities (0-30 mgl^{-1}) of pure piperine in C18 column was found to be 15.4 and the standard curve was drawn (Fig 1). For drawing the standard curve, 5, 10, 15, 20, 25 and 30 mg l^{-1} standard piperine solutions were prepared. From the standard curve, piperine from the callus extract was calculated based on the following relation.

Area of peak produced by piperine = 49171x Concentration (ppm) of piperine

Therefore, concentration of piperine in the given sample (ppm) = Peak area /49171.

For estimation of piperine from callus samples, three cultures were used from each treatment and the mean value and standard error were calculated as detailed below.

3.2.3.3. Statistical analysis

Mean

Mean value of piperine content from three samples were calculated by using the following formula

$$\bar{X} = \frac{\sum X}{n}$$

Where, $\bar{X}$ = Mean
$\sum X$ = Sum of all the value
n = Total no of sample

Standard Error

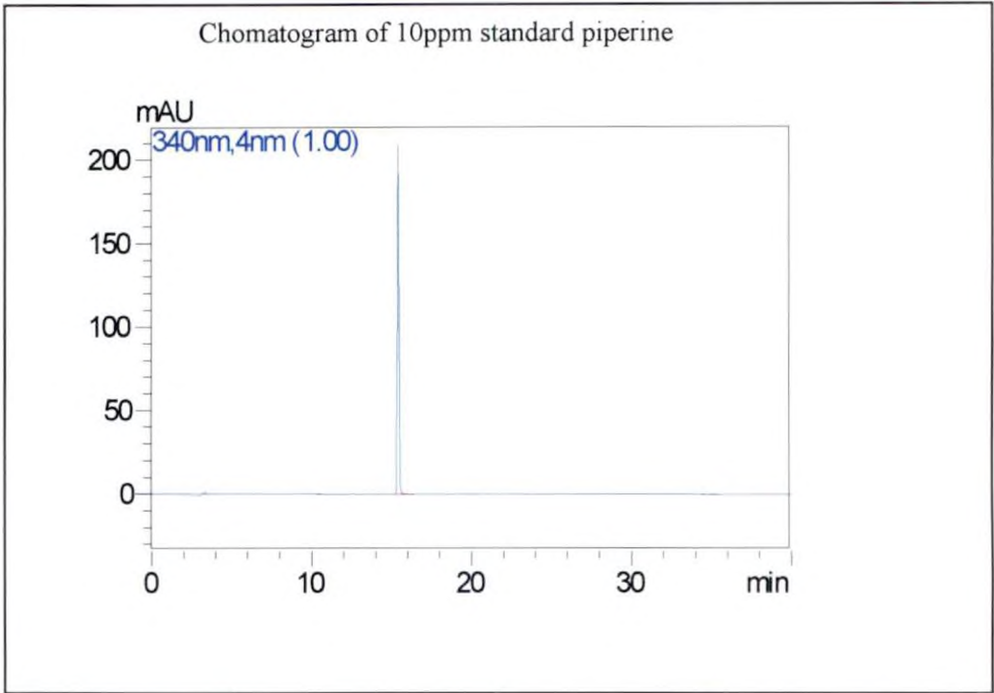
Standard error was calculated by using the following formula

$$SE_m = \frac{s}{\sqrt{n}}$$

Where,

S= standard deviation

n= Total no of sample



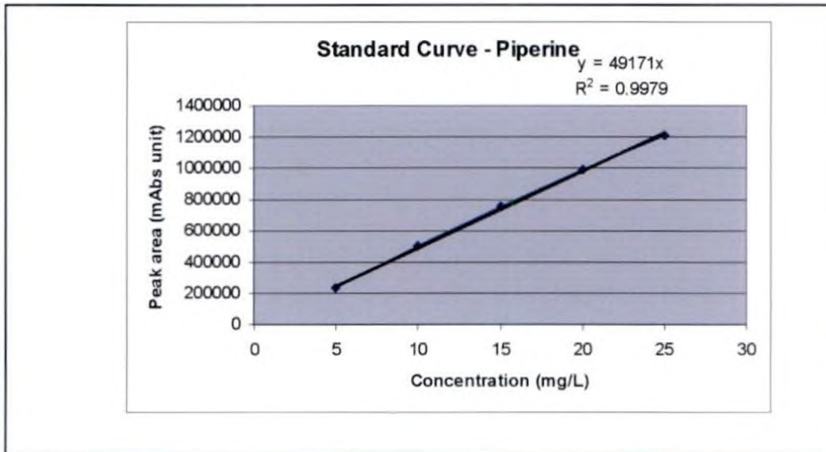
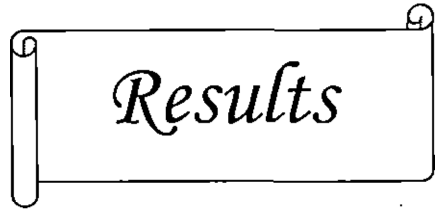


Fig 1. Chromatogram and standard curve drawn from HPLC analysis of pure standard piperine


Results

RESULTS

The results of the studies entitled 'Detection and quantification of piperine from *in vitro* cultures of black pepper (*Piper nigrum* L.)' which was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, are presented in this chapter.

The results of the various experiments conducted are arranged under the following two major sections:

4.1. *In vitro* culture initiation

4.2. Modification of culture medium and estimation of piperine content using HPLC

4.1. *In vitro* culture initiation

4.1.1. Pretreatment and surface sterilization of explants

As the culture inoculation was commenced during the rainy season, fungal contamination was very high especially when berries were used as explants, though the mother plants were kept in net house and were regularly sprayed with plant protection chemicals. After collecting the explants, washing in running tap water, wiping with 70 per cent alcohol and dipping in bavistin (0.1 %) for 10 min was found to be effective in reducing the rate of contamination.

Result of surface sterilization of pretreated explants with 0.1 per cent mercuric chloride ($HgCl_2$) for varying durations *viz.*, 5 min, 7 min and 10 min is given in Table 3. Among the treatments tried, treating leaf explants with 0.1 per cent mercuric chloride ($HgCl_2$) for 5 min was effective in yielding maximum percent of contamination free cultures (95%). On enhancing the duration of surface sterilization to 7 min scorching was observed in the leaf explants and the 20 per cent of the cultures later dried whereas at 10 min duration all the leaf

pieces were blackened and dried. Treatment period of 10 min was better for nodal cuttings (80%), berry (55%) and epicarp (40%), than the lower durations whereas for seed, 7 min treatment resulted in 50 per cent survival of the cultures (Table 3).

4.1.2 Effect of medium on callus induction and proliferation

Two different media were tried for initiation and proliferation of callus from different explants of black pepper [Medium1: ½ MS +IAA ($1 mgl^{-1}$) +BA ($1 mgl^{-1}$); Medium2: ½ MS+2, 4-D ($3 mg l^{-1}$)]. The results indicate that medium1 was suitable for callus induction effectively in leaf and immature berry explants while nodal explants, epicarp and seed failed to produce any callus in this medium (Table 4). The observations recorded for the leaf and berry derived callus cultures (Table 5) shows that calli were initiated in about 21 days after inoculation in 90 per cent of leaf explants while, berry explants took about 40 days to initiate callus in 30 per cent berry explants (Plate 2).

In the second medium tried, the auxin 2, 4-D ($3 mg l^{-1}$) alone was used for initiating callus in different explants (Table 4). This medium was unable to initiate calli in leaf explants. Scorching of leaf samples was observed in 20 per cent of the cultures survived while rest of them remained in the medium without any sign of callusing. However this medium stimulated initiation of calli in about 40 days after inoculation in 35 per cent of epicarp explants and 25 days after inoculation in 20 per cent of seed explants (Table 5). The effect of this medium on berries could not be studied as the set of berry cultures inoculated in the medium were lost due to fungal contamination and since the season was over, berries were not available for further inoculation. The nodal explants failed to produce any callus in this medium.

Since there was no callus induction from nodal explants this was not included in further studies. Also none of the calli produced any shoot /root regeneration in both media tried. Hence observation on length of shoot from

Table 3. Effect of duration of surface sterilization on various plant parts of black pepper used as explants

Explant		Per cent survival of explants in different durations of treatment with 0.1% mercuric chloride		
		5 min	7 min	10 min
Leaf	*Panniyur2	20	25	-
	Panniyur5	95	80	
Nodal cutting	Panniyur2	-	40	80
	Panniyur5	-	42	85
Berry	Panniyur2	-	30	55
Epicarp	Panniyur2	-	30	40
Seed	Panniyur2	-	50	45

*Reduced survival due to fungal contamination on mother plant

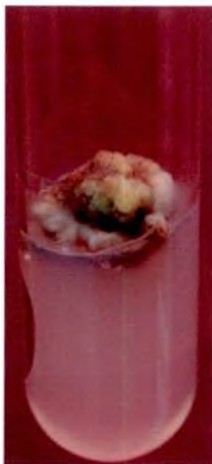
Table 4: Response of different explants of black pepper in the culture initiation media

	Medium1: ½ MS+IAA ($1.0 mgl^{-1}$) + BA($1.0 mgl^{-1}$)				**Medium2:** ½ MS + 2, 4-D ($3 mg l^{-1}$)			
Explant	No. of cultures inoculated	No of cultures survived	No of cultures callusing	Culture with callus score 4	No. of cultures inoculated	No of cultures survived	No of cultures responded	Culture with callus score 4
Leaf	72	69	62	25	72	66	0	0
Berry	72	40	12	7	72	0**	-	-
Nodal cutting	72	57	0	0	72	55	0	0
Epicarp	72	28	0	0	72	30	12	6
Seed	72	36	0	0	72	40	10	0

**Lost due to fungal contamination

a. Shoot from nodal cutting

b. Callus from leaf



c. Callus from berry

d. Callus from epicarp

e. Callus from seed

Plate 2: *In vitro* response from different explants

Table 5. Growth of callli derived from different explants in culture initiation media

Observations	Medium 1 ½ MS+IAA (1.0mgl^{-1}) + BA(1.0mgl^{-1})		Medium2 ½ MS + 2, 4-D (3 mg l^{-1})	
	Leaf calli	Berry calli	Epicarp calli	Seed calli
Number of days for callus initiation	21	40	40	25
Culture showing callus formation (%)	90	30	35	20
Average callus score	2.91	2.8	2.0	0.8
Callus index	261.9	98	70	1.6
Number of shoots/roots from callus	0	0	0	0
*Fresh weight of callus (g)	1.08	-	-	-
*Dry weight of callus (g)	0.20	-	-	-
*Dry matter content (%)	18.52	-	-	-

*Mean of 10 cultures with fully grown callus (score 4). Data recorded only for leaf calli as there was not enough number of cultures for sub culturing as well as to record this observation for the rest of the explants

callus could not be recorded. For recording the fresh weight and dry weight of the calli, a minimum of 10 calli with callus score-4 has to be obtained. This was available only for calli derived from leaf (Table 4).

The average fresh weight and dry weight recorded for leaf calli were 1.08 g and 0.2 g respectively and the dry matter content was 18.52% (Table 5). For berry, epicarp and seed derived calli this observation could not be recorded as the number of calii available for sub culturing into various modifications of media to study the effect on piperine production was limited (Table 4).

4.1.3 Comparative performance of explants in callus induction and proliferation

Callus cultures originating from leaf explants of the variety Panniyur 5 recorded higher values with respect to the various growth parameters (Table 5). Among the cultures obtained without contamination, 90 per cent started callusing in about 21 days. Also highest callus growth rate (average callus score 2.91) and callus index value (261.9) were recorded for leaf cultures growing in ½ MS media supplemented with 1 mg l^{-1} IAA and 1 mg l^{-1} BA. Few calli obtained from leaf of the variety Panniyur 2 also recorded similar performance, but the data were not sufficient to compare as many cultures lost due to the fungal contamination in the initial stage itself. Berry derived calli of the variety Panniyur 2 was next with respect to induction and proliferation of calli, whereas epicarp and seed did not show any response in the medium.

In the medium with 2, 4-D, 35 per cent epicarp started callusing within 40 days. Seeds started callusing earlier (within 25 days of inoculation) but the number of cultures producing callus and average size of calli were less (Table 5). 2, 4-D (3 mgl^{-1}) was found harmful for the leaf explants. Berries inoculated in this medium were contaminated with fungus and hence could not record the response in this medium.

4.2 Modification of culture medium for enhancing *in vitro* piperine production

The effect of various modifications of the culture initiation medium on the piperine synthesis was studied by estimating piperine from fully grown calli obtained in each treatment (Table 6).

Since sufficient number of leaf calli was not available from Panniyur 2 to be sub cultured into all the fifteen modifications of the culture medium, leaf calli from Panniyur 5 was used for the purpose, to have uniformity. As only limited number of berry calli and epicarp calli from Panniyur 2 attained the callus score 4 (Table 4), the berry calli were used to study the effect of growth regulators and epicarp calli for studying the effect of L- lysine (precursor for piperine) in the medium in addition to leaf calli from the variety Panniyur 5.

4.2.1 Modification of sucrose content in the culture initiation medium

The leaf calli (Panniyur 5) proliferating in the culture initiation medium (control) viz- ½ MS+ IAA ($1.0 mgl^{-1}$) +BA ($1.0 mgl^{-1}$) + sucrose (3%) were subcultured into media with 5, 6, 7 and 8 per cent sucrose. After 60 days of sub culturing into these media (Table 6), three fully grown calli were selected from each of the cultures growing in the four media for piperine estimation. The chromatogram and spectrum view of the peak is provided as Fig 2. The results are provided in Table7.

Increasing the level of sucrose concentration in the culture initiation medium up to 7 per cent showed a steady increase in the production of piperine in the calli from explants, in comparison to the control medium with 3 percent sucrose (Fig 3). Maximum piperine (0.135%) was obtained from callus grown in medium supplemented with 7 per cent sucrose (Plate 3a) as compared to control (0.018 %). Media supplemented with 8 per cent sucrose showed a decline in piperine (0.015%) production.

Table 6: Modifications of the culture initiation medium (control) for enhancing *in vitro* production of piperine from calli of *Piper nigrum*

No	Treatment type	Basal medium	Sucrose (%)	Mannitol (%)	Growth regulators (mgl^{-1})	Precursor L-lysine (mgl^{-1})
	Control	½ MS	3	-	IAA – 1.0 BA- 1.0	-
I	Increasing sucrose conc.					
1		½ MS	5	-	IAA – 1.0 BA- 1.0	-
2		½ MS	6	-	IAA – 1.0 BA- 1.0	-
3		½ MS	7	-	IAA – 1.0 BA- 1.0	-
4		½ MS	8	-	IAA – 1.0 BA- 1.0	-
II	Reducing N&P					
1		½ MS with ¼ NO_3 &PO_4	3	-	IAA – 1.0 BA- 1.0	-
2		½ MS with ¼ NO_3 &PO_4	7	-	IAA – 1.0 BA- 1.0	-
III	Growth regulators					
1		½ MS	3	-	2,4-D -3.0	-
2		½ MS	3	-	2,4-D –3.0 BA - 1.0	-
3		½ MS	3	-	2,4-D - 0.5 BA - 1.0	-
4		½ MS	3	-	2,4-D – 1.0 BA -1.0	-
IV	Addition of Mannitol					
1		½ MS	3	4	IAA – 1.0 BA- 1.0	-
2		½ MS	7	4	IAA – 1.0 BA- 1.0	-
V	Addition of L-lysine					
1		½ MS	3	-	IAA – 1.0 BA- 1.0	5
2		½ MS	3	-	IAA – 1.0 BA- 1.0	50
3		½ MS	3	-	IAA – 1.0 BA- 1.0	100

4.2.2 Reduction of nitrate and phosphate concentration

The leaf calli (Panniyur 5) proliferating in the culture initiation medium (control) viz., ½ MS+ IAA (1.0mgl^{-1}) +BA (1.0mgl^{-1})+sucrose (3%) were subcultured into media with ¼ th of nitrate and phosphate content. The effect of this modification alone as well as in combination with the optimum level of sucrose (7%) was also assessed.

Reducing the nitrate nitrogen content of the MS basal medium to one fourth of its original strength and phosphate to one fourth of its original strength supplemented with 3 per cent and 7 per cent sucrose exerted an inhibitory effect on piperine production in *in vitro* calli from leaf explants. The chromatogram of the piperine is provided as Fig 4 and the results are provided in Table 8. Reduction in nitrate nitrogen and phosphorus content in the medium, even when supplemented with 7 per cent sucrose which was found inhibitory for piperine production, registered a decrease in piperine content (0.003%) (Fig 5). Medium containing reduced nitrate and phosphorus with 3 per cent sucrose (Plate 3b) also showed a negative effect on piperine synthesis (0.013%) compared to control medium with 3 per cent sucrose (0.018%).

4.2.3 Effect of supplementing of sucrose with mannitol on piperine production

Leaf calli (Panniyur 5) proliferating in the culture initiation medium (control) viz. ½ MS+ IAA (1.0mgl^{-1}) +BA (1.0mgl^{-1}) + sucrose (3%) were sub cultured into media with 4 per cent mannitol as well as into medium with 4 per cent mannitol and 7 per cent sucrose. The chromatogram and spectrum view of the peak is provided as Fig 6 and the results are provided in Table 9.

Enhanced piperine synthesis (0.107%) content was obtained from callus grown in culture initiation medium containing 4 per cent mannitol (Plate 3c).

Table 7: Effect of modifying sucrose concentration on piperine production in *in vitro* grown leaf calli of *Piper nigrum* (Variety Panniyur 5)

Sl. No.	*Sucrose content in the medium (%)	Piperine (%) (Mean ± SE)
1	3 (Control)	0.018 ± 0.011
2	5	0.025 ± 0.005
3	6	0.070 ± 0.009
4	7	0.135 ± 0.003
5	8	0.015 ± 0.002

* ½ MS + 1 mg l^{-1} IAA +1 mg l^{-1} BA

Table 8: Effect of reduced nitrate and phosphate concentration on piperine production in *in vitro* grown leaf calli of *Piper nigrum* (Variety Panniyur 5)

Sl. No.	*Nitrate and phosphate content and Sucrose in the medium	Piperine (%) (Mean ± SE)
1	½ of that in MS + 3% sucrose (Control)	0.018 ± 0.011
2	¼ th of that in MS + 3% sucrose	0.013 ± 0.002
3	¼ th of that in MS+ 7% sucrose	0.003 ± 0.001

* ½ MS + 1 mg l^{-1} IAA +1 mg l^{-1} BA

a. Callus growth in ½ MS+ 1mg l^{-1} IAA +1mg l^{-1} BA + sucrose (7%)

b. Callus growth in ½ MS with reduced nitrate and phosphate+1mg l^{-1} BA + 1mg l^{-1} IAA + sucrose (3%)

c. Callus growth in ½ MS+ 1mg l^{-1} IAA +1mg l^{-1} BA + Sucrose (3%) + mannitol (4%)

Plate 3: Growth of leaf derived calli in different modified media for *in vitro* synthesis of piperine

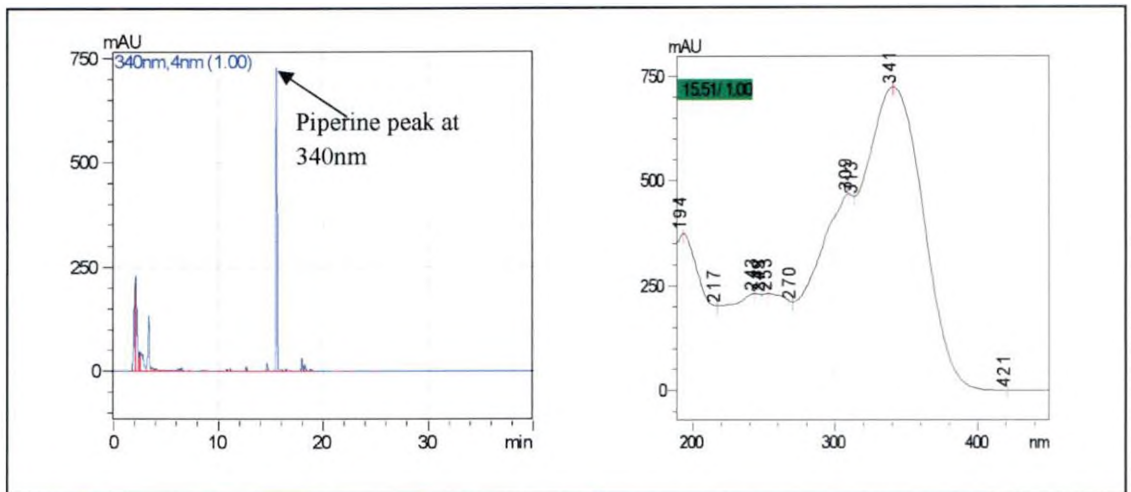


Fig 2. Chromatogram and spectrum view of piperine from leaf derived callus grown in medium with 7 per cent sucrose

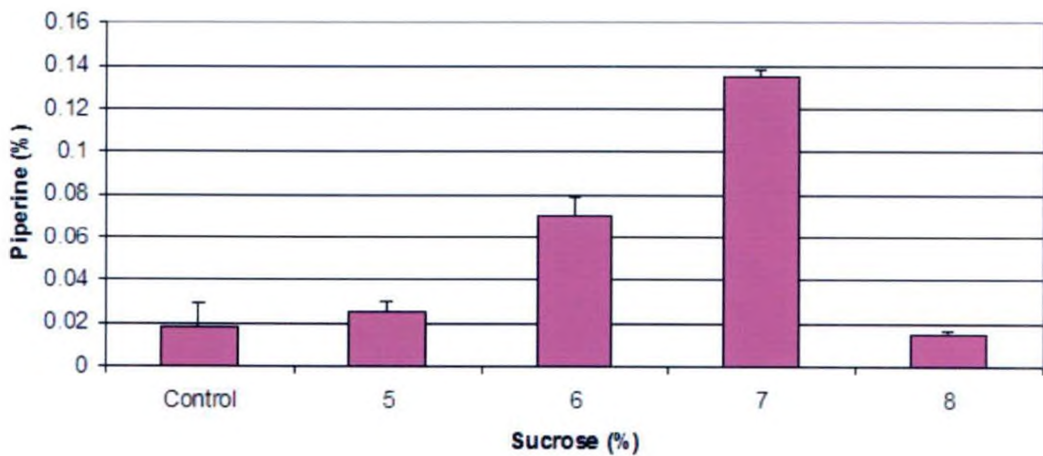


Fig 3. Effect of enhanced sucrose concentrations on piperine yield in *in vitro* leaf derived calli of *Piper nigrum* (variety Panniyur 5)

Data represents Mean ± SE of three replicates

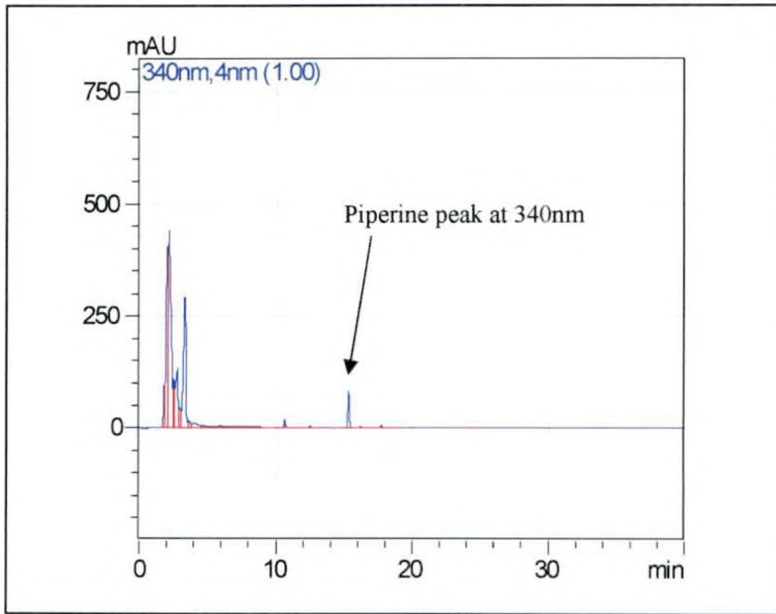


Fig 4. Chromatogram of piperine from leaf derived callus grown in medium with 7 per cent sucrose and reduced phosphate and nitrate concentrations

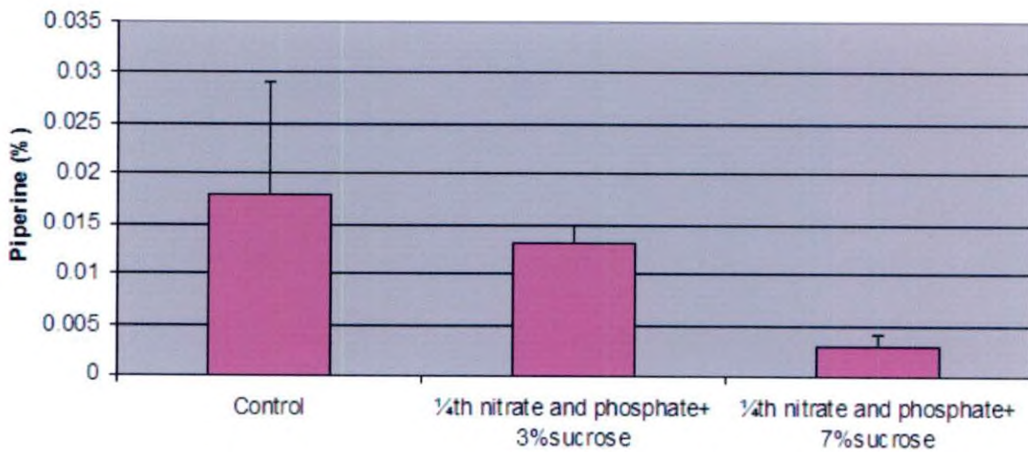


Fig 5. Effect of reduced phosphate and nitrate concentrations on piperine yield in *in vitro* leaf derived calli of *Piper nigrum* (variety Panniyur 5)

Data represents Mean ± SE of three replicates

which is higher than that in the control value (0.018%), while the least piperine (0.007%) was obtained from the medium containing 4 per cent mannitol supplemented with 7 per cent sucrose (Fig 7).

4.2.4 Effect of growth regulators on piperine production

Leaf calli (Panniyur 5) proliferating in the culture initiation medium (control) viz., ½ MS+ IAA (1.0mg l^{-1}) +BA (1.0mg l^{-1}) + sucrose (3%) were sub cultured into media with various concentrations of auxin (2, 4-D) and cytokinin (BA). The results are provided in Table 10.

Callus grown in medium containing 2, 4-D (1 mg l^{-1}) and BA (1 mg l^{-1}) recorded higher piperine (0.042 %) (Plate 3d). The chromatogram and spectrum view of the peak is provided as Fig 8. Medium containing 2, 4-D (3 mg l^{-1}) and BA (1 mg l^{-1}) registered lower concentration (0.002%) than that with 2, 4-D (0.5 mg l^{-1}) and BA (1 mg l^{-1}) (0.023%). The results are given as Fig 9.

As berry derived calli were available from the variety Panniyur 2, the effect of growth regulators was recorded on berry calli also (Table 11). Media containing 2, 4-D (3 mg l^{-1}) was found to enhance piperine production (0.042%) in berry derived callus (Plate 4a). The chromatogram and spectrum view of the peak of piperine for the callus with higher piperine content is provided as Fig 10. The results are given as Fig 11.

Media containing both 2, 4-D (3 mg l^{-1}) and BA (1 mg l^{-1}) resulted in callus with 0.017 per cent piperine (Fig 11). Only one callus growing in the control (½ MS +1 mg l^{-1} IAA and 1 mg l^{-1} BA + 3% sucrose) was available for HPLC analysis and the piperine content recorded for this was very low (0.004%).

Table 9: Effect of mannitol in combination with sucrose on piperine production from *in vitro* grown leaf calli of *Piper nigrum* (Variety Panniyur 5)

Sl. No.	* Mannitol and Sucrose content in the medium	Piperine (%) (Mean ± SE)
1	7% sucrose + 4% mannitol	0.007 ± 0.001
2	3% sucrose + 4% mannitol	0.107± 0.003
3	3% sucrose (control)	0.018 ± 0.011

*½ MS + 1 mg l^{-1} IAA +1 mg l^{-1} BA

Table 10: Effect of growth hormonal concentrations on piperine production in *in vitro* grown berry calli of *Piper nigrum* (Variety Panniyur 2)

Sl. No.	* Growth regulators (mg l^{-1})	Piperine (%) (Mean ± SE)
1	2,4-D(3 mg l^{-1})+BA(1 mg l^{-1})	0.017 ± 0.003
2	2,4-D (3 mg l^{-1})	0.042 ± 0.005
3	Control**	0.004

*½ MS + 3% sucrose

** ½ MS+ 1 mg l^{-1} IAA +1 mg l^{-1} BA + 3% sucrose

Table 11: Effect of growth hormonal concentrations on piperine production in *in vitro* grown leaf calli of *Piper nigrum* (Variety Panniyur 5)

Sl. No.	*Growth regulators (mg l^{-1})	Piperine (%) (Mean ± SE)
1	2,4-D(3mg l^{-1})+BA(1mg l^{-1})	0.002 ± 0.001
2	2,4-D(1mg l^{-1})+BA(1mg l^{-1})	0.032 ± 0.003
3	2,4-D(0.5mg l^{-1})+BA(1 mg l^{-1})	0.023 ± 0.004
4	Control**	0.018 ± 0.011

*½ MS + 3% sucrose

** ½ MS+ 1 mg l^{-1} IAA +1 mg l^{-1} BA + 3% sucrose

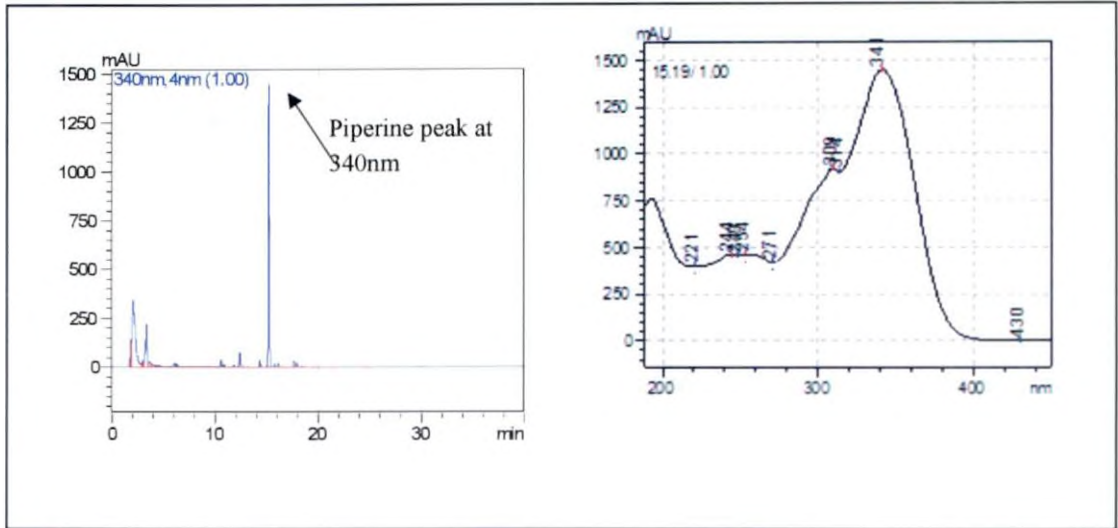


Fig 6. Chromatogram and spectrum view of piperine from leaf derived callus grown in medium with 4 per cent mannitol and 3 per cent sucrose

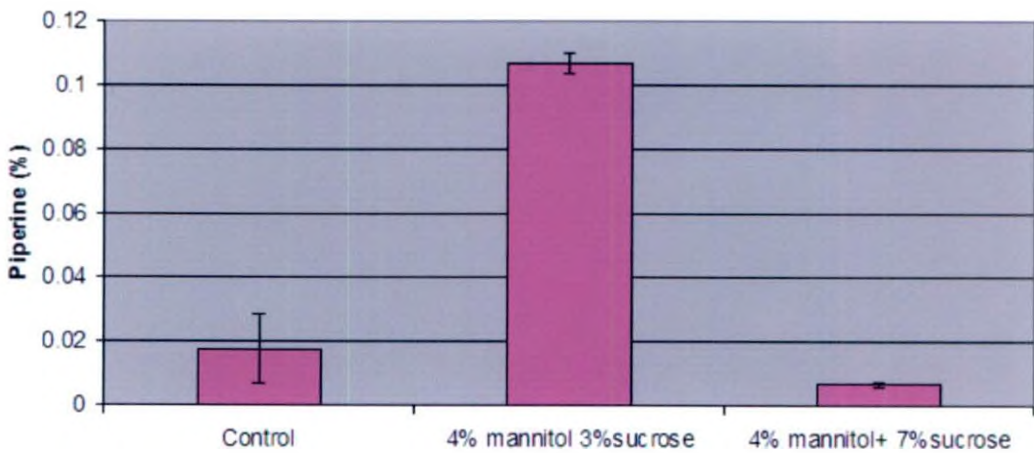


Fig 7. Effect of mannitol along with sucrose on piperine yield in *in vitro* leaf derived calli of *Piper nigrum* (variety Panniyur 5)

Data represents Mean ± SE of three replicates

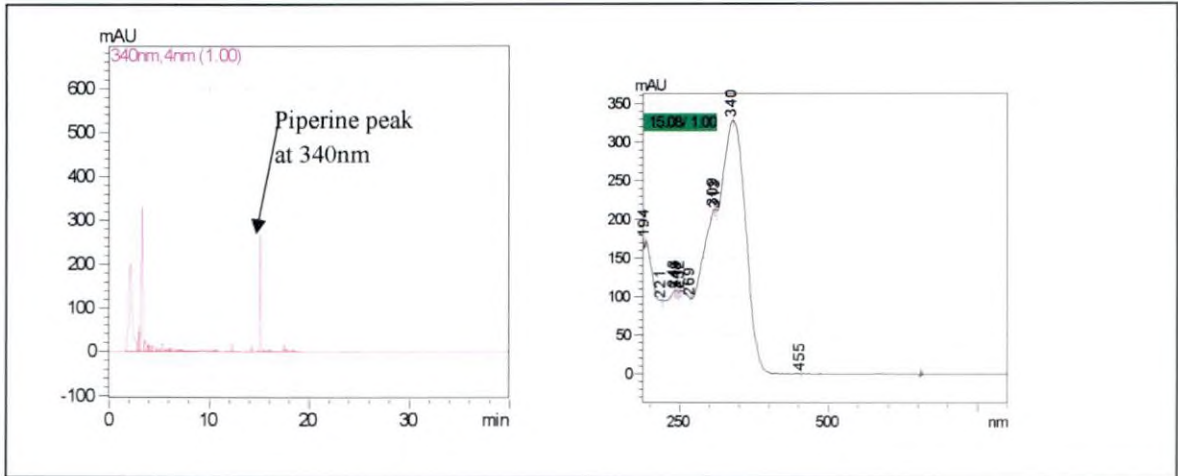


Fig 8. Chromatogram and spectrum view of piperine from leaf derived callus grown in medium with 2, 4-D (1) and BA (1)

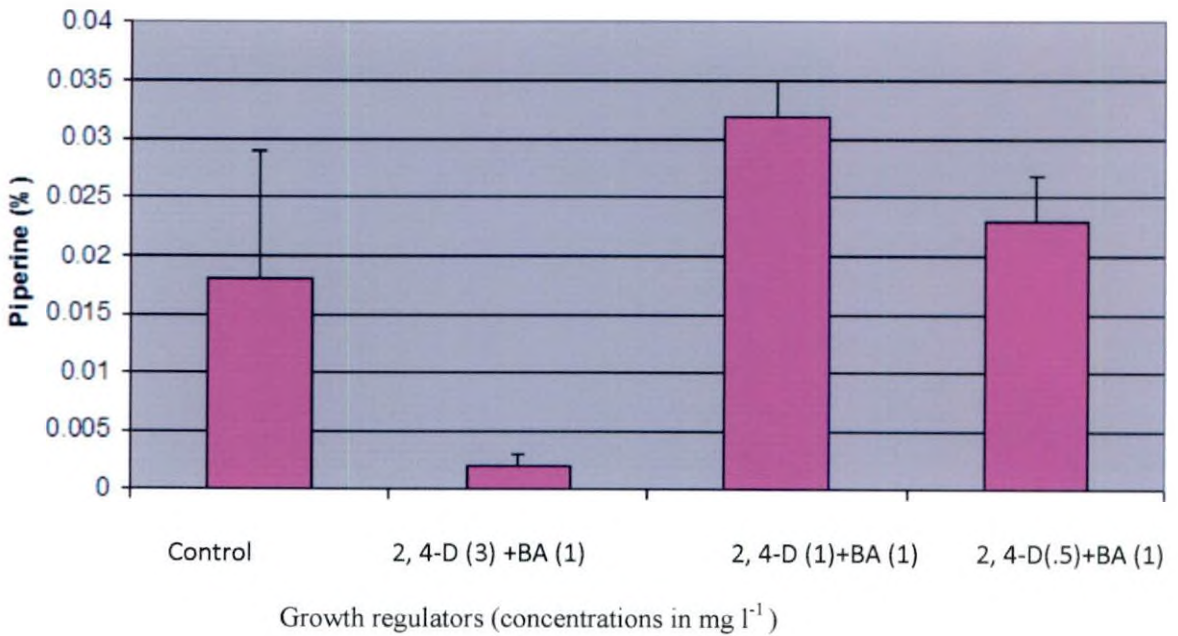


Fig 9.Effect of growth regulators on piperine yield *in vitro* leaf derived calli of *Piper nigrum* (variety Panniyur 5).

Data represents Mean ± SE of three replicates

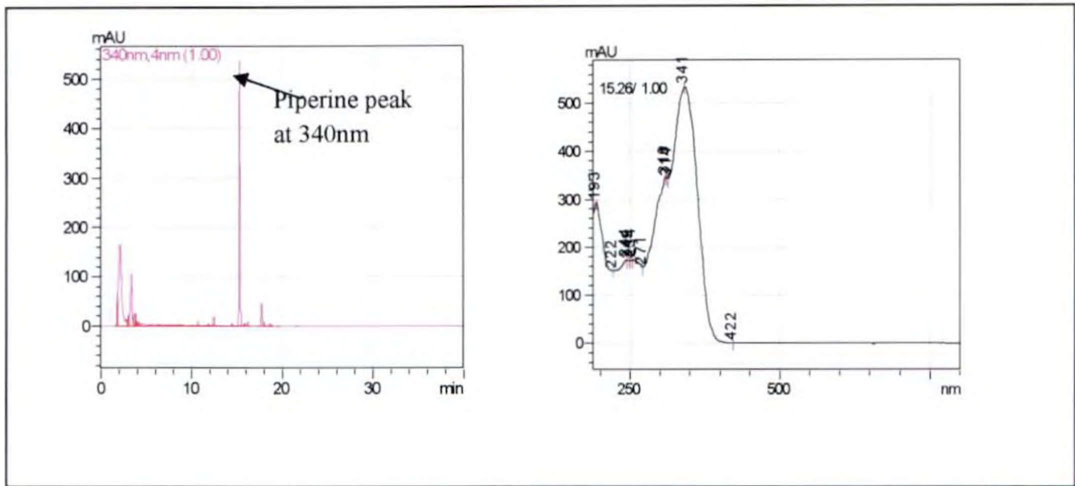


Fig 10. Chromatogram and spectrum view of piperine from leaf derived callus grown in medium with 2, 4-D (3)

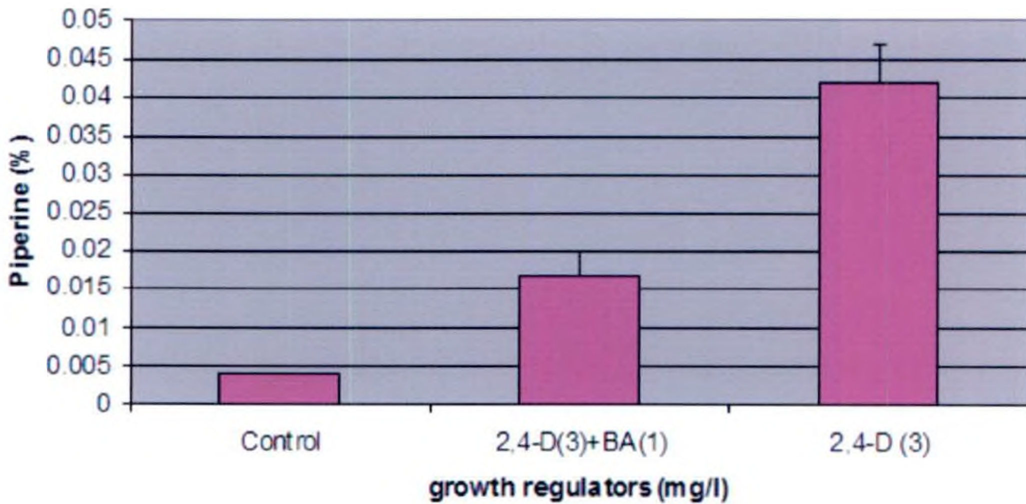


Fig 11.Effect of growth regulators on piperine yield *in vitro* berry derived calli of *Piper nigrum* (variety Panniyur 2).

Data represents Mean ± SE of three replicates

4.2.5 Addition of precursor (L-lysine) into the culture medium

Leaf calli (Panniyur 5) proliferating in the culture initiation medium (control) viz., ½ MS+ IAA (1.0 mgl^{-1}) +BA (1.0m gl^{-1}) + sucrose (3%) were sub cultured into media with 5, 50 and100 mg l^{-1} of L-Lysine which is a precursor of piperine.

Effect of precursor, L- lysine on the stimulation of piperine production in *in vitro* cultures of leaf derived calli are presented in Table 12. Here, the callus grown in medium (Plate 3e) containing higher concentration (100 mg l^{-1}) of L-lysine gave maximum piperine content (0.066%) (The chromatogram is provided as Fig 12) compared to the control medium which recorded 0.018 per cent piperine. The other two media with 5 mg l^{-1} and 50 mg l^{-1} L-lysine, recorded lower piperine concentrations (0.041% and 0.053% respectively) (Fig 13).

The epicarp derived calli (Panniyur 2) were also sub cultured to these media. Effects of the various concentrations of L- lysine on the stimulation of piperine production are presented in table 13. Medium containing 5 mg l^{-1} L-lysine (Plate 4b) produced higher amounts of piperine (0.016%) in comparison to the control medium, which gave 0.002 per cent piperine. Chromatogram is provided as Fig 14. Higher concentrations of L-lysine resulted in decrease of piperine (0.007%) production (Fig 15) in this case.

d.Callus growth in ½ MS+ 1mg l^{-1} 2, 4-D + 1mg l^{-1} BA + Sucrose (3%)

e.Callus growth in ½ MS+1mg l^{-1} IAA + 1mg l^{-1} BA
+ sucrose (3%) + L-lysine (100 mg l^{-1})

Plate 3: Growth of leaf derived calli in different modified media for *in vitro* synthesis of piperine

a.Callus growth in ½ MS+ 1mg l^{-1} 2, 4-D + sucrose (3%)

b.Callus (epicarp) growth in ½ MS+1mg l^{-1} IAA + 1mg l^{-1} BA + sucrose (3%) + L-lysine (5 mg l^{-1})

Plate 4: Growth of berry derived calli in different modified media for *in vitro* synthesis of piperine

Table 12: Effect of L-lysine concentration on piperine production in *in vitro* grown leaf calli of *Piper nigrum* (Variety Panniyur 5)

Sl. No.	* L-lysine content (mg l^{-1}) in the medium	Piperine (%) (Mean ± SE)
1	Control	0.018 ± 0.011
2	5	0.041 ± 0.004
3	50	0.053 ± 0.002
4	100	0.066 ± 0.002

*½ MS + 1 mg l^{-1} IAA +1 mg l^{-1} BA +3% sucrose

Table 13: Effect of L-lysine concentration on piperine production in *in vitro* grown epicarp calli of *Piper nigrum* (Variety Panniyur 2)

Sl. No.	*L-lysine content (mg l^{-1}) in the medium	Piperine (%) (Mean ± SE)
1	Control	0.002 ± 0.001
2	5	0.016 ± 0.011
3	50	0.007 ± 0.003
4	100	0.007 ± 0.004

*½ MS + 1 mg l^{-1} IAA +1 mg l^{-1} BA +3% sucrose

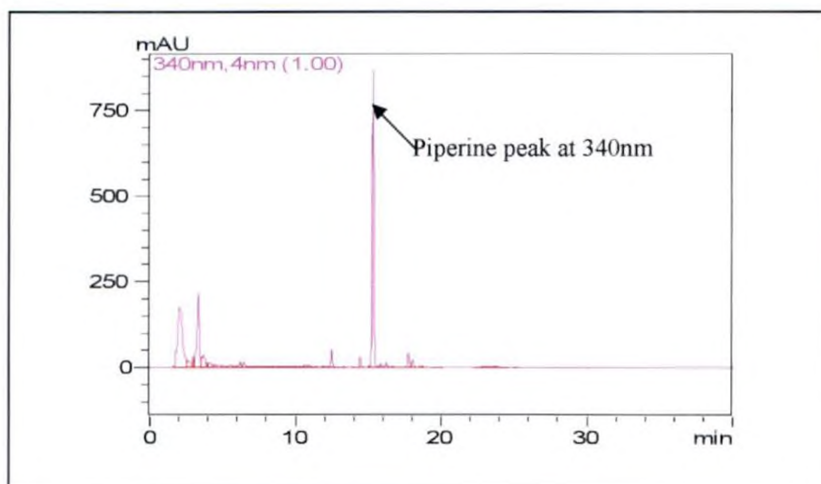


Fig 12. Chromatogram of piperine from leaf derived callus grown in medium with 100 mg l^{-1} L-lysine

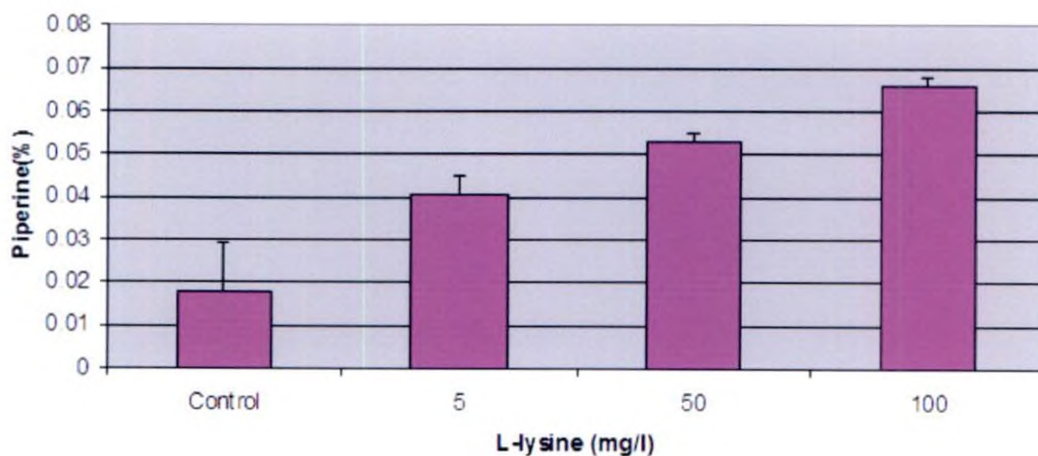


Fig 13. Effect of precursor on piperine yield *in vitro* leaf derived calli of *Piper nigrum* (variety Panniyur 5).

Data represents Mean ± SE of three replicates

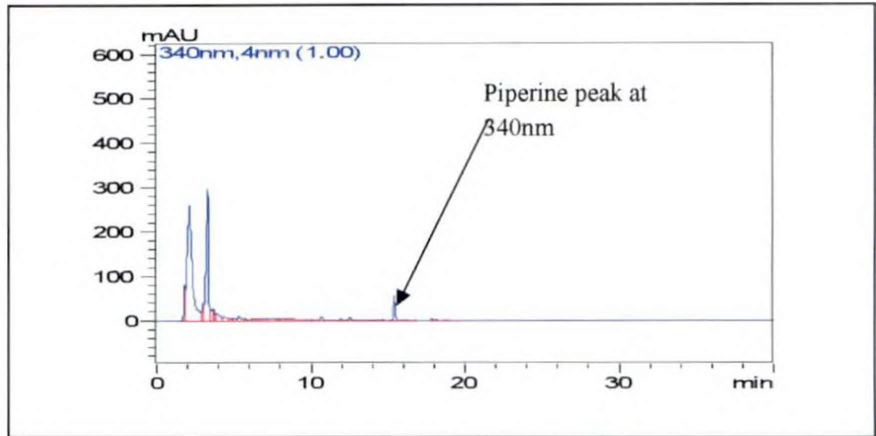


Fig 14. Chromatogram of piperine from epicarp derived callus grown in medium with 5 mg l^{-1} L-lysine

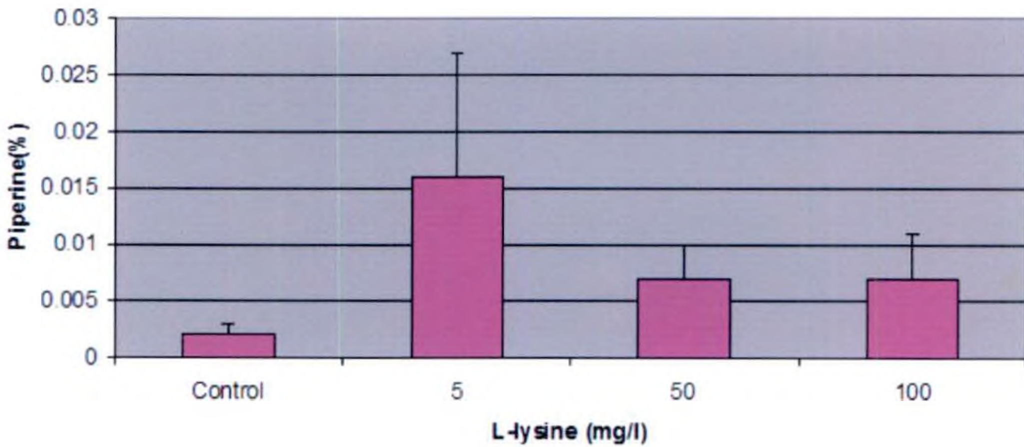
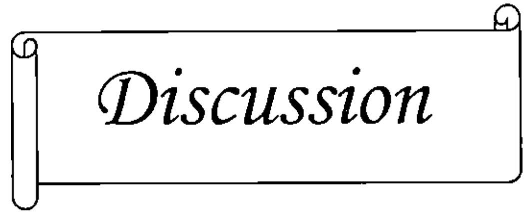


Fig15. Effect of precursor on piperine yield *in vitro* epicarp derived calli of *Piper nigrum* (variety Panniyur 2).

Data represents Mean ± SE of three replicates


Discussion

4. DISCUSSION

Black pepper (*Piper nigrum*) belonging to the plant family Piperaceae is the most recognized culinary spice in the world. Western Ghats in Kerala is the centre of origin for the crop with around fifteen different species. All of the commercial and political attention that black pepper has received throughout the centuries has been due to the pungency and aroma. Pungency is primarily due to the presence of a yellow crystalline alkaloid, called piperine. Whereas the spicy aroma is due to the essential oil oleoresin. The pepper oleoresin has both the aroma and pungency. The cultivars show variation from 2 to 7.4 per cent with respect to the piperine content in berries. Quality attributes like oil and oleoresin contents of the tissue culture derived plants established in the field were reported to be good and comparable with that of the conventional propagules whereas in somaclones produced by callus cultures, these traits showed more variation (Nybe, 2001).

Stage of harvest, hormone application, post harvest handling etc. affects the spice quality in pepper. Hormonal spray (100 ppm NAA or 200 ppm 2, 4-D) to the full grown plants increased the mean weight of spikes, mean weight of 1000g berries and mean volume of 1000g berries but reduced the number of spikes. The oleoresin content was reported to increase upto 14.21per cent when NAA (150 ppm) was applied (Geetha and Nair, 1990).

Hence, the present investigation was carried out with the objective to standardize the protocol for *in vitro* synthesis of piperine in black pepper and to detect and quantify the *in vitro* synthesized piperine.

5.1. *In vitro* culture initiation

5.1.1 Pretreatment and surface sterilization of explants

Maintenance of aseptic conditions is essential for successful tissue culture procedures. To maintain an aseptic environment, all culture vessels, media and instruments used in handling tissues, as well as explants itself must be sterilized. The importance is to keep the air, surface and floor free of dust. All operations should be carried out in laminar air flow sterile cabinet (Chawla, 2003). Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explant to the various sterilants and the sequences of using these sterilants has to be standardized to minimize explant injury and achieve better survival.

In the present study, a two step process of sterilization was carried out, consisting of pretreatment of explants with ethanol followed by dipping in a fungicide followed by surface sterilization. Pretreatment of explants such as wiping with ethanol (70%) and dipping in bavistin (0.1%) was effective in reducing the rate of fungal contamination. Several reports are there describing the treatment with bavistin as effective in reducing contamination (Tiwary *et al.*, 2010). Sood and Chauhan (2009) also reported that shoot apices of pot grown *Picrorhiza kurroa* plants were pre treated with 0.5 per cent bavistin.

Mercuric chloride is a very strong sterilant but it is toxic to the plant part also. As the leaves used as explants were very tender, treatment above 5 min resulted in scorching and death of the cultures. Preethi *et al.* (2011) reported lower concentration and lesser time of treatment with $HgCl_2$ (0.01% for 1-2 min) for leaf explants of Stevia. Nodal cuttings, berries and epicarp needed longer duration (10 min) of treatment for effectively reducing the fungal contamination but

consequently the rate of survival also was reduced for berry (55%) and epicarp (40%). This may be because, the spikes were having thickly set berries so that the chance of occurrence of fungal spores on the surface of berries is more and to remove this, longer period of treatment (10 min) is required. But as the berry is immature, this may also damage the outer layer of tissue. However, for nodal cuttings, 80 per cent survival was found under this treatment. Rajmohan *et al.* (2010) also reported 10 min duration for treating nodal explants of black pepper with $HgCl_2$ (0.01%). Surface sterilization with mercuric chloride (0.1%) for 10 min was most effective in reducing microbial contamination in all the explants of *Tinospora cordifolia* registering 73-74 per cent (Kalimuthu, 2002).

5.1.2 Effect of medium on callus induction and proliferation

In the present study, medium containing a combination of auxin (IAA, 1 mgl^{-1}) and cytokinin (BA,1.0 mgl^{-1}) resulted in a reasonably high percentage of callusing and callus growth score from leaf explants. Sujatha *et al.* (2003) also reported that IAA and BA at a concentration of 1.0 mg l^{-1} were good at initiating callus from leaf explants in *Piper nigrum*. Similar reports are there from *Coscinium fenestratum* leaf explants (Sindhu, 1999). Immature berries also showed callusing but the number of cultures producing the callus and callus growth score was lower than that in leaf explants. Medium containing 2, 4-D (3.0 mg l^{-1}) as the only growth regulator was successful in initiating callus from 35 per cent epicarp and 20 per cent seeds inoculated, but it was harmful in the case of tender leaf. Preethi *et al.* (2011) reported that 2, 4-D along with BA was a potent hormonal combination for stimulating callus induction from leaf explants in *Stevia rebaudiana*.

Lower concentrations of 2, 4-D (0.25 mg l^{-1}) along with BA (3.0 mg l^{-1}) supported both organogenic callus proliferation and shoot regeneration in *Epimedium alpinum* culture (Mihaljevic and Vrsek, 2009). However, Ali *et al.* (2008) reported that 3.0 mgl^{-1} of 2, 4-D was best for maximum callus induction

and proliferation of leaf explants from *Saccharum officinarum*. Sarker and Biswas (2002) reported a higher concentration of 2, 4-D (6 mg l^{-1}) as the best concentration for callus induction from seeds and mature embryos of wheat.

Reports are also available which show that the addition of adequate levels of 2, 4-D into the basal medium resulted in proliferation of callus from a variety of rice explants (Lee *et al.*, 2002).

5.1.3 Comparative performance of explants to callus induction and proliferation

Cultures originating from leaf explants of the variety Panniyur 5 were better performing with respect to earliness in callusing, number of cultures producing callus, growth rate of callus etc. in half MS media supplemented with auxin (1 mg l^{-1} IAA) and cytokinin (1 mg l^{-1} BA). The berry of the variety Panniyur 2 was next with respect to induction and proliferation of calli.

Though from the same variety, epicarp and seed failed to produce any calli in this medium. In ½ MS supplemented with 3 mg l^{-1}2, 4-D) more number of epicarp cultures started callusing (35nos.) as compared to seed cultures (20nos.) but seeds took only 25 days to start callusing, whereas epicarp required almost double the time (40 days). Similar results were reported in *Saccharum officinarum* (Ali *et al.*, 2008) where rate of callus formation was 100 per cent in leaf, 90 per cent in meristem and 60 per cent in pith. Differences in explant performance with respect to callusing are evident in many crop plants. Sutan *et al.* (2010) reported that significant differences in callus induction frequency was observed between explants in strawberry in which petiole explants gave highest percentage of callus formation in comparison to leaf explants cultured on the same MS basal medium. Amudha and Shanthi (2011), reported that among the three explants tested (leaf, internode and node) for callus induction from *Acmella calva,* leaf explants produced considerably more callus than other explants. These results showed that

nature of explants is an important factor in determining the rate of success in such tissue culture experiments. This difference in callus induction may be due to the difference in cellular totepotency (Niedz *et al.*, 1985).

5.2 Modification of culture medium for enhancing *in vitro* piperine production

Leaf calli from the variety of Panniyur 5 were subcultured into five different modifications (total 15treatments) of the culture initiation medium (Table 6) *viz.*, increased sucrose concentration (5, 6,7 and 8%), 1/4th nitrate and phosphate concentration in the MS stock solution (with 3 and 7% sucrose), different hormone combinations (0.5, 1.0, 3.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1}BA in four combinations), stress inducing agent (4% mannitol in combination with 3 and 7 % sucrose), L-lysine which is the precursor of piperine (5, 50 and 100 mg l^{-1}). The effect of each component on the piperine synthesis was studied by estimating the mean piperine content from three fully grown calli obtained in each treatment. Due to heavy loss of cultures raised from the variety of Panniyur 2, calli obtained from different explants like berry and epicarp could be used in only limited treatments.

5.2.1 Modification of sucrose content in the culture initiation medium

Sucrose is the carbon source for *in vitro* grown plantlets. It influences not only the growth but secondary metabolism as well. It is reported that secondary metabolite production is closely related to applied carbohydrate (Kim *et al.*, 1995). Among the various concentrations of sucrose (5–8 per cent) including 3 per cent sucrose used as control in the present study, 7 per cent sucrose was found suitable for piperine production in leaf callus of the variety Panniyur 5. Increasing the sucrose concentration upto 7 per cent showed a positive influence on synthesis of piperine *in vitro*. At 7 per cent sucrose, leaf derived calli synthesized 0.135 per cent piperine in comparison to the control (0.018%).

Increasing the sucrose concentration in the medium may increase the osmotic stress to the culture. Increase in osmotic stress is one of the factors favoring secondary metabolite production up to a specific limit, which may differ from plant to plant.

Similar reports are there in other crops also wherein, 7.5 per cent sucrose favored the production in rosmaric acid yield in *Coleus blumei* culture (Misawa *et al.*, 1988). Kim *et al.* (2010) reported maximum increase in paclitaxel in *Taxus chinensis* culture media containing high sucrose concentration (6 %). Zenk *et al.* (1975) reported that 5 per cent sucrose concentration was best for anthraquinone yield in cultures of *Morinda citrifolia.*

The media supplemented with 8 per cent sucrose showed a decline in piperine (0.015 percent) production in the present study whereas it favored the indole alkaloid accumulation in cell cultures of *Catharanthus roseus* (Knobloch and Berlin, 1980). Beyond the optimum limit, the osmotic stress might have adversely affected the growth and physiological functioning of the tissue.

In contrast to these results, lower concentrations of sucrose than the normal three per cent and very high concentrations of other sugars were also reported to increase secondary metabolite production in some plants. Three per cent sucrose favored the ginsenoside production in *Panax ginseng* cell suspension culture (Lian *et al.,* 2002). In *Bacopa monnieri* shoot cultures, 2 per cent sucrose favored biomass accumulation, whereas sucrose free medium accumulated maximum amount of bacoside A content (Naik *et al.,* 2010). The culture growth of *Azadirachta indica* decreased 3.5 times with increasing glucose concentrations from 30 to 120 g l^{-1} (Prakash and Srivastava, 2006).

5.2.2 Reduction of nitrate and phosphate concentration

Maintaining phosphate and nitrate levels below the recommended level in the MS medium is often employed as a strategy to provide a good growth limiting environment in *in vitro* systems that stimulate valuable secondary metabolite production. However, in the present study, reduction of phosphate and nitrate, to one fourth of the original concentration did not exert a beneficial influence on synthesis of piperine *in vitro*. Also it nullified the beneficial effect of higher concentration of sucrose as evidenced by the reduced piperine content (0.003%) in the calli grown in this nutrient limiting media (1/4th of nitrate and phosphate than MS) supplemented with 7 per cent sucrose compared to that in control (½ of nitrate and phosphate than MS) supplemented with 3 per cent sucrose(0.018%).

This modification was tried in the present study as there are reports in many crops where, such nutrient limitations produced favourable effects. Sujanya *et al.* (2008) reported increase in azadirachtin production in media containing reduced level (half of the original media) of phosphate in *Azadirachtia indica* cultures. Decreased phosphate has been correlated with an increase in alkaloids, anthocyanins and phenolics in *Catharanthus roseus*, and increased alkaloid content in *Peganum harmala* (Zenk *et al.,* 1977). Reduced level of nitrate also stimulated increased rhein production in callus cultures of *Rheum ribes* (Sepehr and Ghorbani, 2002).

But similar to the results of the present study, lower phosphate concentrations decreased the level of production of psilostachyinlides and altamisine production and reduced level of nitrate concentration resulted in a decrease on the level of production of psilostachyin B and C in callus cultures of *Ambrosia tenufolia* (Goleniowski and Trippi, 1999). Shinde *et al.* (2009) reported that, when nitrate concentration was reduced to half, a 3-fold decrease in phytoestrogens production was observed. The limitation of phosphate supplied to culture medium tends to suppress growth and in some systems, diminished secondary product contents (De-Eknambul and Ellis, 1985).

5.2.3 Effect of addition of mannitol along with sucrose on piperine production

In the present study, incorporation of 4 per cent mannitol into the culture initiation medium stimulated piperine synthesis (0.107 %) compared to the control medium. However, when this was used in combination with the optimum sucrose concentration used in the initial treatment (i.e., 7%), the calli recorded lower level of piperine (0.007 %). This may be due to the combined osmotic stress produced by mannitol and sucrose as mannitol itself is inducing high osmotic stress to the plant, in combination with 3 per cent sucrose, the plant may be able to withstand the stress but when sucrose content increase further to 7 per cent along with the 4 per cent mannitol, the osmotic stress might be beyond the tolerance limit of the tissue. In other crops also, increase in total alkaloids was reported.

When mannitol was added into the medium, high percentage of vinblastin and vincristine as relative to that in intact plant were recorded with leaf derived calli cultures from MS medium which was supplemented with 8 per cent of mannitol alone (Taha *et al.*, 2009). Babu *et al.* (2006) reported that MS media supplemented with 5 per cent sucrose and 1 per cent mannitol largely influenced the azadirachtin-A alkaloid production in *Azadirachta indica* culture.

5.2.4 Effect of growth regulators on piperine production

Growth regulator concentration is often a crucial factor in secondary product accumulation (Di-Cosmo and Towers, 1984). Mantell and Smith (1984) reported that the type and concentration of auxins and cytokinins or the auxin/cytokinin ratio, dramatically alters both the growth and the secondary metabolite production in cultured plant tissue.

In the present investigation, berry derived callus of variety Panniyur 2 grown in the medium 2, 4-D at 3 mg l^{-1} synthesized more piperine (0.042 %) in

comparison to all other cultures. For leaf derived callus from the variety of Panniyur 5, highest piperine was obtained in medium with 2, 4-D (1 mg l^{-1}) and BA (1 mg l^{-1}) which recorded 0.017 per cent piperine. But the difference in the result may also be attributed to the genotype of other plant and the type of explants used. But, in general, 2, 4-D is found to have a favourable effect on piperine production than using IAA as auxin. Rhodes *et al.* (1994) also reported that flavone yield increased 30 times in suspension cultures of *Morinda citrifolia* (Rubiaceae) when 2, 4-D was substituted for NAA. Xu *et al.* (1995) reported that increasing the 2, 4-D content of the growth medium resulted in a larger increase in the salidroside content of *Rhodiola sachalinesis*. Masoumian *et al.* (2011), reported higher production of flavonoids (8.29 mg g^{-1} DW) in the presence of 2, 4-D (2 mg l^{-1}) medium in *Hydrocotyle bonariensis* callus cultures. But, Zhao *et al.* (2001) reported that 2, 4-D generally has a negative effect on synthesis of secondary metabolite.

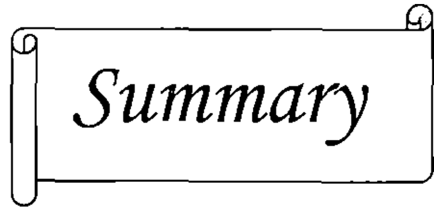
5.2.5 Effect of precursor feeding

Ouyang *et al.* (2005) reported that excess precursors may cause feedback inhibition to the metabolic pathway. It is very important therefore to determine the appropriate precursor concentration in precursor-feeding trials.

In the present study, L-lysine, the amino acid precursor of piperine, at concentration of 100 mg l^{-1}, had a beneficial influence on piperine synthesis. For leaf derived calli of the variety Panniyur 5, higher concentration (100 mg l^{-1}) of L-lysine registered maximum of piperine (0.066 %). Similarly, Shin and Chi (1989) reported that in case of *Rubia cordiafolia* callus cultures, addition of 100 mg l^{-1} of alfa- keto glutaric acid enhanced the total pigment synthesis. For *Catharanthus roseus* cultures, it was found that increased indole alkaloid production was obtained after feeding with L- tryptophan at higher concentration. The addition of 500 mg l^{-1} of L- tryptophan resulted in a 2.85 fold increase in alkaloid production (Zenk *et al.*, 1977). Callus of *Artemisia absinthium* revealed the presence of

artemisinin by the addition of 12.5 mg l^{-1} glutamine as the precursor of artemisinin (Zia *et al.*, 2007).

However, the present study on the effect of the precursor feeding on piperine content in epicarp derived calli showed a different result. Here, L-lysine in lower concentration (5 mg l^{-1}), registered maximum of piperine (0.016 %) in epicarp derived calli. The result is similar to the study conducted by Kiong *et al.* (2005), where highest yield of asiaticoside was obtained in media supplemented with lower concentration of squalene (0.16 mg l^{-1}) in callus culture of *Centella asiatica*. Fett-Neto *et al.* (1994) reported that, paclitaxel production was stimulated about 4.5 fold and 4 fold compared to the control, when phenylalanine (8.25 mg $l^{-1)}$ and benzoic acid (6.1 mg l^{-1}) were added to cultures of *Taxus cupisidata* and decreased when the concentration exceeded 8.25 and 6.1 mg l^{-1}, respectively. The highest flavonoid production was attained in calli treated with 4 mg l^{-1} of naringenin and was 19.72 percent higher compared with the control in *Hydrocotyle bonariensis* callus culture (Masoumian *et al.*, 2011).


Summary

5. SUMMARY

The present investigation entitled 'Detection and quantification of piperine from *in vitro* cultures of black pepper (*Piper nigrum* L.)' was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Thrissur during the period 2009 to 2011. The study was aimed to standardize the protocol for *in vitro* synthesis of piperine in black pepper and to detect and quantify the *in vitro* synthesized piperine. The salient findings of the investigation are summarized below.

1. Among the various explants used (*viz.*, tender pale green leaf and single nodded cuttings from the variety of Panniyur 5 and leaf, immature berry, epicarp of berry and seed from the variety of Panniyur 2), young leaves were found to be the most suitable for callus induction in black pepper. Ninety per cent of cultures from leaf explants exhibited callusing with the highest mean callus growth score (2.91) and mean callus index (261.9).

2. Pretreatment of the explants with 0.1 per cent bavistin followed by surface sterilization with 0.1 per cent mercuric chloride for 5 minute for young leaf and 10 minute for nodal cuttings and immature berry and 7 minute for seed explants were most effective in reducing microbial contamination of *Piper nigrum* cultures registering healthy culture.

3. Half strength MS medium enriched with IAA (1mg l^{-1}) and BA (1mg l^{-1}) and $CuSO_4$ (15 mgl^{-1}) and sucrose (3%) recorded highest percentage (90 %) of calli initiation from leaf explants of the variety Panniyur 5. The same medium registered callusing in 30 per cent from berry explants of the variety Panniyur 2. But, epicarp and seed explants did not produce any callus in this medium.

4. ½ MS with 2, 4-D (3mg l^{-1}) singly was used for inoculation of epicarp, seed, and leaf explants and proved effective in callusing 35 per cent berry and 20 per cent seed explants. But this medium was unable to initiate callus in leaf explants.

5. The study revealed that increasing the concentration of sucrose up to 7 per cent in the callus initiation medium increased the piperine content from 0.018 per cent to 0.135 per cent whereas reducing the nitrate nitrogen and phosphate content of the basal medium to $1/4^{th}$ of its original strength in MS exerted an inhibitory effect on piperine production (0.003%) in leaf derived calli of the variety Panniyur 5.

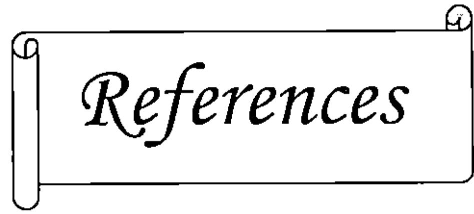
6. The stress inducing agent, mannitol when supplemented in the callus initiation medium at 4 per cent (W/V) resulted in enhanced *in vitro* synthesis of the target compound (0.107 %) in leaf derived calli of the variety Panniyur 5.

7. Among the growth regulator combinations tried in the study, supplementing the media with 2, 4-D (3.0 mg l^{-1}) alone enhanced the *in vitro* synthesis of piperine to 0.042 per cent from berry derived calli of the variety Panniyur 2 whereas, the medium containing 2, 4-D (1.0 mg l^{-1}) and BA (1.0 mg l^{-1}) registered maximum piperine (0.032%) from leaf derived calli of the variety Panniyur 5.

8. Favourable influence of the precursor of piperine (L-lysine) on *in vitro* synthesis of piperine in the leaf derived calli, was evident at 5, 50 mgl^{-1} and 100 mg l^{-1}. L-lysine at 100 mg l^{-1} registered maximum piperine (0.066 %) synthesis in comparison to other two concentrations (5 and 50 mgl^{-1}). For the epicarp derived calli, maximum piperine (0.016%) was obtained from the medium containing 5 mg l^{-1} of L-lysine.

9. The maximum piperine content estimated from the various treatments was from the leaf calli (0.135%) than berry calli (0.042%). This piperine production from leaf calli (0.135%) was higher than that in the leaf used as explant (0.01%) as reported by Sujatha (2010). Berry calli registered very less amount of piperine (0.042%) compared to the reported piperine content of fresh immature berry (2.7%) used as explant (Sujatha, 2010).

The results indicated that *in vitro* synthesis of piperine may be exploited by further modifications of the culture media.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Appendices

APPENDIX I

Composition of MS basal medium

Major salts	mg l^{-1} medium	Stock solution	Concentration
NH_4NO_3	1650	I	50X
KNO_3	1900		
$MgSO_4.7H_2O$	370		
KH_2PO_4	170		
$CaCl_2.2H_2O$	440	II	50X
Na_2EDTA	0.25	III	100X
$FeSO_4 \cdot 7H_2O$	27.8		
$MnSO_4 . 4H_2O$	22.3	IV	100X
H_3BO_3	6.2		
$ZnSO_4.4H_2O$	8.6		
$Na_2Mno_4 \cdot 2H_2O$	0.25		
KI	0.83		
$CoCl_2.6H_2O$	0.025		
$CuSO_4.5H_2O$	0.025		
Thiamine (HCl)	0.1	V	100X
Nicotinic acid	0.5		
Glycine	2.0		
Pyrodoxine (HCl)	0.5		

MS basal medium (for 1 liter)

Stock solution I : 10 ml
II : 10 ml
III : 5 ml
IV : 5 ml
V : 10 ml

Sucrose = 30 g

Myo-inositol = 100 mg

Copper sulphate = 15 mg

Stock solutions of plant growth regulators = 1000X

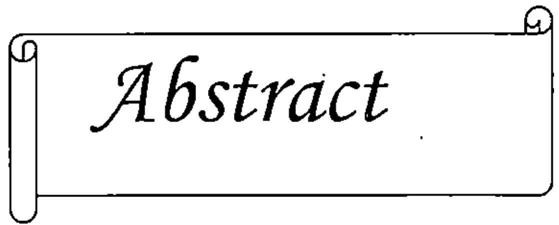
APPENDIX II

Composition of modified basal MS stock 1

Major salts	mg l^{-1} medium	Stock solution	Concentration
*NH_4Cl	1,650	I	50X
KNO_3	1900		
$MgSO_4.7H_2O$	370		
**KH_2PO_4	85		
KCl	85		

* ½ reduction in NO_3 nitrogen concentration as NH_4NO_3 is replaced by NH_4Cl

**½ reduction in PO_4 concentration


Abstract

DETECTION AND QUANTIFICATION OF PIPERINE FROM *IN VITRO* CULTURES OF BLACK PEPPER (*Piper nigrum* L.)

By

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(2009-11-103)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the
requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

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2011

ABSTRACT

The present study on "Detection and quantification of piperine from *in vitro* cultures of black pepper (*Piper nigrum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, during the period 2009-2011. The objective of the study was to standardize the protocol for *in vitro* synthesis of piperine in black pepper and to detect and quantify the *in vitro* synthesized piperine.

Callus cultures from tender, pale green leaf from the variety Panniyur 5 and immature berry and epicarp of berry from the variety Panniyur 2 were established successfully. Surface sterilization with mercuric chloride (0.1 %) for 5 min was effective in initiating healthy, contamination free callus from leaf and 10 min were effective in initiating healthy callus from berry explants. It was observed that half strength MS (Murashige and Skoog, 1962) medium supplemented with IAA (1.0 mg l^{-1}) along with BA (1.0 mg l^{-1}) was suitable for initiation and proliferation of calli. Leaf derived cultures were superior with respect to callus initiation and proliferation.

Leaf calli were sub cultured into five different modifications of the culture initiation medium viz., (I) increased sucrose concentration (5 different concentrations), (II) 1/4th nitrate and phosphate concentration in the MS stock solution, (III) different hormone combinations (2, 4-D and BA in four combinations), (IV) stress inducing agent (mannitol) and (V) precursor of piperine (L-lysine) in three different concentrations. The effect of these modified media on piperine production was studied by detecting the presence of piperine and estimating the quantity by High Performance Liquid Chromatography in the respective calli, callus growing in the culture initiation medium (as control) and the calli growing in these modified media.

Piperine was extracted from 1.0 g callus by grinding in 5 ml methanol and the centrifuged crude extract was used for High Performance Liquid Chromatography analysis (Shimadzu, Japan). Gradient elution system using 0.1% ortho-phosphoric acid and 90 % acetonitrile in 0.1% ortho-phosphoric acid was employed for detection of piperine.

The study revealed that increasing the concentration of sucrose up to 7 per cent in the callus initiation medium increased the piperine content from 0.018 % to 0.135% in leaf derived calli whereas, reducing the nitrate nitrogen and phosphorus content of the basal medium to $1/4^{th}$ of its original strength exerted an inhibitory effect on piperine production (0.003%). Similarly, supplementing the callus initiation medium with mannitol at 4 per cent along with 3 per cent sucrose, resulted in enhanced *in vitro* synthesis of the target compound (0.107 %). Similarly the medium with 2, 4-D (3.0 mgl^{-1}) enhanced the *in vitro* synthesis of piperine to 0.042 per cent in berry calli. Precursor feeding with L-lysine at higher concentration (100 mgl^{-1}) also exerted a favorable influence on piperine (0.066 %) synthesis in comparison to other concentration (5 and50 mgl^{-1}).

Among the various explants studied, young leaves were found to be the most suitable for callus induction in black pepper. *In vitro* piperine production from leaf calli (0.135 %) was higher than that in the leaf used as explant (0.01%). Berry calli produced very less amount of piperine (0.042 %) compared to that of fresh berry used as explant (2.7%). The results indicated that *in vitro* synthesis of piperine may be exploited by further modifications of the culture media.