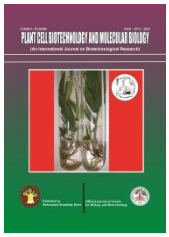

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# ‘Spicing up’ with Biotechnology: Trends and Developments in Black Pepper (*Piper nigrum*) Research

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***Authors’ contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

Black pepper, often referred to as “Black Gold” is primarily a culinary spice and is commonly used in traditional medicine. A large and diverse black pepper germplasm is available in and around the centre of origin, the tropical evergreen forests of the Western Ghats in India. As the full bearing age in black pepper is 6-7 years, the time and effort required to bring about a tangible trait improvement through conventional breeding is huge and it often takes decades for the development of a new variety. Needless to say, combining different traits like biotic and abiotic stress tolerance with industry-acceptable yield and quality traits, thus becomes a daunting task and a distant dream. Biotechnological interventions in plant breeding have proven their potential to facilitate speedy crop improvement. The present review provides a fair account of biotechnology research that happened in black pepper in recent times. It underlines the scope of biotechnological tools as ways and

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means to produce high-yielding, superior-quality and stress-resilient varieties. This article focuses on the studies in gene expression and regulation in black pepper during different stages of crop and environmental stimuli. Molecular insights from such studies are expected to contribute considerably to increase crop resilience and productivity.

*Keywords: Black pepper; biotechnology; whole genome sequencing; transcriptome; gene expression; cloning.*

## 1. INTRODUCTION

Spices have been a vital ingredient of food all over the world since time immemorial. Among the spices, black pepper (*Piper nigrum*, 2n=52) is the most widely used and commercially the most important one, aptly known as 'the king' of spices [1]. It was the primary spice in early trade between Europe and Asia. Its production, transportation and consumption dictated the international relationship for centuries. The crop originated from the pristine tropical evergreen forests of the Western Ghats of South India and is now cultivated primarily in Vietnam, Indonesia, Brazil, India, Sri Lanka, China, Malaysia and Cambodia [2]. In the year 2022, the world's total black pepper production was around 5,20,000 tones and exports were to the tune of 21,882 tones [3]. Black pepper is a perennial, woody climbing vine belonging to the family *Piperaceae*. In black pepper, dried berries are the economically important part. The typical pungency of the black pepper berries is due to the lysine-derived alkaloid Piperine and its flavour is attributed to volatile oils [1,4]. Black pepper mainly used as a spice to flavour food, also finds use as an ingredient in traditional medicines, preservatives and perfumery [5]. Piperine is reported to possess cytotoxic activity on tumor cell lines [6]. Moreover, its antipyretic, analgesic and anti-inflammatory activities are also shown to protect against chemical carcinogens [7]. Piperine is known to stimulate digestive enzymes in the stomach and thus enhance digestion [8]. Therefore, black pepper has been an important ingredient in food preparations in different parts of the world constituting a major share of the global spice trade of 12 billion USD (http://www.investindia.gov.in).

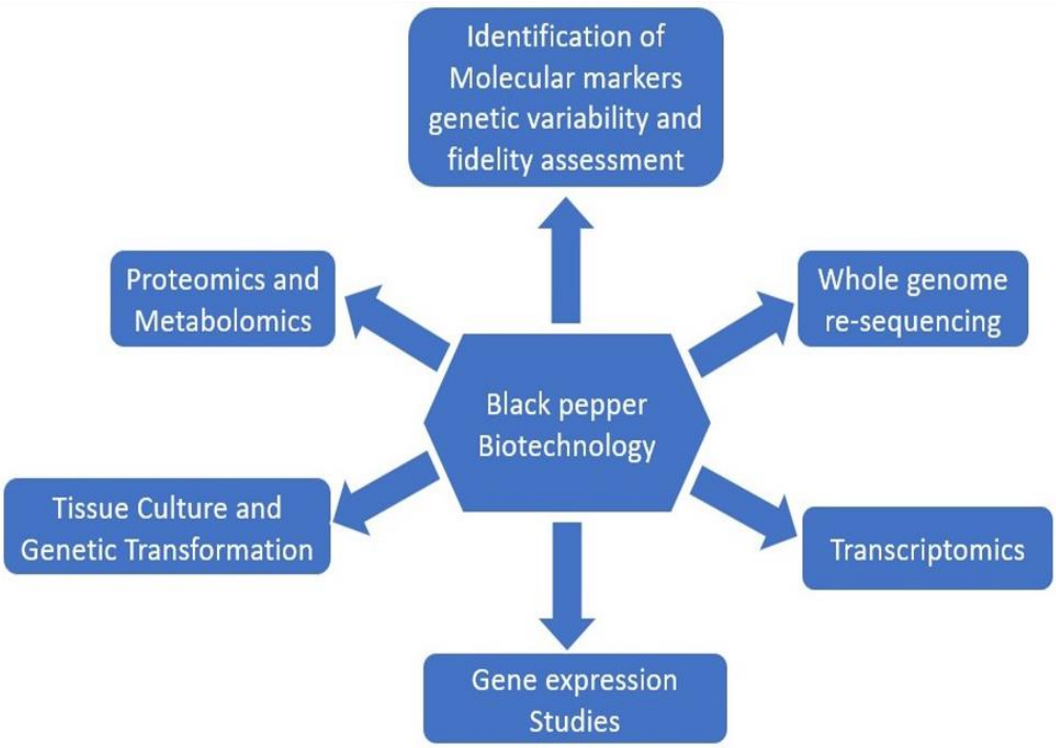


**Fig. 1. Different facets of biotechnology research in black pepper**

The available genetic diversity of black pepper in and around the Western Ghats, the centre of origin, remained largely untouched owing to the long breeding cycle of 10-20 years through conventional methods. Conventional breeding utilizes domestic crop cultivars and related genera as a source of genes for the improvement of existing cultivars, and this process involves the transfer of a set of genes from the donor to the recipient. Molecular breeding has proven potential for trait improvement in various crops, even though the effectiveness is dependent on genetic background and Quantitative Trait Loci (QTL) characteristics in question [9,10,11,12].

This review presents studies on various fields of biotechnology research in black pepper (Fig. 1) worldwide and details gene expression profiling and regulation under different contexts of internal and external stimuli.

## 2. MOLECULAR MARKER-BASED STUDIES

In black pepper molecular markers have been employed in diversity analysis, breeding, varietal identification and genotypic fidelity testing even though to a limited extent.

In black pepper there have been many attempts to use molecular markers for assessing the genetic diversity in germplasm from India [13,14], Hainan Island, China [15,16] as well as from different parts of the world [17] mainly using Expressed Sequence Tag-Simple Sequence Repeat (EST-SSR) and Randomly Amplified Polymorphic DNA (RAPD) markers. At the Indian Institute of Spices Research under the Indian Council of Agricultural Research at Kozhikode, India, 23 Piper species of Indian and exotic origins were differentiated using SSR marker-based genetic diversity analysis [18].

In a hybridization program involving black pepper genotypes as female parent (*P. nigrum* L.) and *Piper colubrinum* as male parent molecular markers like RAPD and SSRs were quite efficient in showing the species-level differences between parents and establishing the hybridity among the putative hybrids [19]. Negi et al*.,* identified polymorphic SSR markers in black pepper through genotyping-by-sequencing (GBS) approach. The polymorphic markers were obtained from 29 genotypes of black pepper, including wild relatives, germplasm accessions and released varieties. Based on these findings a web genomic resource BlackP2MSATdb (Black Pepper Polymorphic Microsatellite Database) was made available for aiding in molecular breeding, MAS, QTL identification and evolutionary studies in black pepper [20]. Marker-assisted breeding was sparingly visited due to constraints in mapping population development. However, a marker associated with resistance to *Phytophthora capsici* has been identified which can be used in marker-assisted breeding in black pepper [21].

Every crop improvement program relies on information about the genetic relatedness of varieties. In one study, many EST-SSR markers in black pepper have been developed and validated in about 35 varieties and cultivars of India [22]. In yet another study, about nine black pepper varieties of Indonesia were assessed for their phylogenetic relationship using RAPD and SSR markers [23]. Apart from these, DNA based markers like Inter Simple Sequence Repeat (ISSR), SSRs and barcodes were developed for varietal identification of 13 black pepper varieties of Indian origin (Fig. 2).

For the detection of plant-based adulterants and food authentication molecular marker-based method is more reliable, quick and easy when compared to conventional analytical methods like microscopy, spectrometry and Thin Layer Chromatography [24, 25, 26, 27]. In one such study, three barcoding loci viz., psbA-trnH, rbcL, rpoC1 were used to detect adulteration of traded black pepper powder [28]. Two-loci barcode approach was found to be robust in identifying *P. nigrum* from its related adulterant species belonging to *Piper galeatum* and *P. attenuatum*. The loci considered for this study were rbcL, matK, psbA-trnh and rpoC1 [29]. Markers like SSRs and RAPDs are employed in genetic fidelity testing of tissue culture-raised plants as well. In black pepper, genetic fidelity testing using SSR markers on somatic embryo-derived plants showed genetic uniformity among themselves and with the explant [30].

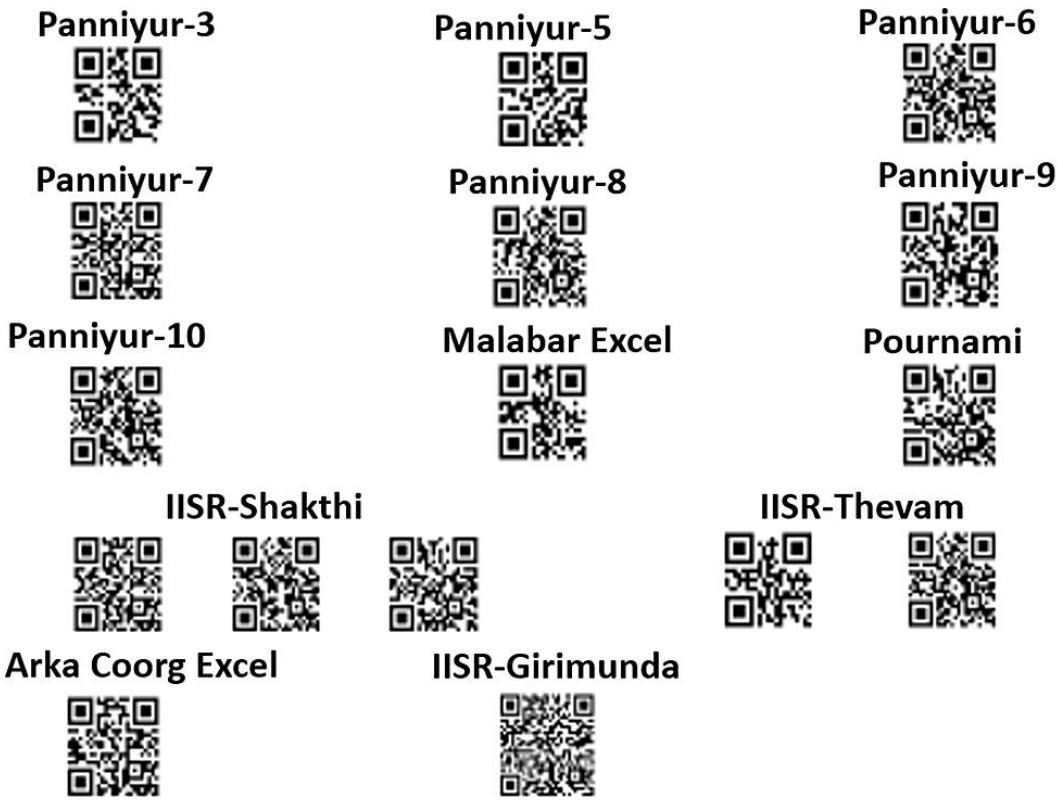


**Fig. 2. Barcodes generated for different varieties based on polymorphic markers**

## 3. GENE EXPRESSION STUDIES

### 3.1 Flower and Fruit Transcriptomes

Advances in next generation sequencing (NGS) technology have made transcriptomic studies very popular among researchers. Comparative transcriptomics facilitates the identification of a considerable number of molecular players acting during a particular condition an organism is exposed to, such as biotic and abiotic stress or developmental stage. A comparative transcriptomic study between three different black pepper varieties, Semengok Aman, Kuching and Semengok having different fruit characteristics provides valuable insights into gene regulation of flower and fruit development in black pepper. In this study, numerous carbohydrate metabolism-related genes were differentially expressed and analysis suggests that these genes are developmentally regulated. This study was extended to Piperine biosynthesis genes and suggested that the transportation of lysine-derived products happens only in the early stages of fruit development [31]. The genes involved in the biosynthesis of piperine and other bioactive compounds unravelled in such studies are valuable candidates for enhancing the medicinal properties of black pepper through genetic manipulation. Another fruit transcriptome analysis of black pepper has identified many Simple Sequence Repeats (SSRs), lysine/ornithine related genes and housekeeping genes [32].

### 3.2 Root Transcriptome

Root transcriptome provides a better understanding of water and nutrient uptake mechanisms and their use efficiency characteristics. An early study using NGS SOLiD platform sequenced the root transcriptome of *P. nigrum* and identified 615 candidate genes which most likely define the plant's root pattern. This analysis also identified many SSRs that would be useful in developing genetic panels suitable for root characteristics improvement in black pepper [33].

### 3.3 Phytophthora Resistance

Foot rot is a devastating disease of black pepper caused by *P. capsici*. A comparative transcriptome analysis of the Phytophthora-resistant species *Piper flaviflorum* and a susceptible species *P. nigrum* cv. Reyin-1 provided insights on foot rot susceptibility

mechanisms in black pepper. This study also revealed the upregulation of the phenylpropanoid pathway genes involved in secondary metabolite production during *P. capsici* infection [34].

Several studies on the gene expression patterns during Phytophthora infection have been taken up in black pepper. A report by Suraby et al, identified upregulation of Resistance Gene Analogs (RGAs) during Phytophthora infection in two moderately resistant (IISR Sakthi and 04-P24-1) and one susceptible (Subhakara) black pepper genotypes. The identified RGAs of black pepper belonged to the non-TIR R gene class having NBS motifs [35].

Differential expression of three important genes encoding pathogenesis-related proteins (PR proteins) viz. β-1,3-glucanase (PR-2), Osmotin (PR-5) and Cytosolic ascorbate peroxidase (cAPX, PR-9) were carried out in Phytophthora susceptible (Sreekara) and resistant (04-P24) black pepper lines compared to un-inoculated plants using qPCR. The expression of β-1, 3-glucanase and Osmotin genes showed higher levels on 5 DAI, whereas the cAPX gene expression was high on 3 Days After Inoculation (DAI). *P. capsici* soil inoculation also affected the transcriptional activity of these genes in black pepper stem tissue giving vital proof for the induction of Systemic Acquired Resistance (SAR) against the pathogen [36].

Genotype-specific differential expression of PR protein and R genes in resistance and susceptible varieties of black pepper along with a genome-wide survey in *P. nigrum* identified eleven PR-1 gene homologs that mapped to different genome scaffolds. Black pepper transcriptome from plants infected with *P. capsici* showed the expression of PR-1 genes from all the mapped loci. A thorough in silico analysis also revealed cis-regulatory elements such as transcription activator binding sites, phytohormone responsive sites, etc. in the promoter regions [37].

## 3.4 Flavour Pathways

A transcriptomic study using Illumina platform has helped researchers to identify Terpene synthases (TPS) and the subsequent tBLAST searches using amorpha-4,11-diene synthase sequence as a query identified 19 sesquiterpene synthases (sesqui-TPSs). Terpene synthases (TPS) are the family of enzymes which are responsible for the characteristic flavour of black pepper [38].

In a report by Schnabel et al., the piperine synthase gene (piperoyl-CoA:piperidinepiperoyl transferase) belonging to the BAHD-type of acyltransferases family was reported based on a differential RNA-Seq analysis of immature black pepper fruits [39]. Following that a fruit-specific cytochrome P450 enzyme that catalyzes the formation of the 3,4-methylenedioxy group in the aromatic part of the piperine molecule was identified based on the RNA sequencing data from different black pepper organs and through gene expression studies in yeast [40].

In black pepper immature fruits and leaves, several piperoyl-CoA ligases capable of converting piperic acid to piperoyl-coenzyme A (piperoyl-CoA) have now been described. Piperine synthase has been identified as an important enzyme in the piperine biosynthesis pathway owing to its differential expression in flowers, fruits and leaves, with the highest expression in young fruits [41].

In another study, the black pepper berry transcriptome revealed the presence of the entire terpene synthase family genes responsible for the biosynthesis of the flavour-imparting volatiles in black pepper berries. About 98 terpene synthase genes belonging to various terpene synthesis pathways were identified by sequencing and three important monoterpene synthases were further validated [42]. In a cross-species comparative transcriptome study of fully mature berries from *P. nigrum* and *Piper longum* by Illumina and nanopore sequencing platforms, identified gene families belonging to Piperine and other secondary metabolite biosynthetic pathways [43]. De novo transcriptome of unripe green berries of *P. nigrum* presents the details of phenylpropanoid biosynthesis in immature berries. As the bioactive compounds found in unripe berries are used in Ayurvedic medicines, understanding the regulatory framework of the biosynthesis of these bioactive compounds could lead to the development of high-quality custom-designed varieties for Ayurvedapharmaceuticals and also facilitate the production of such biomolecules through metabolic engineering.

## 3.5 Drought-Responsive Studies

Drought has a tremendous impact on crop production in black pepper. Negi et al., identified 4914 differentially expressed genes from

drought-affected black pepper through high-throughput transcriptome sequencing of leaves by Illumina Hiseq 2000 platform. The study also identified 2110 transcription factors involved in drought tolerance [44]. Earlier studies on expression analysis of drought-responsive genes in black pepper showed that genes like dehydrin and osmotin were upregulated and aquaporin and the transcription factor bZIP genes were downregulated [45].

## 4. STUDIES ON GENE REGULATION

A critical step towards understanding the molecular basis of any trait is to study the molecular events in the context of gene regulation. In plants, small RNAs (sRNAs) such as miRNAs are crucial in gene regulation during growth, development and environmental stress [46,47,48,49]. In black pepper, initial in silico analysis suggested that miR166 and miR171, as well as their targets, are evolutionarily conserved [50]. Later 303 conserved miRNA families were identified from the black pepper sRNAome data and from these, eight were found to be differentially expressed through stem-loop qRT-PCR experiments [51].

The presence of SSRs in miRNAs is a relatively less studied aspect of miRNA mediated gene regulation. Analysis of SSRs in miRNA genes from 171 organisms revealed the presence of Splice Regulatory Element (SRE), near the SSRs in pre-miRNA sequences, suggesting their putative role in the regulation of alternate splicing [52]. In black pepper, primary-miRNA transcripts having (CT) dinucleotide SSRs were identified using SMART strategy [53]. De novo leaf transcriptome analysis also led to the discovery of miRNA candidates harbouring microsatellites. The analysis revealed that about 0.033% of the black pepper transcriptome constituted 'pre-miRNA candidates bearing SSRs' [54].

It was found that some tRNA-derived sRNAs play a vital role in conferring plant immunity [55]. The tRNA-derived fragments (tRFs) are also found to be associated with argonaute proteins, which are vital for the RNA silencing mechanism to function [56]. The up-regulation of tRF during stress conditions such as pathogen infection [57] and drought [58] has been reported in plants. The predominance of the 5′tRFs in Phytophthora-infected black pepper leaf and root was revealed by a high-throughput analysis of the small RNAome (sRNAome). The presence of 5′tRFs in high numbers in the sRNAome and their potential targets being defense-responsive genes, points towards their regulatory role during stress response in black pepper [59].

According to Asha et al., high-throughput studies have identified the existence of highly expressed unique rRNA-derived fragments (rRFs) from the 5' terminus of 5.8S rRNA. These small RNAs are specific to the Piperaceae family and have shown differential expression and cleavage during Phytophthora infection. In addition to its role during pathogen stress the particular small RNA has regulatory roles in the RNAi pathway and has the potential to carry out taxonomic profiling [60].

## 5. MOLECULAR BIOLOGY INVESTIGATIONS

Piperine and phenolamide in black pepper have numerous applications in traditional and current pharmacological interventions [61,62]. A bioinformatic analysis of genes associated with Piperine Biosynthetic pathway has identified 19 sesquiterpene synthase genes (sesqui-TPSs) of which three (PnTPS1 – PnTPS2) have been cloned, expressed and characterized. Based on in vitro enzyme assays, TPS1 was identified as caryophyllene synthase (PnCPS) PnTPS2 as cadinol/cadinene synthase (PnCO/CDS) and PnTPS3 as germacrene D synthase (PnGDS) [38]. A cloned piperic acid CoA ligase gene produced a putative precursor of piperine with high substrate specificity to piperic acid (41). Later, two genes PipBAHD1 and PipBAHD2 were cloned and characterized after a transcriptomic study by the same group [39]. Cloning and characterization of genes from black pepper gained momentum only in recent times, whereas the protocol of genetic transformation in black pepper was standardized using Agrobacterium-mediated method and GUS marker constructs years back [63,64]. From the black pepper variety IISR Sakthi, 23 ORFs having high similarity to RGAs Resistance Gene Analogs have been cloned [35].

Proteomics is a revolutionary field in molecular biology and only a miniscule of proteomics research has been carried out in black pepper. Transcriptome-assisted label-free quantitative proteomics strategy was used to study the molecular response of black pepper to *P. capsici* infection. Certain unique proteins were found to be differentially expressed in inoculated tissues after black pepper, among which oxidoreductase were the most populated category of proteins expressed during Phytophtora infection [65]. In

another study, the label-free quantitative proteomics method brought out crucial evidence for the systemic response induced by *Trichoderma harzianum* in the black pepper phytopathosystem [66]. Knowledge of antimicrobial peptides (AMPs) from black pepper has also been unravelled through proteomic AMP signature profiling experiments [67].

## 6. WHOLE GENOME SEQUENCING OF BLACK PEPPER

Reference genome assembly of black pepper was reported by integrating different NGS platforms [68]. The assembled genome was organized into 26 pseudo chromosomes having about 55% repetitive sequences. Around 5082 transcription factors identified belong to 75 gene families accounting for 8% of the protein coding genes. Berry-specific genes like Lysine decarboxylase (LDC) genes involved in lysine metabolism; Glycosyl transferase (GTF), Cytochrome P450 (CYP) and Hydroxycinnamoyl transferase (HCT) genes in the phenylpropanoid pathway, BAHD acyl transferase (BAHD-AT) and serine carboxypeptidase-like acyltransferases (SCPL-AT) genes were also discovered. The particular study provides valuable leads that may serve as a foundation for future research on piperine biosynthesis and Piperales taxonomy, leading to a better understanding of the evolution, phytochemistry and ecology of the Piper genus.


**Fig. 3. Germplasm accessions of black pepper maintained at ICAR-Indian Institute of Spices Research**

To assess the genetic diversity and underlying genetic structure and also for identification of trait linked markers, 39 accessions of black pepper with various traits taken from the germplasm collection maintained at ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India were identified and the genomic DNA was extracted and sent for whole genome re-sequencing. This includes released varieties (21 Nos.), farmers' varieties (2 Nos.), wild *P. nigrum* and *P. colubrinum* (3 Nos.). Unique genotypes for traits like high piperine, bold berry, long spike, Phytophthora resistance, berry shape, etc. were also shortlisted and used for re-sequencing (Fig. 3). The screening of the resultant sequences with bioinformatic tools led to the identification of 27,657,631 high-confidence molecular markers which consisted of 8,139,898 SSRs, 17,665,899 SNPs and 1,851,834 InDels in total for all the 39 accessions (Sheeja et al, un published data).

Kumari et al., sequenced the draft genome of the black pepper (*P. nigrum*) using the Illumina, IRYS and PacBio sequencing platforms. As a result, the first NGS based genomic SSRs in black pepper was brought out and this indeed serves as a valuable resource for genetics and plant breeding studies, genetic map construction, QTL identification, etc. The draft genome of black pepper comprised 916 scaffolds with genome coverage of 80X. The sequence information was used for genome-wide mining and characterization of SSRs in black pepper. The study reported a total of 69,126 SSRs from the assembled genomic sequence of *P. nigrum*. From this, a small subset consisting of 85 SSRs was further tested for cross-amplification in nine Piper species [69].

The chloroplast genome of black pepper deciphered recently was found to be 161,522 bp in size, showing a quadripartite structure with both a large single copy (LSC) region (89,153 bp) and a small single copy (SSC) region (18,255 bp); both these regions were separated by inverted repeats (IRs), of size 27,057 bp in length. The genome consists of 131 genes of which 81 were protein-coding genes, 37 were tRNAs, 4 were rRNAs, and 1 was a pseudogene [70].

## 7. CONCLUSION

Biotechnology of black pepper gained attention with molecular breeding efforts and progressed to the development of protocols for micropropagation, somatic embryogenesis, *In vitro* conservation, protoplast isolation and genetic transformation. The demand for disease-free planting materials and the need for in vitro conservation of germplasm has led to the development of tissue culture protocols for the regeneration of black pepper during the late 90s itself [71].

In recent times, high-throughput sequencing experiments have yielded a large repertoire of molecular information on the differentially modulated genes under different spatiotemporal and environmental conditions.

In concert with marker-assisted plant breeding practices, faster methods of genetic manipulation such as genetic engineering and gene editing that genetically alter crop performance is a commendable contribution of biotechnology [71,72]. Genetic engineering involves the delivery and integration of defined genes or genomic regions into plant cells and the expression of such genes, while gene editing allows genetic modifications without transgene integration, tremendously reducing the hurdles for biosafety compliance [73]. Both the techniques have potential to enhance the varietal development process by bypassing the year-long process of crossing and selection through either conventional or marker-assisted methods. Black pepper, being a high-value crop, presents a huge scope for exploitation by molecular breeding as well as fast-evolving gene editing tools.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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