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RECENT PROGRESS IN BIOTECHNOLOGICAL INTERVENTIONS IN *Piper* SPECIES: A REVIEW

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ABSTRACT

Medicinal plants have tremendous potential for the development of new drugs molecule effective against various diseases. Genus *Piper* is the largest genus in family *Piperaceae* and indigenous to India with one of the most widely used spice in the world. Biotechnological innovations in *Piper* species have lead to the development of plant-derived molecules which exhibit a promising effect in therapeutics. The present paper reviews the genetic diversity analysis of major *Piper* species through DNA markers, to establish *in vitro* protocol for mass multiplication of elite clone of different species, phytochemical compounds extracted in various solvent systems in *Piper* species and comparative analysis of active compounds associated with *Piper* species through HPTLC analysis as well as testing their antimicrobial activities. The activity has been tested against bacteria like *E. coli, Micrococcus luteus, Streptomyces epidermidis,* etc. and fungi like *Aspergillus niger, A. flavus* and *Rhizoctonia solani* using agar well diffusion method. The review also highlights the use of *in vitro* strategy in genetic manipulation of transgenic traits using specific promoters to accomplish high tissue specific protein production and overcome biotic stresses.

Key words: Antimicrobial properties, genetic diversity, *Piper* species, secondary metabolites, genetic engineering

1. INTRODUCTION

Plants play major role in the existence and survival of mankind. As a source of food and shelter, they produce chemical substances that have potential therapeutic value. Medicinal plants are used by human beings as a source of medicine and food. *Piper* species are world-wide distributed in tropical and subtropical regions and have high commercial and economic value. The genus *Piper* is the largest genus in family *Piperaceae* and has more than 3000 species (Rahiman and Nair, 1983). Among these, 60 species were recorded in China only, and 11 to 12 species in Hainan Island (Cheng *et al.*, 1999). The North East India in Eastern Himalaya forms mega biodiversity area of Indian *Piper*. All the species, with the exception of *Piper betle* and *P. nigrum*, occur wild. Out of the 1000 species of *Piper* in India, *P. nigrum* is largely cultivated due to its high economic value. Black pepper (*P. nigrum* L.), an important commercial species, was introduced into Hainan, China in 1947 from Indonesia. In India *P. nigrum* is confined to Western-Ghats of South India. These species are also cultivated in Malaysia, Indonesia, Brazil, SriLanka and West Indies. *P. nigrum* is found in high altitudes and shows greater adaptability to a wide range of environmental conditions. Most of the *Piper* species occur in semi-evergreen and moist deciduous forests of Western Ghats of

India. Wild *Piper* species are resistant to foot rot disease, caused by *Phytophthora capsici*, so may be useful in variety improvement of black pepper. Moreover, the fruits and roots of most *Piper* species are widely used in Chinese and Indian medicines. The leaves of *P. betle* along with other ingredients are used as "chewing gum" by local people and the leaves of *P. sarmentosum* are used as vegetable and spice. Betel (*Piper betle*) is a spice whose leaves have medicinal properties. It is native of Central and Eastern Malaysia and spread over tropical Asia and later to Madagascar and East Africa. In India, betel is widely cultivated in Orissa, Maharashtra, Tamil Nadu, Madhya Pradesh, West Bengal and Uttar Pradesh. The present review provides an insight into the progress in *in vitro* conservation and propagation, analysis of phytochemical and antimicrobial properties, genetic diversity assessment through molecular and phytochemical markers and genetic improvement of *Piper* species.

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2. *In vitro* PLANT REGENERATION

In vitro techniques offer rapid clonal multiplication of elite germplasm thereby allows the production of genetically stable progeny. This technique has played a vital role in clonal propagation, germplasm conservation and plant improvement of various *Piper* species particularly black pepper (Bhat *et al*., 1995). Combination of cryopreservation and *in vitro* propagation techniques helped in conservation of germplasm diversity in black-pepper. Successful micropropagation of various *Piper* species have been achieved by using shoot tip and leaf explants (Nazeem *et al*., 1992; Philip *et al*., 1992; Babu *et al*., 1993). During early stages of callus formation, the parenchyma cells of mesophyll tissue near the vascular bundles in leaf explants resume meristematic activity due to wounding and produce primary callus having undifferentiated cell mass. Callus differentiation initiates when peripheral meristematic activity and formation of centers of cell division deeper in tissue. On these meristematic regions, characterized with small cells having densely stained nuclei, meristemoids are produced. The scattered nature of vascular bundles is a distinguishing feature of genus *Piper*. Formation of vascular nodules in callus cultures may represent or be associated with early stage of shoot meristem development. Bhat *et al.* (1995) reported that root, leaf, node and internode explants of *P. nigrum* have high morphogenetic potential. Successful micropropagation of black-pepper has been reported using mature shoot-tip explants (Nazeem *et al.*, 1992; Babu *et al.*, 1993; Joseph *et al.*, 1996), leaf explants (Sujatha *et al.*, 2003), nodes explants and seeds (Nair and Gupta, 2006). Though the plantlets regenerated from the seedlings derived from callus and shoot apices have been reported, yet most attempts to regenerate plants from mature tissues were unsuccessful (Mathews and Rao, 1984). The explants source is vital in determining the regenerative potential which is influenced by the physiological conditions of donor plant. Maintaining donor plants in clean and controlled environmental conditions delivers healthy and sterile explants. The physiological age, type and size of explant are other factors influencing the formation of organogenesis (Rout *et al.*, 2000). At higher concentrations (5-10 mg L^{-1}) of 6-benzylaminopurine (BAP), only 40% nodal explants of *P. nigrum* responded. Kinetin (Kn) failed to induce shoot buds in *P. nigrum* (Bhat *et al.*, 1995). Cytokinins like BAP and activated charcoal have a significant effect on the number of shoots. Shoot tip and nodal explants failed to stimulate bud break and shoot formation on cytokinin-free medium.

Leaf explants cultured on modified MS (Murashige and Skoog, 1962) medium supplemented with 1 mg L^{-1} indole-3-acetic acid (IAA) and 1 mg L^{-1} BAP produced creamy white to pale green friable calli. The addition of 5-15 mg L^{-1} silver nitrate in culture medium enhanced shoot-regeneration frequency. Either BAP alone or in combination with IBA and adenine sulphate (Ads) helped in the initial proliferation of shoot tip explants, BAP >5 mg L^{-1} suppressed growth of shoots and proliferation while 1.5 mg L^{-1} BAP and 3 µM IBA produced higher response (Philip *et al.*, 1992). Increasing the concentration of BAP (upto 3.0 mg L^{-1}) enhanced the growth response in both types of explant. Higher concentrations of BAP and Kn suppressed the number of shoot formation. Upto 88% response was observed for 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} Kn (Anand and Rao, 2000).

Maximum shoot organogenesis has been achieved in medium having Kn and IAA, while BAP and IAA combinations proved considerably ineffective (Rubluo and Barroso, 1992). Direct somatic embryogenesis from micropylar tissues of germinating seeds of black-pepper and its scaling up through high-frequency cyclic secondary somatic embryogenesis have been reported (Nair and Gupta, 2003). Secondary embryos formed from radicular end of primary somatic embryos on growth regulator-free nutrient medium in the absence of light. The process of *in vitro* root initiation, development and elongation normally require medium containing auxin. Philip *et al.* (1992) achieved root induction from excised micro-shoots of *P. nigrum* when transferred to ½ strength MS basal medium having 1.0 µM naphthalene acetic acid (NAA). The *in vitro* raised plants were grown normally as compared to the field grown plants (Philip *et al.*, 1992). All the regenerated plants were successfully established in soil (Bhat *et al.*, 1995). The cultures incubated in dark culture favoured higher rooting response. The rooted plants were transferred to field and grown normally (Anand and Rao, 2000). The combination of cytokinins and auxins also favoured positive response on shoot proliferation and multiplication from apical and axillary meristems. In medium having 1.5 mg L^{-1} BAP along with 0.5 mg L^{-1} IAA showed the highest rate of shoot growth and proliferation in *Piper longum*. Increase in the concentrations of either BAP or IAA did not show any more positive response by the culture.

In *P. longum*, maximum shoot proliferation of 83.3% was noticed in MS medium supplemented with 1.5 mg L^{-1} BAP, 0.5 mg L^{-1} IAA and 3% sucrose within 8 weeks of culture. The average number of multiple shoots per culture varied from 1.0 to 3.4 in different treatments (Fig. 1A-B). In *P. betle*, maximum response on shoot multiple was 40% on MS medium supplemented with 1.5 mg L^{-1} BAP along with 1.5 mg L^{-1} IAA and 0.2 mg L^{-1} NAA. The average number of shoots per culture varied from 1.1 to 1.8 shoots per culture. In *P. nigrum* the maximum percentage of shoot proliferation was 34.2% on MS medium supplemented with 2.0 mg L^{-1} BAP + 2.0 mg L^{-1} IAA + 0.2 mg L^{-1} NAA. The average shoot number varied from 1.0 to 1.4 shoots per culture. Reports confirm that cytokinin and auxin help in shoot multiplication of different medicinal plant species (Joseph *et al.*, 1996; Philip *et al.*, 1992; Thomas *et al.*, 2003; Nair and Gupta 2005; Sencie-Tarazona *et al.*, 2015). The elongated shoots were rooted in ½ strength MS medium supplemented with 0.25 mg L^{-1} IAA and subsequently transferred to field for acclimatization (Fig. 1C). The rooted plants were survived and grown normally and morphology with uniform appearance (Fig. 1D, E).

Fig. 1: *In vitro* shoot multiplication of *Piper longum* A) Shoot proliferation on MS medium supplemented with 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} Kn + 100 mg L^{-1} Ads + 3% sucrose after 4 week of culture (Bar = 10 mm); B) Induction of multiple shoots on MS medium + 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA after 4 weeks of culture (Bar = 10 mm); C) Induction of rooting from micro-shoot on ½ MS medium + 0.25 mg L^{-1} IBA + 2% sucrose (Bar = 20 mm); D) *Invitro* raised plantlets established in poly-bags with soil manure after 1 month of transfer (Bar = 100 mm).

3. GENETIC DIVERSITY ASSESSMENT

Genetic diversity assessment is essential to identify genotypes which can be effectively used by breeders, geneticists and conservationists. In early days, the classification and evaluation of members of piper were based on morpho-physiological characteristics which, however, are easily influenced by environment. The genetic information provides reliable and consistent classification. With limited knowledge of genetic differentiation among genotypes, it is difficult to retain uniform quality of cultivated piper species. Genetic studies using molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and inter simple sequence repeats (ISSR) have been used to identify genetic constituent and evolutionary status of different piper species (Pradeepkumar *et al.*, 2003; Joy *et al.*, 2007). ISSR has proved highly useful in estimating genetic diversity and assessing genetic linkage as it is simple, fast, cost-effective, reliable and highly discriminating (Uysal *et al.*, 2010; Zhang and Liu, 2011). Varghese and Bhat (2011) reported genetic fidelity test of embryogenic mass-derived plantlets by RAPD using 23 random primers and found no genetic variation in progenies and parents. Jiang and Liu (2011) used ISSR markers to evaluate genetic variation in *Piper* spp. collected from China and found overall genetic diversity in *Piper* spp. high with mean Shannon information index (I) of 0.2843 and mean Nei's genetic diversity (H) of 0.1904. The genetic similarity coefficient varied from 0.548 to 0.976 within 74 individual plants; and within-species genetic distance ranged from 0.104 to 0.28. They found 247 polymorphic bands out of 248 (99.6%) obtained from 74 individual *Piper* species. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram showed *P. kadsura* as most divergent and most distant of 11 species, and *P. hainanense* and *P. bonii* were closely related as well as *P. sarmentosum* and *P. betle*. The diversity analysis unambiguously distinguished all piper species. Ahmad *et al.* (2013) used HPLC fingerprint analysis to identify active principle piperine content in *P. nigrum*. Chowdhury *et al.* (2014) used RAPD markers to assess genetic diversity and phenetic relationships in six piper species collected from North East India and found that 4 arbitrary 10-mer oligonucleotide primers produced 195 DNA fragments, of which 159 were polymorphic. On the basis of Jaccard's coefficient analysis, different accessions of piper showed high genetic variation. Anupama *et al.* (2015) used microsatellite marker based cross species amplification and genetic diversity analysis in genus *Piper*.

Singh and his associates evaluated genetic relationship through ISSR markers between 8 piper species/accessions collected from Eastern India (personal communication). Of the 40 ISSR primers tested, 15 ISSR primers generated 138 clear and scorable bands with mean of ~ 9 bands/ primer. Of these, 114 bands were polymorphic revealing 82.6% polymorphism. The banding pattern by ISSR primers USB-838, USB-810, USB-841 and USB-835 are presented in Fig. 2 which indicated ISSR marker efficient enough to distinguish these species/ accessions; thereby revealing molecular relationship among themselves. The similarity matrix of ISSR data after multivariate analysis using Nei and Li's coefficient has been presented in Table 1. The similarity value ranged morphologically distinct from the rest of 7 species/ accessions and grouped into single group. At the

Table 1: Similarity matrix of 8 species/accessions of *Piper* generated by ISSR markers

Species	P1	P2	P3	P4	P5	P6	P7	P8
P1	1.00							
P2	0.71	1.00						
P3	0.48	0.50	1.00					
P4	0.66	0.60	0.41	1.00				
P5	0.55	0.60	0.45	0.48	1.00			
P6	0.64	0.90	0.50	0.57	0.60	1.00		
P7	0.62	0.88	0.48	0.55	0.59	0.88	1.00	
P8	0.62	0.88	0.48	0.55	0.59	0.98	1.00	1.00

P1 - *Piper* sp. var. accession-1, P3 - *Piper* sp. var. accession-2, P4 - *Piper longum*, P5 - *Piper nigrum*, P6 - *Piper betle* var. Utkal Sudhama, P7- *P. betle* var. Astarangi Balunga, P8 – *P. betle* var. Godi Balunga

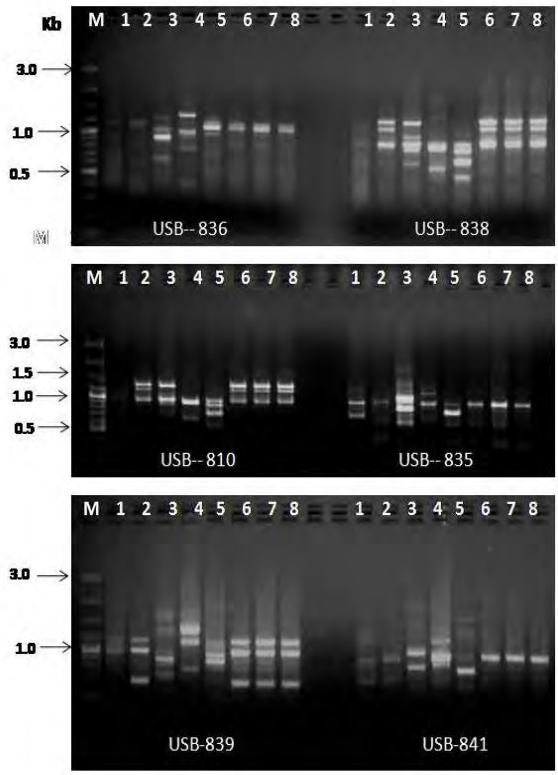

Fig. 2: ISSR bending pattern in 8 species/ accessions of *Piper* obtained from PCR amplification

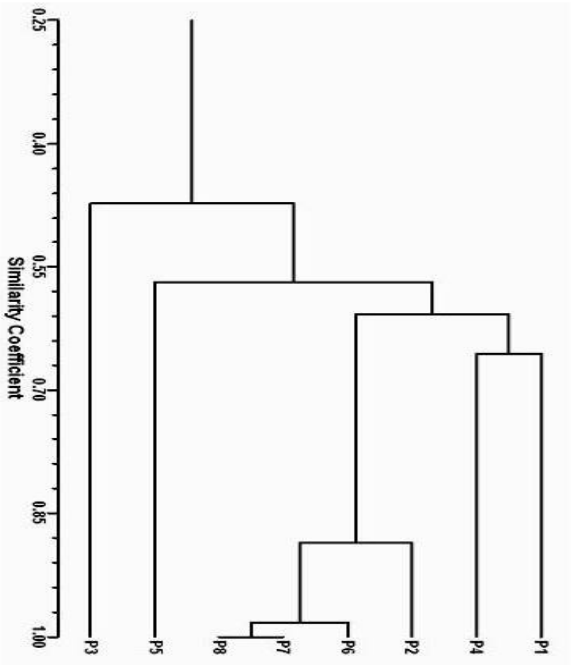

Fig. 3: Dendrogram showing cluster analysis of 8 specie/accessions of *Piper* using ISSR markers; P1 – *Piper chaba;* P2 - *Piper* sp. var. accession-1; P3 – *Piper* sp. var. accession–2; P4 – *P. longum;* P5 - *P. nigrum*; P6 - *P. betle* var. Utkal Sadhama; P7 - *P. betle* var. Astarangi Balunga; and P8 - *P. betle* var. Godi Balunga

molecular level *Piper* specie accession-1 had unique ISSR bands. The remaining 7 species/ accessions positioned in group II and again divided into 2 clads. The 1st clad has 6 species/ accessions (*P. chaba*), *P. longum*, *Piper* sp. accession-1, *P. betle* var. Utkal Sudhama, *P. betle* var. Astarangi Balunga and *P. betle* var. Godi Balunga and other clad having one species *P. nigrum*, *P. betle* var. Godi Balunga and *P. betle* var. Astarangi Balunga are grouped together with 100% similarity at genetic level, whereas *P. betle* var. Utkal Sudhama had 97% similarity with P7 and P8. Morphologically, they were very close with each other. The genotype like *Piper* sp. accession-1 formed single cluster with 88% similarity with *P. betle* var. Astarangi Balunga and var. Godi Balunga. The dendogram showed that there was distant variation within *P. chaba* and *P. longum* and 65% genetic similarity with *Piper* sp accession-1, *P. betle* var. Utkal Sudhama, Astarangi Balunga and Godi Balunga. This high variability reflected a relatively large genetic diversity in piper plantations where these accessions originated. Apart from genetic drift, inbreeding depression may also be one of the factors leading to genetic variation (Sherwin and Moritz, 2000). Inbreeding can be reduced in all the accessions of piper as the plants are dioecious, although within population, gene exchange between plants is unavoidable. The wide range of variation observed might also be due to several evolutionary forces, which include pollen flow and local selection pressures. Pollen can be dispersed over large distances; this long-term reciprocal movement of pollen must also have contributed to the variation. The local selection pressures may be due to the effects of environmental factors. The wide spread occurrence of wind pollination and breeding systems that promotes out crossing may lead to higher genetic diversity. It is believed that mutations, genetic drift due to finite population size and natural selection lead to genetic diversification in local populations and that the movement of gametes and individuals (gene flow) oppose that diversification. The lack of gene flow and effect of genetic drift due to restricted population size might have caused the

accessions of piper to differentiate genetically among themselves. The high degree of genetic variation or differentiation recorded due to the transfer of germplasm between different locations should be avoided to ensure adoption of genetic material to local conditions and it will help to develop better conservation strategies.

4. ANTIMICROBIAL ACTIVITIES

Piper species are important as they possess high medicinal values. *Piper betle* contains terpinine, ρ-cymene, chavicol, carvacrol and its derivatives, allyl catechol, eugenol, estragol, oxalic acid, malic acid and amino acids. Leaves contain good amounts of vitamins particularly nicotinic acid, ascorbic acid and carotene as well as contain significant amounts of essential amino acids, except lysine, histidine and arginine. Large concentrations of asparagines are present while glycine and proline occur in good amount. Essential oil of leaf gives it aromatic flavour. ß-sitosterol is present in roots. Krishnakumar *et al*. (2001) reported that betel leaf extracts with betel-nut have positive effect on anesthetic activities in rabbit and Guianese pigs. Betel leaf showed dose-dependent infiltration anesthetic activity comparable with xylocaine. As a surface anesthetic, the onset was as quick as xylocaine and the duration was shorter than xylocaine. Betel nut significantly reduced the infiltration activity and abolished the surface anesthetic activity of betel leaf. Arambewela *et al*. (2005) investigated antidiabetic activity of *Piper betle* leaves, tested in diabetic rats using oral administration of hot water extract (HWE) and cold ethanolic extract (CEE). Singh *et al*. (2011) studied mitochondrial activity of sperm, after treating semen with various concentrations of leaf extract of *P. betle*. The mitochondrial activity was also evaluated after incubating semen samples for various time periods. The authors tested leaf and root extract of *P. longum*, *P. nigrum*, *P. betle* var. Utkal Sudhama, *P. betle* var. Astarangi Balunga, *P. betle* var. Godi Balunga against pathogenic bacteria namely *Escherichia coli*, *Micrococcus luteus*, *Streptomyces epidermidis* and fungi like *Aspergillus niger, A. flavus* and *Rhizoctonia solani* at various concentrations (0, 20, 40 and 60 mg mL^{-1}) and found that plant extracts showed antibacterial activity against all the bacteria tested. The inhibition zone was high at concentrations of 60 mg mL^{-1} of root extract of *P. betle* i.e. 22 mm in *M. luteus*, 18 mm in *S. epiderdimis* and 14 mm in *E. coli* (Table 2). Agarwal *et al*. (2012) too observed similarly while evaluating cold aqueous, methanolic and ethyl acetate extracts of dried leaves of four varieties of *P. betle* at a final concentration of 500 mg mL^{-1} against pathogenic bacteria

Table 2: Antibacterial activity of different *Piper* species [The data represent the mean value of two independent experiment**]**

Plant species	Plant extract used	Conc. (mg mL^{-1})	Zone of inhibition (mm)		
			Micrococcus luteus	*Streptomyces epidermidis*	*Escherichia coli*
Piper betle var. Utkal Sudhama	Leaf	0	0	0	0
		20	7	10	8
		40	16	13	9
		60	17	15	10
	Root	0	0	0	0
		20	9	12	11
		40	20	16	13
		60	22	18	14
P. longum	Leaf	0	0	0	0
		20	5	8	6
		40	10	12	9
		60	14	13	12
	Root	0	0	0	0
		20	8	10	11
		40	10	13	13
		60	16	18	15
P. nigrum	Leaf	0	0	0	0
		20	7	9	8
		40	11	13	10
		60	12	14	12
	Root	0	0	0	0
		20	8	10	8
		40	14	13	10
		60	18	16	112

like *Pseudomonas aeruginosa, Staphylococcus aureus* and *E. coli*. Bacteria are prokaryotes with thin cell wall and relatively simple genetic system, which enhance easy penetration of bioactive substances, leading to manipulation of genetic system as a result of bioactive interruption. In addition, microorganisms show variable sensitivity to chemical substances related to different resistance levels between strains. The zone of inhibition was observed in fungal strains such as *A. flavus* and *R. solani*. There was no zone of inhibition in *A. niger*. The organic extracts particularly alcoholic exhibited better antibacterial principles either polar or non-polar are effectively extracted only through organic solvent medium (Essawi and Srour, 2000). The anti-bacterial activity of plant extracts was not likely due to a single active constituent but due to the combined action of other compounds. The aqueous extracts appeared to have less antibacterial activity than any of the organic extracts. Organic solvent is more beneficial for antimicrobial activity. Hydroxychavicol from *P. betle* leaves has antifungal activity against pathogenic fungi (Singburaudom, 2015).

Lupina and Crrips (1987) reported that secondary metabolites derived from *P. nigrum* play defensive role against infections by insects, microbes and animals. Piperamides extracted from *P. nigrum* showed activities particularly against insect (Scott and Albert, 2005; Boff *et al.*, 2006; Scott *et al.*, 2008). ß-caryophyllene extracted from *P. nigrum* showed anesthetic activity. Nerolidol is a very famous secondary metabolite of *P. nigrum*, used to control mites. Another important component of pepper volatile oil is piperine, which is a famous odorants. Black-pepper is anti-microbial and anti-mutagenic (EI-Hamas *et al.*, 2003), a free-radical scavenger (Gulcin, 2005), immuno-modulator, anti-tumor (Sunila and Kuttan, 2004), anti-depressant (Lee *et al.*, 2005), anti-apoptotic (Pathak and Khandelwal, 2007), anti-metastatic, anti-thyroid, hepato-protective, immunostimulator (Pathak and Khandelwal, 2009), anti-diarrheal and anti-spasmodic (Bajad *et al.*, 2001). Black-pepper is reportedly used to treat pulmonary diseases (Ravindran, 2000), fever, cold, colic disorder and gastric ailments (Parmar *et al.*, 1997). Anti-spermatogenic and infertility effect in mice were reported by Mishra and Singh (2009).

5. PHYTOCHEMICAL SECONDARY METABOLITES STUDIES

Natural products are the main constituents of bioactive molecules and play a vital role in discovery of new compounds. The important properties of medicinal plants are due to the presence of various secondary metabolites such as alkaloids, glycosides, phenols, saponins, flavonoids, sterols, etc. The preliminary screening is useful in detection of bioactive principles which may subsequently lead to the drug discovery and development. *P. nigrum*, *P. betle* and *P. longum* are extensively cultivated in India. *P. nigrum* is regarded as king of Indian spice. *P. betel* contains various biologically active compounds whose concentration depends on the variety. Chemical compositions of essential oil constitute safrole present in leaf, stalk, stem and root and ß-phellandrene present in fruit. The aroma of *P.betle* leaf is due to the presence of essential oils, comprising of phenols and terpenes. Younger leaves reportedly yield more essential oil than mature leaves (Menon *et al.*, 2000). Leaf and other plant parts have yielded active compounds like hydroxychavicol, hydroxychavicol acetate, allypyro-catechol, chavibetol, piper-betol, methyl-piperbetol, piperol-A and piperol-B (Menon *et al*., 2003). The active ingredient of *P. betle* oil, obtained from leaves, are primary class of allyl benzene compounds, chavibetol (betle-phenol; 3-hydroxy-4-methoxyallylbenzene), chavicol (ρ-allylphenol; 4-allyl-phenol), estragole (ρ-allylanisole; 4-methoxy-allylbenzene), eugenol (allyl-guaiacol; 4-hydroxy-3-methoxy-allylbenzene; 2-methoxy-4-allyl-phenol), methyl eugenol (eugenol methyl ether; 3,-dimethoxy-allylbenzene) and hydroxycatechol (2,4-dihydroxy-allylbenzene) as reported earlier (Reshmi *et al*., 2010).

Piperine, the major active compound of black pepper, is closely related in structure to the known natural carcinogens - safrole, estragole and methylengenol which are also widely distributed in spices and plant oils. Piperine is a major alkaloid of other members of *Piperaceae* family like *P. longum*, *P. guineense*, etc. It is an important bioenhancer and can increase the bioavailability of substances including curcumin, selenium, β-carotene, amino acids and certain antibacterial compounds.

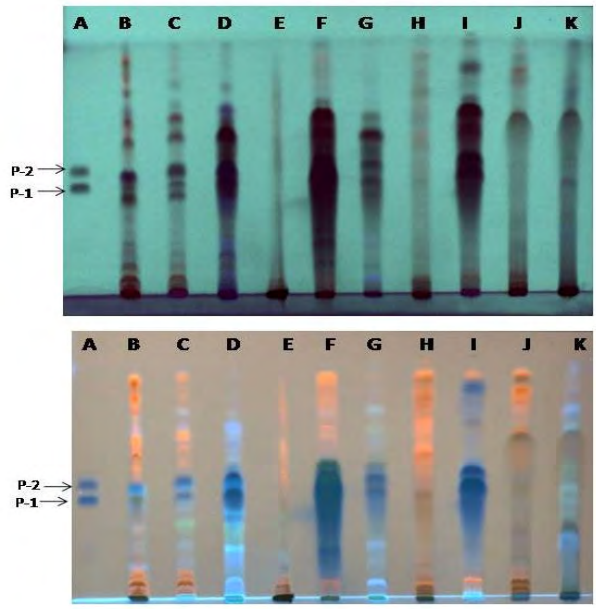

Fig. 4: TLC analysis of piperine content (P1 & P2) in leaf, root and fruit of different *Piper* species; A– standard piperine, B– *P. longum* (leaf extract), C- *P. longum* (fruit), D- *P. longum* (root), E- *P. nigrum* (leaf), F- *P. nigrum* (fruit), G- *P. nigrum* (root), H- *P. chaba* (leaf), I- *P. chaba* (fruit), J- *P. betle* (leaf), K- *P. betle* (root).

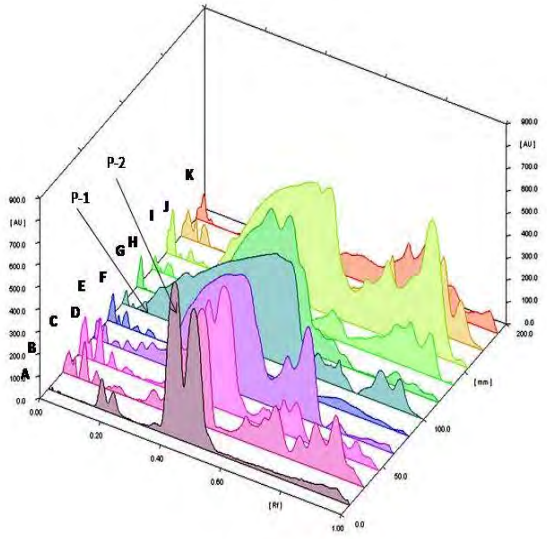

Fig. 5: Graphical representation of piperine content (P1 & P2) in leaf, root and fruit of different *Piper* species; A– standard piperine, B– *P. longum* (leaf extract), C- *P. longum* (fruit), D- *P. longum* (root), E- *P. nigrum* (leaf), F- *P. nigrum* (fruit), G- *P. nigrum* (root), H- *P. chaba* (leaf), I- *P. chaba* (fruit), J- *P. betle* (leaf), K- *P. betle* (root).

It also has anti-mycobacterial, anti-hyperlipidemic, anti-androgenic, immunoregulatory and anti-tumor properties. The fruits of *P. nigrum* contain 3-9% piperine on dry weight basis. The biologically important phytochemicals have been extracted from *P. nigrum* such as alkaloids, amides, propenyphenols, lignans, neolignans, dihydrochalcones, brachyamide, terpenes, steroid, kawapyrones, piperolides and chalcones, dihydropipericide, 3,4-dihydroxy-6(N-ethyamine), benzamide, (2E,4E)-neicosadienoyl pereridine, guineensine, piperamide, piperamine, piperettine, N-transferuloyltryamine, N-formyl-piperidine, pipericide, piperine, piperolein, trichostachine, sarmentine, sarmentosine, tricholein, retrofractamide (Bourgaud *et al.*, 2001). The major components of essential oil obtained from aerial parts of *P. nigrum* were gluulol, α-pinene, ß-caryophyllene and α-terpinene. Saraf and Saraf (2013) reported antimicrobial activity in the fruit extract of *P. longum*. They found various compounds including 6 essential oils, 3 bitter principles, 13 arbutin, 7 steroids, 4 coumarins, 4 alkaloids and 4 sterols. The piperine content examined in various species of piper through TLC and HPTLC analysis revealed variation from species to species and from explants to explants source. The roots had higher content of piperine as compared to leaves or stem of *P. longum* and *P. nigrum* (Fig. 4 & 5). The piperine content was higher in *P. chaba* (fruit) as compared to other species. However, piperine-2 content was more in *P. nigrum* (fruit) than in other species.

6. GENETIC TRANSFORMATION STUDIES

Efficient *in vitro* propagation strategy through somatic embryogenesis was used to enable genetic manipulations of this plant for specific traits including abiotic and biotic stress tolerance. Often, improvement of transgenic traits in plants is achieved by using specific promoters to accomplish high tissue specific protein production in transgenic plants. Scanty report is available on geneticengineering of *Piper* species (Varghese and Bhat (2011). A PCR-based suppression subtractive hybridization (SSH) technique was used to generate a leaf-specific subtracted cDNA

library of *P. nigrum*. Native promoters were known to mimic the expression of endogenous genes more effectively and did not affect by gene silencing (Alex *et al.*, 2008). SSH techniques have efficiently been used to identify many differentially expressed plant genes and also to generate equalized and enriched tissue-specific cDNA library (Alex *et al.*, 2008). One of the advantages of using SSH technique, which is highly efficient in removing the transcripts common to driver and tester population, is enriched expression of cDNA in subtracted population. Suppression subtractive hybridization was used to generate a defense gene enriched subtracted cDNA library successfully in resistant wild pepper. SSH was used to identify a set of genes differentially expressed in the leaves of *P. nigrum*, which could facilitate targeted engineering of valuable crop. The efficiency of subtraction was confirmed by PCR analysis using housekeeping gene actin (Abbasi *et al.*, 2010). On sequence analysis, almost 30% clones showed homology to metallothionein type-2 gene. The predominance of metallothionein transcripts in leaf was further confirmed using real-time PCR analysis and northern blotting. The possible role of metallothionein type-2 homologues in leaf has been discussed along with the feasibility of using SSH techniques for identification of more number of tissue-specific genes from *P. nigrum* (Alex *et al.*, 2008). Typically, plant metallothioneins are Cys-rich proteins and further categories such as type 1 and 2 have been designated on the basis of predicted location of Cys residues (Alex *et al.*, 2008). Metallothioneins type-2 transcripts have been detected primarily in mature leaves. High level expression of type-2 metallothioneins in leaves, especially in trichomes is correlated to the secretory function of trichomes to exude excess heavy metals accumulating preferentially in leaves (Garcia-Hernandez *et al.*, 1998) and leaf trichomes produce secretions supposedly provide a first line of defense against pests and pathogens (Wang *et al.*, 2002). A comparative study on the accumulation of Cd, Fe, Cu, Mn and Zn in commonly used tropical spices indicated that highest concentration of these metals in leaves and seeds of *P. nigrum* (Ozkuthu *et al.*, 2006). Varghese and Bhat (2011) developed an efficient *Agrobactertium*-mediated transformation of black pepper plants through somatic embryogenesis. Embryogenic mass developed from primary somatic embryos that were obtained from the micropylar region of mature germinating seeds of black pepper was found ideal tissue for genetic transformation. They observed that cefotaxime at 100 µg mL^{-1} was optimum to control *Agrobacterium* infection besides its ability to promote somatic embryo development. The transformants that survived

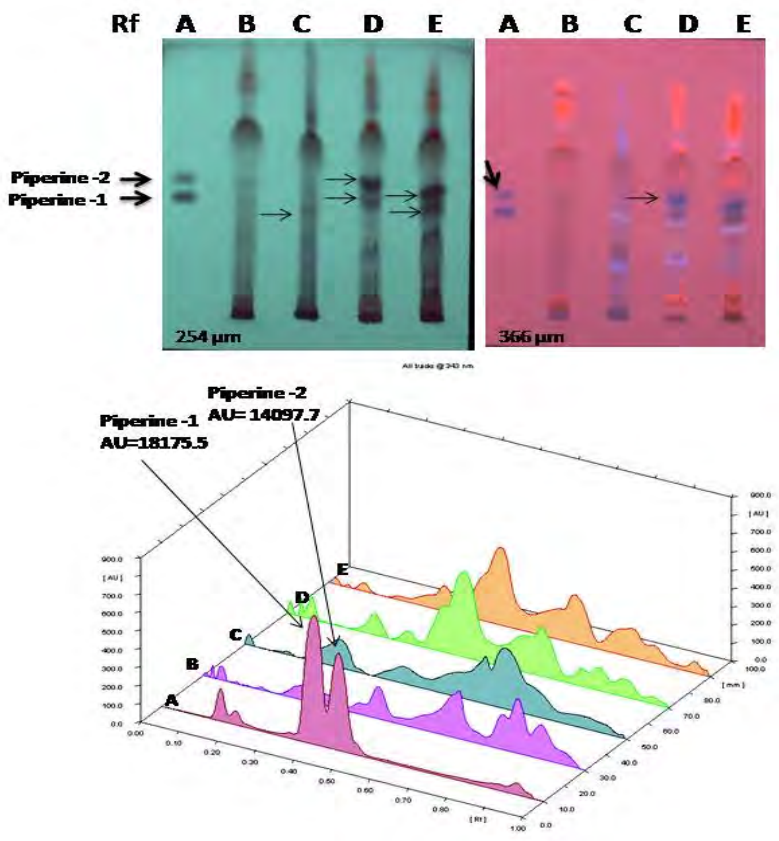

Fig. 6: Analysis of piperine content in leaf, root, transformed callus and non-transformed callus of *Piper betle* through TLC and HPLC analysis; A– standard of piperine 1 (AU = 18175.5) & piperine 2 (AU = 14097.7); B– leaf extract, C- root extract, D- transformed callus; E- non-transformed callus

Table 3: Comparison of piperine content in leaf, root, transformed and non-transformed calli of *Piper betle* [The data represent the mean value of two independent experiments**]**

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

in selection medium were hardened in greenhouse (Sasikumar and Vehuthambi, 1996). An average of 900 putative plantlets g^{-1} embryogenic mass were obtained. Screening of transgenic by PCR revealed some PCR-positive plants which were further tested by dot blot and Southern hybridization for expression. Dot blot analysis confirmed the presence of NPT II gene in 62 of the putative transgenic plants out of 74 plants screened (Sasikumar and Vehuthambi, 1996). Further, the study compared two secondary compounds piperine-1 and piperine-2, obtained from leaf and root, transformed and non-transformed callus extract of *Piper betle* with standard. TLC analysis showed the R_f values of piperine-1 and piperine-2 ranged from 0.39-0.40 and 0.42-0.46, respectively (Table 3). HPTLC analysis showed that the transformed callii (transformed with A4 strain) had higher piperine content (0.56%) with unit of 10187.4 AU as compared to the root, leaf and non-transformed calli extracts. The piperine-2 content was more in transformed calli extract than in non-transformed calli, leaf and stem extract. Piperine, the major active principle of black pepper, is closely related in structure to the known natural carcinogens-safrole, estragole and methylengenol which are also widely distributed in spices and plant oils.

Epilogue: The understanding of the basis of host target tissue infection with *Agrobacterium* and evaluation of strategies such as inclusion of anti-bactericidal compounds during *Agrobacterium*-infection process, adapting explants of appropriate physiological age from younger donor plants, and combining the critical factors affecting optimal T-DNA delivery could lead to the establishment of efficient *Agrobacterium*-mediated transformation system in aromatic plants. Selection strategies allow the recovery of transformed tissues expressing antibiotic resistance with relatively high efficiency. Studies are ongoing to extend this transformation technology to incorporate desirable genes into existing cultivars and begin the testing of functional genes.

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REFERENCES

Abbasi, B.H., Ahmad, N., Fazal, H. and Mahmood , T. 2010. Conventional and modern propagation activity. *Journal of Ethnopharmacology*, **70:** 343-349.

Agarwal, T., Singh, R., Shukla, A.D., Waris, I. and Gujrati, A. 2012. Comparative analysis of antibacterial activity of four *Piper betle* varieties. *Advances in Applied Science Research*, **3**: 698-705.

Ahmad, N., Abbasi, B.H., Rahman, I. and Fazal, H. 2013. *Piper nigrum*: Micropropagation, antioxidative enzyme activities, and chromatographic fingerprint analysis for quality control. *Applied Biochemistry and Biotechnology*, **169**: 204-215.

Alex, S.M., Dicto, J., Purushothama, M.G., and Manjula, S. 2008. Differential expression of metallothionein type-2 homologues in leaves and roots of black pepper (*Piper nigrum* L.) *Genetics and Molecular Biology*, **31**: 551-554.

Anand, A. and Rao, C.S. 2000. A rapid *in vitro* propagation protocol for *Piper barberi* gamble. A critically endangered plant. *In vitro Cellular and Developmental Biology - Plant*, **36**: 61-64.

Anupama, K., Cyriac, A., Saji, K.V. and Nirmal Babu, K. 2015. Microsatellite marker based cross species amplification and genetic diversity analysis in the genus piper. *International Journal of Advanced Research*, **3**: 184-191.

Babu, N.K., Lukose, R. and Ravindran, P.N. 1993. Tissue culture of tropical spices. pp. 257-267. **In**: *Proceedings of International Symposium on Genetic Engineering and Tissue Culture for Crop Pest and Disease Management* (ed. Vidhyasekharan). Daya Publishing House, New Delhi, India.

Bajad, S., Bedi, K.L., Singla, A.K. and Johri, R.K. 2001. Antidiarrhoeal activity of piperine in mice. *Planta Medica*, **67**: 284-287.

Bhat, S.R., Chandel, K.P.S. and Malik, S.K. 1995. Plant regeneration from various explants of cultivated *Piper* species. *Plant Cell Reports*, **14**: 398-402.

Bourgaud, F., Gravot, A., Milesi, S. and Gontier, E. 2001. Production of plant secondary metabolites: A historical perspective. *Plant Science*, **161**: 839-851.

Cheng, Y.Q., Xia, N.H. and Gilbert, M.G. 1999. Piperaceae. pp. 110-131. **In**: *Flora of China Vol. 4*. (eds. Z. Wu and P.H. Raven), Beijing Science Press, St Louis, USA.

Chowdhury, U., Tanti, B., Rethy, P. and Gajurel, P.R. 2014. Analysis of genetic diversity of certain species of *Piper* using RAPD-based molecular markers. *Applied Biochemistry and Biotechnol*ogy, **174**: 168-173.

El Hamas, R., Idaomar, M., Alonso-Moraga, A. and Muñoz, S.A. 2003. Antimutagenic properties of bell and black pepper. *Food and Chemical Toxicol*ogy, **41**: 41-47.

Essawi, T. and Srour, M. 2000. Screening of some Palestinian medicinal plants for antibacterial

Garcia-Hernandez, M., Murphy, A. and Taiz, L. 1998. Metallo-thioneins 1 and 2 have distinct but overlapping expression patterns in *Arabidopsis*. *Plant Physiology*, **118**: 387-397.

Gulcin, I. 2005. The antioxidant and radical scavenging activities of black pepper seeds. *International Journal of Food Science and Nutrition*, **56**: 491-499.
its antiproliferative activity. *African Journal of Pharmacy and Pharmacology*, **4**: 562-573.

Jiang, Y. and Liu, J.P. 2011. Analysis of genetic diversity of *Piper* spp. in Hainan island (China) using inter-simple sequence repeat ISSR markers. *African Journal of Biotechnology*, **10**: 14731-14737.

Joseph, L., Nazeem, P.A., Thampi, M.S., Philip, S. and Balachandran, M. 1996. *In vitro* techniques for mass multiplication of black pepper (*Piper nigrum* L.) and *ex vitro* performance of the plantlets. *Journal of Plant Breeding and Crop Science*, **24**: 511-516.

Joy, N, Abraham, Z. and Soniya E.V. 2007. A preliminary assessment of genetic relationships among agronomically important cultivars of black pepper. *BMC Genet*ics, **8**: 42.

Krishnakumar, S., Geetha, V.S. and Kuruvilla, A. 2001. Determination of local anesthetic action of betel leaf extract alone and with betel nut using infiltration and surface anesthesia. *Journal of Natural Remedies*, **1**: 28-32.

Lee, S.A., Hong, S.S., Han, X.H., Hwang, J.S., Oh, G.J., Lee, K.S., Lee M.K., Hwang B.Y. and Ro, J.S. 2005. Piperine from the fruits of *Piper longum* with inhibitory effect on monoamine oxidase and antidepressant-like activity. *Chemical and Pharmaceutical Bulletin (Tokyo)*, **53**: 832.-835.

Lupina, T. and Cripps, H. 1987. The photo isomers of *Piperine. Journal of Analytical Chemistry*, **70**: 112-113.

Mathews, V.H. and Rao P.S. 1984. *In vitro* responses in black pepper (*Piper nigrum*). *Current Science*, **53**: 183-186.

Menon, A.N., Padmakumari, K.P. and Jayaleksmy, A. 2003. Essential oil composition of four major cultivars of black pepper (*Piper nigrum* L.). III. *Journal of Essential Oil Research*, **15**: 155-157.

Menon, A.N., Padmakumari, K.P., Jayaleksmy, A., Gopalakrishnan, M. and Narayanan, C.S. 2000. Essential oil composition of four popular Indian cultivars of black pepper (*Piper nigrum* L.). *Journal of Essential Oil Research*, **12**: 431-434.

Mishra, R.K. and Singh, S.K. 2009. Antispermatogenic and antifertility effects of fruits of *Piper nigrum* L. in mice. *Indian Journal of Experimental Biology*, **47**: 706-714.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiology*, **15**: 473-497.

Nair, R.R. and Gupta, S.D. 2003. Somatic embryogenesis and plant regeneration in black pepper (*Piper nigrum* L.). *Journal of Horticultural Science and Biotechnol*ogy, **78**: 416-421.

Nair, R.R. and Gupta, S.D. 2005. Effect of explants and genotype on primary somatic embryogenesis in black pepper (*Piper nigrum* L.). *Cytologia*, **70**: 195-202.

Nair, R.R. and Gupta, S.D. 2006. High frequency plant regeneration through cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum* L.) *Plant Cell Reports*, **24**: 699-707.

Nazeem, P.A., Joseph, L., Geetha, C.K. and Nair, G.S. 1992. *In vitro* cloning of black pepper (*Piper nigrum* L.). *Journal of Plantation Crop*, **20**: 257-259.

Ozkuthu, F., Sekeroglu, N. and Kara, S.M. 2006. Monitoring of cadmium and micronutrients in spices commonly consumed in Turkey. *Research Journal of Agriculture and Biological Sciences*, **2**: 223-226.

Parmar, V.S., Jain, S.C., Bisht, K.S., Jain, R., Taneja, P., Jha, A., Tyagi, O.D., Prasad, A.K., Wenge, l.J., Olsen, C.E. and Boll, P.M. 1997. Phytochemistry of the genus *Piper*. *Phytochemistry*, **46**: 597-673.

Pathak, N. and Khandelwal, S. 2009. Immuno-momodulatory role of piperine in cadmium induced thymic atrophy and splenomegaly in mice. *Environmental Toxicology and Pharmacology*, **28**: 52-60.

Pathak, N. and Khandlewal, S. 2007. Cytoprotective and immunomodulating properties of piperine on murine splinocytes: An *in vitro* study. *European Journal of Pharmacol*ogy, **576**: 160.

Philip, V.J., Joseph, D., Triggs, G.S. and Dickinson, N.M. 1992 Micropropagation of black pepper (*Piper nigrum* L) through shoot tip culture. *Plant Cell Reports*, **12**: 41-44. Pradeepkumar, T., Karihaloo, J.L., Archak, S. and Baldev, A. 2003. Analysis of genetic diversity in *Piper nigrum* L. using RAPD markers. *Genetic Resources and Crop Evolution*, **50**: 469-475.

Rahiman, B.A. and Nair, M.K. 1983. Morphology, cytology and chemical constituents of some *Piper* species from India. *Journal of Plantation Crop*, **11**: 72-90.

Reshmi, S.K., Sathya, E. and Suganya Devi, P. 2010. Isolation of piperdine from *Piper nigrum* and

Rout, G.R., Samantary, S. and Das, P. 2000. *In vitro* manipulation and propagation of medicinal plants. *Biotechnology Advances*, **18**: 91-120.

Rubluo, A. and Barroso, A.L. 1992. *In vitro* morphogenetic responses and cytokinin-auxin interaction for callus production in pepper. *Anales del Instituto de Biologia Universidad Nacional Autonoma de Mexico Serie Botanica*, **63**: 195-201.

Saraf, A. and Saraf, A. 2013. Phytochemical and antimicrobial studies of medicinal plant *Piper longum* Linn. *International Journal of Pharmacognosy and Phytochemical Research*, **6**: 213-222.

Sasikumar, B. and Veluthambi, K. 1996. Transformation of black pepper (*Piper nigrum* L.) using *Agrobacterium* mediated Ti plasmid based vectors. *Indian Perfumer*, **40**: 13-16.

Scott, D. and Albert, L.L. 2005. Maize metallothionein promoter. *Patent Publication Number. WO/2005/063997*.

Scott, I.M., Jensen, H.R., Philogene, B.J.R. and Arnason, J.T. 2008. A review of *Piper* spp. (Piperaceae): Phytochemistry, insecticidal activity and mode of action. *Phytochemistry Rev*iew, **7**: 65-75.

Sencie-Tarazona, A., Rojas-Idrogo, C., Kato, M.J. and Delgado-Paredes, G.E. 2015. *In vitro* rapid clonal propagation in static liquid medium and acclimatization of *Piper solmsianum* and *P. tuberculatum*. *International Journal of Pure and Applied Bioscience*, **3**: 1-10.

Sherwin, W.B. and Moritz, C. 2000. Managing and monitoring genetic erosion. pp. 9-34. **In**: *Genetics, Demography and Viability of Fragmented Populations.* (eds. A.G. Youn and G.M. Clarke), Cambridge Press, United Kingdom.

Singburaudom, N. 2015. Hydroxychavicol from piper betel leave is an antifungal activity against plant pathogenic fungi. *Journal of Bioscience*, **8**: 82-92.

Singh, A., Kala, S., Kapoor, D.N., Gupta, R., Virk, A., Singh S. and Chaudhary, J. 2011. Effect on human sperm mitochondrial activity by *Piper betle* and *Calendula officinalis*. *Annals of Biological Research*, **2**: 622-627.

Sujatha, R., Babu L.C. and Nazeem, P.A. 2003. Histological of organogenesis from callus cultures of black pepper (*Piper nigrum* L.). *Journal of Tropical Agriculture*, **41**: 16-19.

Sunila, E.S. and Kuttan, G. 2004. Immunomodulatory and antitumor activity of *Piper longum* Linn. and piperine. *Journal of Ethnopharmacology*, **90**: 339-346.
techniques in *Piper nigrum. Journal of Medicinal Plants Research*, **4**: 7-12.

Thomas, T.V., Rema Shree, A.B., Nabeesa, E., Neelakandan, N. and Nandakumar, S. 2003. *In vitro* propagation of *Terminalia arjuna* Roxb. A multipurpose tree. *Plant Cell Biotechnology and Molecular Biology*, **4**: 95-98.

Uysal, H., Fu, Y.B., Kurt, O., Peterson, G.W., Diederichsen, A. and Kusters, P. 2010. Genetic diversity of cultivated flax (*Linum usitatissimum* L.) and its wild progenitor pale flax (*Linum bienne* Mill.) as revealed by ISSR markers. *Genetic Resources and Crop Evolution*, **57**: 1109-1119.

Varghese, J.M. and Bhat, A.I. 2011. An efficient *Agrobacterium*-mediated transformation protocol for black pepper (*Piper nigrum* L.) using embryogenic mass as explant. *Journal of Crop Science and Biotechnology*, **14**: 247-254.

Wang, W., Gan, S. and Wagnaer, G.J. 2002. Isolation and characterization of the CYP71D16 trichome-specific promoter from *Nicotiana tabacum* L. *Journal of Experimental Botany*, **53**: 1891-1897.

Zhang, L.J. and Dai, S.L. 2010. Genetic variation within and among populations of *Orychophragmus violaceus* (Cruciferae) in China as detected by ISSR analysis. *Genetic Resources and Crop Evolution*, **57**: 55-64.