ALLELIC DIFFERENCE IN THE PUTATIVE GENE *ipk1* SEQUENCE AND PHYTIC ACID (INSP6) CONTENT IN BLACK PEPPER (*Piper nigrum* L.)

By

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(2017-11-079)

THESIS

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DEPARTMENT OF PLANT BREEDING AND GENETICS

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KERALA, INDIA

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DECLARATION

I, hereby declare that this thesis entitled **"Allelic difference in the putative gene *ipk1* sequence and phytic acid (InsP6) content in 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 of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Padannakkad
Date: 12/12/'19

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CERTIFICATE

Certified that this thesis, entitled **"Allelic difference in the putative gene *ipk1* sequence and phytic acid (InsP6) content in black pepper (*Piper nigrum* L.)"** is a record of research work done independently by Ms. Gladish Mary Joy (2017-11-079) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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4

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Gladish Mary Joy

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Percentage
°C	Degree celcius
viz.,	Namely
et al.	Co- workers/ Co-authors
Fig.	Figure
g	Gram
i.e.	that is
cDNA	Complementary deoxyribonucleic acid
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxy ribonucleic acid
dNTP	Deoxyribo nucleoside triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed Sequence Tags
GW	Genome Walking
HPLC	High performance liquid chromatography
IMP	Inositol monophosphate phosphatase
Ins	Inositol/ myo inositol
InsP1	Inositol monophosphate
InsP2	Inositol biphosphate
InsP3	Inositol triphosphate
InsP4	Inositol tetraphosphate
InsP5	Inositol pentakisphosphate
InsP6	Inositol hexakisphosphate
IPK1	Inositol pentakisphosphate 2- kinase
IPK2	Inositol pentakisphosphate 3/6- kinase
Kb	Kilobase
kD	Kilodalton
mA	Milli ampere
Mg	Milligram
Min	Minute
MIK	Myo inositol kinase
MIPS	D- *myo* inositol 3 phosphate synthase

Ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information
Ng	Nanogram
NGS	Next generation sequencing
nmole	Nanomole
ORF	Open reading frame
PA	Phytic acid
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pi	Inorganic phosphorus
PVP	Polyvinyl pyrrolidone
RAPD	Random Amplified Polymorphic DNA
RCA	Rolling circle amplification
RNAi	RNA interference
Rpm	Rotation per minute
rRNA	Ribosomal ribonucleic acid
Sec	Second
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TEMED	Tetramethyl ethylenediamine
Tm	Melting temperature
UTR	Untranslated region
V	Voltage
WGA	Whole genome amplification
µg	Micro gram
µl	Micro litre
µM	Micro molar
KAU	Kerala Agricultural University

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.) is one of the oldest and most sought after valuable spice in the world. Due to its immense potential and worldwide importance, it has been titled as 'King of Spices'. It is a tropical flowering vine with Western Ghats as centre of origin. India is one of the leading producers of black pepper in the world followed by Vietnam and Indonesia, accounting for 53,000 tons. The major producers in India are Kerala, Karnataka, Konkan region of Karnataka and Tamil Nadu. Black pepper is the product of dried and matured berries of *Piper nigrum*. Since black pepper is a widely traded crop across the world with huge demand and medicinal values with anticancer, antioxidant, anti-inflammatory and antimicrobial properties, more molecular analysis and genome studies are a requisite for enhancing the crop improvement. The present study involves the sequencing of a putative gene controlling phytic acid synthesis in black pepper and the difference between black pepper genotypes with respect to gene and phytic acid content.

Phytic acid or inositol 1,2,3,4,5,6 hexakisphosphate is the main storage form of phosphorus in cereals, legumes and nuts. Phytic acid is found to be the most abundant form of myo-inositol phosphate in plants. Phytic acid is present in the form of calcium, potassium and magnesium salts, mainly occurring in the aleurone layers of cereals and legumes (Reddy *et al.*, 1982). York *et al.* (1999) has reported that phytic acid is a positive regulator for mRNA transport. The lower polyphospho inositols also have a major role in cell vesicular trafficking, signalling and gene expression (Shi *et al.*, 2005). It also helps in controlling the concentration of inorganic phosphorus present in seeds and seedlings (Strother, 1980) and it is the major storage of phosphorus and myo-inositol for making it available during germination of seeds. Phytic acid has also got many advantageous effects in human health; helps in reduction of glucose level in blood, prevention of cancer and elimination of renal calculi (Lee *et al.*, 2006; Vucenik and Shamsuddin, 2006; Saw *et al.*, 2007).

However, anti-nutritional effects of phytic acid have been reported in various studies. Its chelating effect of binding minerals, protein and ions are found to cause negative effects in organisms. Phytic acid also results in the excretion of phosphorus by ruminant animals, which eventually results in environmental pollutions (Oatway *et al.*, 2001). To minimize the deleterious effects caused by phytic acid, many researchers are trying to identify the genes involved in the biosynthesis of phytic acid and to control the gene expression to eventually produce low-phytic acid mutants. However, extreme reduction in phytic acid content affects the plant development and defense mechanism also. So a better understanding of the genes involved is essential. Till now, the biosynthetic pathway and the major genes involved in phytic acid synthesis are not fully understood and still needs to be explored in various crops. The estimation of phytic acid and other polyphosphoinositols has also need to be done for screening and determining low phytate genotypes. So more works have to be carried out to identify the genes involved and the role played in phytic acid biosynthesis pathway in different crops.

Next generation sequencing methods such as whole genome sequencing methods are greatly helpful in sequencing and studying of plant and animal genomes. Genome walking is a stepwise strategy for finding out the unknown flanking regions of a known sequence. Genome walking procedures are of various kinds such as inverse PCR, ligation mediated PCR, vectorette PCR and panhandle PCR which require restriction and ligation processes. However, there are many limitations including requirement of available restriction sites and more amount of starting material. There are other genome walking strategies which do not require restriction and ligation *viz.*, universal fast walking, targeted gene walking etc. (Guo and Xiong, 2006). A high throughput method utilizing rolling circle amplification (RCA) mechanism with the help of Phi 29 DNA polymerase enzyme reported by Reddy *et al.* (2008) was employed to amplify the whole genome by attachment of walker adaptor primers randomly to the genomic DNA. Directional genome walking performed with walker primers and locus specific

and nested locus specific primers are used to amplify and decipher the unknown 5' and 3' flanking regions of a known gene.

Keeping in consideration of huge economic and medicinal importance of black pepper and the need for understanding of biosynthesis pathway of phytic acid, the present study was undertaken to decipher the unknown flanking region towards 5' end of a unigene *Pnc135* obtained from Expressed Sequence Tags (EST) (Unpublished data, Sujatha, R). When gene ontology studies were conducted, this gene was found to be similar with the putative gene *ipk1* encoding for inositol pentakisphosphate-2-kinase enzyme which is involved in the biosynthetic pathway of inositol 1,2,3,4,5,6-hexakisphosphate or phytic acid (IP6). This inositol pentakisphosphate-2-kinase enzyme is responsible for the phosphorylation of inositol pentakisphosphate to phytic acid (IP6). Later the unigene *pnc135* was partially sequenced and the gene fragment was named as *pnipk1* (1072 bp) by Giridhari (2017). However, only the 3'end was sequenced. Therefore the present study entitled "Allelic difference in the putative gene *ipk1* sequence and phytic acid (InsP6) content in black pepper (*Piper nigrum* L.)" was undertaken during 2017-19 at College of Agriculture, Padanakkad with the following objectives:

- To sequence the 5' region of the putative *ipk1* gene coding for inositol-pentakisphosphate 2- kinase involved in phytic acid biosynthesis in black pepper.
- To screen black pepper genotypes (selected 10 varieties) for allelic difference in the putative *ipk1* gene sequence and estimate the phytic acid content in the selected black pepper varieties.

Review of literature

2. REVIEW OF LITERATURE

2.1 BLACK PEPPER - BLACK GOLD

Black pepper (*Piper nigrum* L.) is a perennial woody climber belongs to the family Piperaceae. It is an important culinary spice crop cultivated for its berries. The records of black pepper history start with that of humankind. Black pepper was believed to be of use in afterlife and therefore it was kept aside of the mummified body of Pharaoh in ancient Egypt. Spices especially the black pepper has been mentioned in the holy books such as Bible, Quran and Vedas. Pepper, as an important ingredient in unique medical formulations, has also been cited in some of the ancient Indian medical texts such as Ashtangahridaya and Samhitas (Nair, 2004).

Black pepper is very well known for its economic and medical importance, thereby known as 'King of Spices'. Centre of origin of this crop is Western Ghats in South Western India. Till the early 19th century, India was the only country growing it (Sim, 1986). Even now, India is one of the largest producers of black pepper after Vietnam and Indonesia. Major states extensively producing black pepper in India are Kerala, Karnataka and Tamil Nadu.

Black pepper is the most extensively traded spice worldwide. The crop is known as 'Black Gold' because of its high demand and value in global market. It has high therapeutic value due to the presence of certain bioactive compounds. Presence of an active ingredient 'Piperine' makes it useful as volatile oil, oleoresins and alkaloids; and also found helping in chemoprevention and controlling tumour growth (Butt *et al.*, 2013).

2.2 BOTANY OF PEPPER

Black pepper is a spreading vine with stem attached to the supports with the help of adventitious roots. Leaves are green, arranged alternatively, oblong, lanceolate, base unequally cordate, with pronounced auricle, and tips are acuminate, 3–4 pairs of lateral ribs arise right from the base. The inflorescence of

black pepper is a catkin-like spike bearing 50-120 flowers (Sim, 1986). Flowers in black pepper may exist as male, female and hermaphrodite flowers. Bracts are peltate, orbicular, glabrous and pedicellate. Flowers are laterally fused with 3-4 stamens, a single carpel with obovate ovary, where style is absent, stigma 3–4 lobed, short and papillate.

The cultivated *P. nigrum* is monoecious, while majority of the wild ones possess dioecious flowers. Pepper is a self-pollinated crop and its pollen dispersal occurs either by rain or dewdrops or through the process of geitonogamy. Outcrossing is ineffective due to the poor pollen transfer mechanism in pepper (Nair, 2004). The fruits are drupes produced in spike, fused laterally, spicy and pungent. Seeds of black pepper are very small. Matured fruits will be yellowish red with a single seed inside and the dried fruits are called as peppercorns.

2.3 GENETIC VARIATION IN BLACK PEPPER

Linnaeus (1753) was the first to introduce genus Piper in his book *Species Plantarum* and identified around 17 species in the Piper family belonging to the same genus. The origin of the genus name Piper is from Greek name for black pepper, *Peperi*. Black pepper belongs to the family Piperacea of the Magnoliid subclass, commonly termed as the basal angiosperms. The genomic data available for such a big family with paramount genetic diversity is very little (Gordo *et al.*, 2012). A high level of polymorphism was detected among cultivars to assess the genetic variation in black pepper.

Joy *et al.* (2011) studied forty genotypes and four different species with Simple Sequence Repeat (SSR) markres and reported that pepper genotypes are highly diverse in both morphologically and genetically. The genus *Piper* comprises about 3000 diverse species of herbs, shrubs and climbers of which 110 are of Indian origin. Sheeja *et al.* (2013) studied 27 *Piper* spp. and classified into six clusters in the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram with the help of Inter Simple Sequence Repeat (ISSR) marker system.

Pradeepkumar *et al.* (2003) characterized 22 cultivars of *P. nigrum* from South India and one accession each of *P. longum* and *P. colubrinum* using RAPD marker sysytem. Twenty four primers generated 372 RAPD markers of which 367 were polymorphic. Jaccrad's similarity between *P. nigrum* cultivars ranged between 0.20 and 0.66; and mean was 0.42. Landraces exhibited larger divergence than advanced cultivars. Landraces grown in southern part of coastal India were grouped in separate clusters of dendrogram. Aimpiriyan, Balankotta, Cheriakaniakadan, Cholamundi, Dodiga, Kalluvally, Karimunda, Malligeswara and Panchami were the landraces studied.

Screening for drought tolerance and transcriptome analysis for identification of differentially expressed up-regulated and down-regulated genes in selected genotypes of black pepper [PRS 64 (Angamaly), PRS 44 (Panniyur-1), PRS 115 (Panniyur-5), PRS 155 (wild type), PRS149 (wild type), PRS160 (hybrid), PRS161 (hybrid), PRS 153(Panniyur-8), PRS156 (*Piper attenuatum*), Karimunda] showed varied response to physiological parameters. PRS 64 (Angamaly) expressed high drought tolerance whereas Panniyur1 showed the least drought tolerance for the same (Pallavi, 2015). When tested with EST-SSR markers, the different varieties of black pepper showed similarity and grouped *in* same clusters, whereas cultivars of black pepper were grouped in another cluster that shows diversity among black pepper genotypes (Jose, 2017).

Wu *et al.* (2016) was the first to study the genetic diversity of black pepper genotypes using EST-SSR markers. A total of 148 accessions were studied and high level of polymorphism was identified in every EST-SSR markers giving an average polymorphism information content of 0.93 indicating a complex genetic background and large genetic diversity in black pepper germplasm.

Sen *et al.* (2010) analysed genetic diversity in eight species of *Piper* using RAPD marker sysytem and reported a total of 149 RAPD fragments, among which 12 (8.05 %) were monomorphic and 137 (91.95 %) were polymorphic with an average gene diversity of 0.33 across all species. Genetic diversity analysis of

seven high yielding lines of black pepper was done using RAPD and morphological features were studied, where primers in the OPKm line exhibited maximum unique bands and lines OPKm, HP-1411 and HP-105 showed distinct morphological features (Sreedevi *et al.*, 2005).

2.4 GENOME WALKING

Genome walking is a method for determining a flanking unknown region like promoter regions from a known region in cloned or uncloned genomic DNA. Mainly there are three PCR based genome walking methods. Genome walking is an efficient method for sequencing of regions of DNA and helps in genome sequencing starting from a known sequence area into an unknown flanking region (Shapter *et al.*, 2014).

Three types of genome walking methods include Restriction-based genome walking method, Primer-based genome walking method and Extension-based genome walking method. In restriction based GW methods, as the name suggests, the genomic DNA is cut into several fragments using restriction enzymes and the fragments are later ligated to DNA cassettes. In primer based GW methods, random generate primers along with specific primers are used for various PCR amplifications. In extension based GW method, the extension of a sequence specific primer occurs and along the same time 3'-tailing of the resulting single strand DNA (ssDNA) will be used as the substrate for further PCR amplifications.

Genome walking methods with the help of next generation sequencing (NGS) have great role in making various useful applications in functional genomics and it may replace classic techniques such as microarray hybridization. It is also found to have immense potential in medicine for various investigations into the integration processes of retrovirus and DNA vector for gene therapy (Volpicella *et al.*, 2012).

Leoni *et al.* (2010) has employed genome walking method for sequencing the flanking regions of cDNA coding for the light harvesting protein Lhcb1 gene in spinach. A genome walking method called universal fast walking (UFW) method was practised which is independent of restriction enzymes and ligations (Myrick and Gelbart, 2002).

2.5 ROLLING CIRCLE AMPLIFICATION

Nelson *et al.* (2002) generated many templates of high quality for DNA sequencing with TempliPhi™ kits that make use of phi 29 DNA polymerase enzyme and rolling circle amplification. RCA is an isothermal process where heating and cooling cycles are avoided to prevent the degradation of biomolecules during this heat and cool cycles in PCR amplifications (Mohsen and Kool, 2016). Guo and Xiong (2006) developed a genome walking method with partially degenerated primers (walker primer) and nested specific primers based on nested PCR technique to amplify the unknown flanking regions of a known sequence in the genomic loci of two highly divergent photosynthetic organisms, *Rhodobacter capsulatus* and *Heliophilum fasciatum*.

A method for genome walking based on rolling circle mode of DNA synthesis by Phi29 DNA polymerase and PCR was developed by Reddy *et al.* (2008) in order to find the unknown flanking regions of putative gene sequences; and is an easy and cost effective method compared to other genome walking tools. RCA is an efficient isothermal amplification to develop several diagnostic methods and to construct multivalent molecular scaffolds and nanostructures in fields *viz.* biology, diagnostics and therapeutics (Ali *et al.*, 2014). Haible *et al.* (2006) reported that RCA with phi 29 DNA polymerase along with restriction fragment length polymorphism (RFLP) is a better and cost effective method than PCR, since no primers allowed reliable a diagnosis of geminiviruses and in all the viruses having small single-stranded circular DNA genome.

RCA method can be used to automatically produce DNA hydrogels with stable horse-radish peroxidise (HRP) like catalytic functions (Huang *et al.*, 2017). RCA is a simple approach for isolation of full length cDNAs, high throughput

isolation of transcripts, isolation of genes and simultaneous isolation of both ends of a transcript (Polidoros *et al.*, 2006). RCA method of whole genome amplification has also helped in the isolation and characterisation of various microorganisms such as viruses and for various vector constructs (Ramesh, 2012).

Johne *et al.* (2009) also reported that RCA technique can be used for efficient amplification of circular DNA viral genomes without the requirement of specific primers. RCA method has already been used for the amplification of six virus families and can now extend this robust technique for whole genome amplification of other virus families. Phi 29 DNA polymerase mediated RCA method can be extensively used as an important tool in virology and has got several applications like virus detection of known virus species, detection of unknown viruses, whole genome analysis of viruses that could not be well propagated through tissue culture methods, detection and isolation of infectious genomic clones directly from clinical specimens and differentiation between linear and circular forms.

RCA method can also be employed in identification of fungal species (Tehrani *et al.*, 2014). Complete sequencing of a tenth human polyomavirus which was found in skin of humans infected with a rare genetic disorder known as warts, hypogammaglobulinemia infections, and myelokathexis (WHIM) syndrome. Rector *et al.* (2004) were the first to put forth the use of RCA with phi 29 DNA polymerase for the identification of viral genomes. Multiply primed RCA technique is a powerful application for detecting the circular DNA of viruses which are not known yet since the requirement of specific primers are not needed (Stevens *et al.*, 2010).

A new sensitive technique for early identification of prognostic markers has been developed through RCA coupled with nicking endonuclease-assisted signal amplification (NESA) and detection of p53 DNA is done using the personal glucose meter (PGM) which has got immense applications in clinical diagnostics and biomedical research (Jia *et al.*, 2019). Dean *et al.* (2001)

employed the method of RCA for amplifying the vectors M 13 and plasmid vectors from single colonies or plaques. It was suggested that multiply primed RCA is not a time consuming, but simple confering high quality template for use in DNA sequencing, probe generation and cloning.

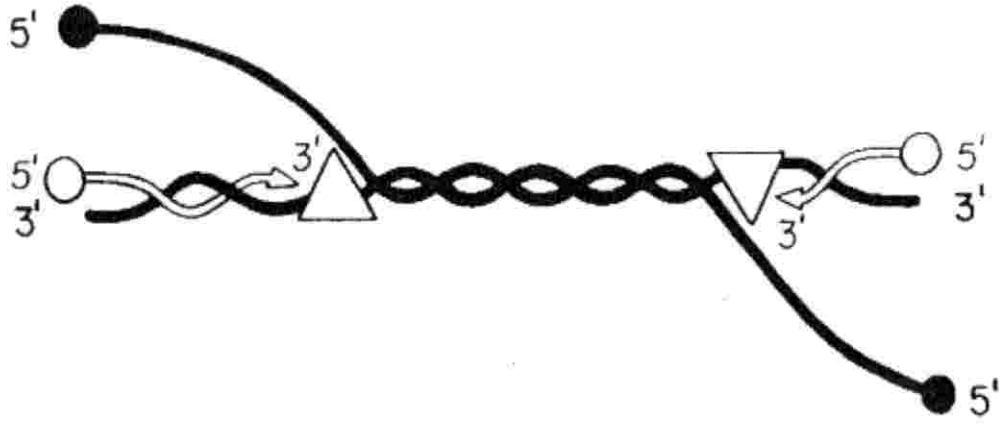
2.6 PHI 29 DNA POLYMERASE ENZYME

Phi 29 DNA polymerase enzyme is a DNA polymerase enzyme used in genome replication in some bacteriophages. These bacteriophages are found to have inverted repeats at terminal ends with an average length between 6 to 300 nucleotides. These polymerases are included in the T4 family of DNA polymerases and they have high 3'-5' exonuclease activity. Phi 29 DNA polymerases can be useful for DNA sequencing with their capability to recognize the site of terminating agents such as dideoxynucleotide and can be well exploited for determining a nucleotide base sequence of DNA molecule (Blanco *et al.*, 1993).

Taq DNA polymerases lack some activities which may be often the limiting causes in genome amplification whereas Phi 29 DNA polymerases possess some special features such as high fidelity and high processivity (Silander and Sareela, 2008). The strand displacement activity of Phi 29 DNA polymerase can be efficiently utilized for multiple displacement amplification for whole genomic amplification and genomic analysis (Dean *et al.*, 2002). Studies conducted by Blanco *et al.* (1989) revealed that two proteins namely the 429 terminal protein p3, which plays a role as a primer for initiation, and the viral DNA polymerase p2 are required for complete and efficient replication of both DNA strands of a double helix. The two remarkable features of Phi 29 DNA polymerases like processivity and multiple strand displacement confers this 66 kD DNA polymerase its high catalytic efficiency. The enzyme also posseses unwinding properties without any need of helical unwinding proteins and even in absence of rNTP breakdown. The Phi 29 DNA polymerase has got a symmetric model of replication unlike other polymerases due to the presence of two

replication origins at both the ends of duplex DNA chromosome which allows a continuous replication.

SYMMETRIC MODEL

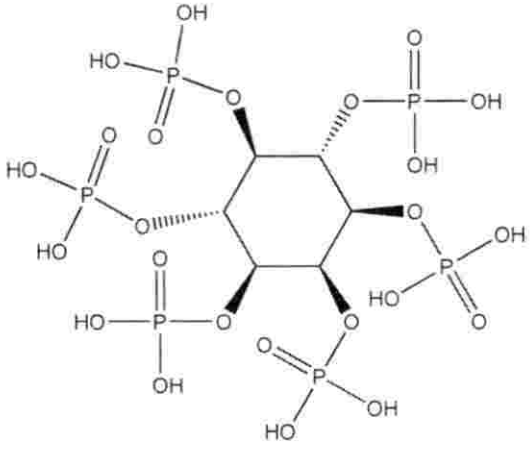


(Blanco *et al.*, 1989)

2.7 PHYTIC ACID

Phytic acid is a cyclic compound (1,2,3,4,5,6 hexakis dihydrogen phosphate myoinositol). Phytic acid is major source of phosphorus, inositol, and cations in cereals, nuts, legumes, and vegetables seeds (Vats and Banerjee, 2004). Phytic acid has got high affinity for metal ions such as calcium, zinc, and iron due to the negative charge it carries at a wide range of pH values. Phytic acid occurs as calcium – magnesium salt in seeds and is a major form of phosphorus typically accounting for 60-90 per cent of total phosphorus, mainly constituting 1-3 per cent in plant seeds (Graf, 1983). Johnson and Tate (1969) determined the molecular structure of phytic acid. Phytate is a major storage for inositol, phosphate, K, Mg, Ca, Mn, Fe and Zn for use by the seedling (Batten and Lott, 1986).

PHYTIC ACID



(Guerrero *et al.*, 2016)

Sasakawa *et al.* (1995) studied the metabolic pathways of InsP5 and InsP6 and recognized their role as precursors for rapidly metabolized inositol pyrophosphates. It was also reported that unique housekeeping functions in iron chelating, modulating receptor sensitivity and internalization. InsP6 promotes dynamine 1- mediated endocytosis in pancreatic ß cells and helps in both activation of protein kinase C and inhibition of phosphoinositide phosphatase for phosphatidylinositol 4,5 – biphosphate formation.

2.8 PECULARITIES OF PHYTIC ACID

Phytic acid is a major source of phosphorus in seeds and present in protein bodies as globoid inclusions (Prattley and Stanley, 1982). Phytic acid functions as a potent antioxidant for seed preservation and can even lower the occurrence of various human disorders like colonic cancer and inflammation by chelating iron and suppressing iron catalysing oxidative reactions (Graf and Eaton, 1993; Graf *et al.*, 1987). Phytate is also present as mixed cation salt of phytic acid in seed thereby functioning as a vital mineral storage compound and is necessary for grain growth and seedling development (Lott *et al.*, 2000).

Phytates perform a vital role in plants, acting as an energy source. Raboy (2003) reported that InsP6 can function as an effector or ligand in insphosphate

and pyrophosphate pathways involved in signalling and regulation. Chlieh *et al.* (2000) reported that InsP6 can prevent the inhibition of $Ik_{i,in}$ through chelating with Ca^{2+} and plays a major role in physiological response in guard cells to ABA. Macbeth *et al.* (2005) reported that IP6 are conserved in some adenosine deaminase that act on transfer RNA (tRNA) and have a major role in *in vivo* and *in vitro* deamination of adenosine 37 of $tRNA^{ala}$ by ADATI.

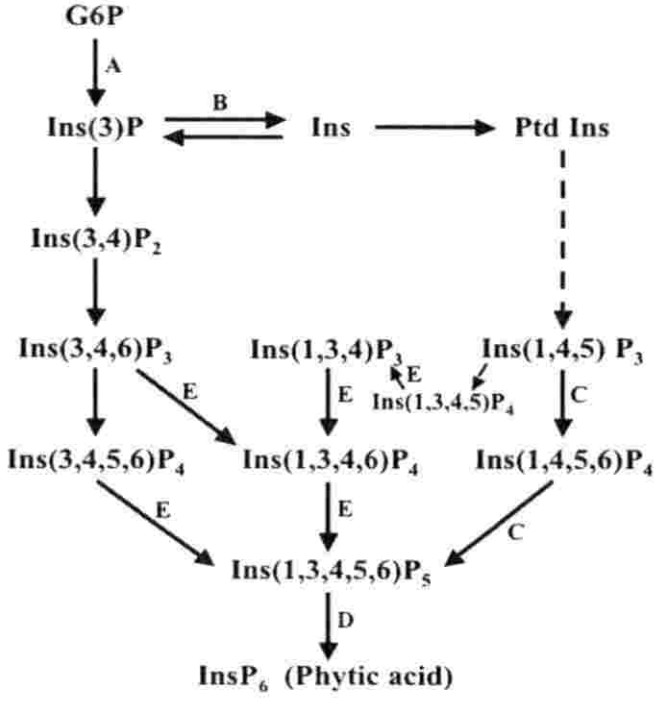
Through microdialysis technique, phytic acid was proved to be a potent inhibitor of iron catalyzed hydroxyl radical (•OH) generation induced by ischemia-reperfusion in rat hearts due to its iron chelating ability (Obata and Nakashima, 2016). Phytic acid helps in chemoprevention of cancer by preventing the formation of highly reactive species from hydrogen peroxide (H_2O_2) by chelating transition metal ions (Midorikawa *et al.*, 2001). Phytic acid acts as a potent absorption enhancer of flavinoid components and influences the integrity of tight junction (TJ) in $CaCO_2$ cell monolayers. A concentration dependant attenuation in the dispersal of tight junction proteins like occludin, ZO-1 and claudin-1 will occur due to the phytic acid activity. Phytic acid helps in opening the tight junction by downregulating proteins and finally the integrity of $CaCO_2$ cell monolayers is lowered (Fu *et al.*, 2015).

Lee *et al.* (2006) performed a study to examine the effect of phytate on the blood glucose level in mice and they found that mices treated with phytates in their diet showed a decreased blood glucose level suggesting that phytate supplements in diets of diabetic patients will help to treat hyperglycaemic like conditions. Administration of phytic acid has enhanced immunity and antioxidant effects and it also reduces the chances of occurrences of cancer in humans (Vucenik and Shamsuddin, 2006). The chelating effect and the extent to which chelation occurs by phytic acid can be determined by the pH during food processing and digestion in gastrointestinal tract since the degradation of phytic acid and generation of lower inositol phosphates ($InsP_{5-3}$) from phytate is dependent upon pH (Sandberg and Scheers, 2016)

York *et al.* (1999) studied a yeast mutant deficient in a factor Gle 1 p, associated with the nuclear pore complex (NPC) and their studies concluded that IP6 played a major role in GLE 1 function and mRNA report. Jenab and Thompson (1998) studied five groups of azoxymethane-treated male Fischer 344 rats and the rats were given a basal control diet (BD) or BD supplemented with either 25% wheat bran (WB), 25% dephytinized WB (DWB), 25 % DWB plus 1.0 % PA or 1.0 % PA for 100 days. The studies revealed that it was plausible for phytic acid in wheat bran to lower early biomarkers of colon cancer risk.

2.9 INSP6 PATHWAY

Phytic acid was first found and reported as a plentiful phosphorus containing compound and referred as phytic acid (Cosgrove, 1980). In higher plants, two parallel types of phytic acid metabolic pathway have been proposed of which one is an inositol lipid-independent pathway and the other is phospholipase C (PLC)-mediated pathway (Brearley and Hanke, 1996; Stevenson-Paulik *et al.*, 2002; Raboy, 2003).



(Suzuki *et al.*, 2007)

The first one occurs through the sequential phosphorylation of 1D-myo-inositol 3-phosphate (Ins(3)P) and in the latter inositol 1,4,5-tris-phosphate (Ins(1,4,5)P3) is sequentially phosphorylated to InsP6. Twelve genes coding for enzymes that could help in the metabolic pathway of phytic acid were identified in rice and the enzymes involved in the pathway were 1D-myo-inositol 3-phosphate synthase (MIPS), inositol monophosphatase (IMP), inositol 1,4,5-trisphosphate kinase/ inositol polyphosphate kinase (IPK2), inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IPK1), and inositol 1,3,4-triskisphosphate 5/6-kinase (ITP5/6K). It was also concluded that four genes, a *MIPS*, an *IPK1*, and two *ITP5/6K*s played important role in phytic acid biosynthesis in the embryo and therefore in developing seeds the lipid-independent pathway mainly occurs. It was also found that genes *MIPS*, an *IMP*, an *IPK2*, and an *ITP5/6K* in anthers are involved in PLC-mediated pathway (Suzuki *et al.*, 2007).

Ives *et al.* (2000) characterized the biochemical and functional parameters of recombinant *scIpk1* gene encoding for 2-kinase enzyme in *Saccharomyces cerevisiae* and reported that production of IP6 predominately occurs through phosphorylation of inositol 1,3,4,5,6 – pentakisphosphate (IP5) by the 2-kinase.

Phytic acid metabolic pathway involving myo-inositol phosphates and glucose-6-phosphate during germination and formation of seeds helps in the generation of ATP and lowering power during the early stages of germination (Biswas *et al.*, 1984). Kuwano *et al.* (2009) has reported in rice that the amount of soluble inorganic phosphate (Pi) can be brought down by the manipulation of the expression of the rice Ins(3)P1 synthase gene *RINO1* using transgenic method. They transformed *RINO1* gene into the rice plants in the antisense orientation. *GluB 1* promoter of rice major protein storage gene was used and analysed Pi and phytic acid in samples with ion chromatographic method and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) to determine the expression patterns of *RINO1* and *GluB 1*.

Total phosphorus in seed was increased by increasing the InsP6 level in transgenic rice through the overexpression of *Ospgk1* gene, which plays a key role in the conversion step from Insp1 to Insp2. It was also observed that overexpression of *Ospgk1* gene has effect on other phytic acid related genes such as *OsMIK, OsITPK2, OsITPK3, OsITPK4*, and *OsITPK6*. On the contrary, total phosphorus was not increased by the influence of first and last steps of InsP6 biosynthetic pathway (Tagashira *et al.*, 2015).

2.10 *IPK1* GENE

Synthesis of inositol 1,2,3,4,5,6-hexakisphosphate (IP_6), also known as phytate, is carried out mainly through phosphorylation of inositol 1,3,4,5,6-pentakisphosphate (IP_5) by inositol 1,3,4,5,6-pentakisphosphate 2-kinase enzyme. *IPK 1* is having role in this stage of phytic acid metabolism. Verbsky *et al.* (2002) reported a human genomic DNA sequence on chromosome 9 encoding for human inositol 1,3,4,5,6-pentakisphosphate 2-kinase (InsP5 2-kinase) based on several studies conducted on conserved sequence motifs among five Ipk1 proteins from different fungal species. Their studies have shown that the expression of human InsP5 2-kinase mRNA generally occur in brain, heart, placenta, and testis. Sun *et al.* (2007) isolated and characterized *ipk1* cDNA and genomic clones from maize and identified a small gene family with two duplicate members ZmIPK1A and ZmIPK1B encoding Ins(1,3,4,5,6) P5 -2 kinase.

Shi *et al.* (2003) identified that a mutation in *Zmipk* gene can greatly reduce the phytic acid content in maize. It was proved that *ZmIpk* gene plays an important role in phytic acid biosynthesis in developing maize seeds and it was also found that *Zmipk* mRNA was expressed in embryo where phytic acid is mostly get accumulated. All these findings combinely makes it plausible that *Zmipk* was responsible for the phosphorylation of Ins(3)P to InsP6.

2.11 PHYTIC ACID AND ITS MYO INOSITOL DERIVATIVES

Lower inositol polyphosphate derivatives are the catabolites of phytic acid. These lower polyphosphates include myoinositol monophosphates, myoinositol

biphosphates, myoinositol triphosphates, myoinositol tetrakisphosphates and myoinositol pentakisphosphates.

Sl. No.	InsPn	Inositol phosphate
1	InsP	Ins(1)P, Ins(2)P, Ins(3)P, Ins(4)P
2	InsP2	Ins(1,2)P2, Ins(1,4)P2, Ins(3,4)P2, Ins(4,5)P2
3	InsP3	Ins(1,2,3)P3, Ins(1,2,6)P3, Ins(1,4,5)P3, Ins(1,5,6)P3, Ins(3,4,6)P3
4	InsP4	Ins(1,2,3,6)P4, Ins(1,2,5,6)P4, Ins(1,3,4,5)P4, Ins(1,4,5,6)P4, Ins(3,4,5,6)P4
5	InsP5	Ins(1,2,3,4,6)P5, Ins(1,2,3,5,6)P5, Ins(1,2,4,5,6)P5, Ins(1,3,4,5,6)P5

(Frank, 2013)

2.11.1 MYOINOSITOL MONOPHOSPHATES

A total of six monophosphates are reported in which four are important as plant metabolites. Its chemical formula is OP $(O)(OH)_2$. The paths for the synthesis of different monophosphates are different. They are mainly found in carrot, lucerne, tobacco, rice, wheat, Madagascar periwinkle, cell suspension cultures, in apple buds etc. (Frank, 2013).

2.11.2 MYOINOSITOL BIPHOSPHATES

Fifteen myoinositol biphosphates are identified in which four are relevant plant metabolites and the chemical formula for biphosphates is $OP(O)(OH)_2$. These are mainly found in barley aleurone tissue, tobacco, lucerne, pea flour, mesophyll cells etc. The synthesis of myo-inositol biphosphates occurs through phosphorylation of D-2,3:5,6-di-O-cyclohexylidene-myoinositol with diphenyl phosphorochloridate or by partial dephosphorylation of D-Ins(1,3,4)P3 with alkaline phosphatise and similar another metabolic pathways (Frank, 2013).

2.11.3 MYOINOSITOL TRIPHOSPHATES

Out of 20 myoinositol triphosphates identified, 5 are important plant metabolites. They mainly occur in barley aleurone tissue, cauliflower hypocotyls, carnation flower petals, apple buds and lucerne. Phosphorylation of 4,5,6-tri-O-benzoyl-myo-inositol with dibenzyl N,N-diisopropylphosphoramidite or by phosphorylation of D-2,3,6-tri-O-benzyl-myo-inositol or by phosphorylation of 1D-5-O-acetyl-1,2-O-cyclohexylidene-myo-inositol with O-xylene or of D-1,2,5-tri-O-benzylmyo-inositol with di-O-benzyl N,N-diisopropylphosphoramidite in the presence of 1H-tetrazole are the different metabolic pathways observed for synthesis of myoinositol triphosphates (Frank, 2013).

2.11.4 MYOINOSITOL TETRAKISPHOSPHATES

Fifteen different types of myoinositol tetrakisphosphates are so far identified. Among the fifteen reported, five are important and they are myoinositol tetrakisphosphates, D-myo-Inositol 1,2,3,6-tetrakisphosphate (Ins(1,2,3,6)P4), D-myo-Inositol 1,2,5,6-Tetrakisphosphate (Ins(1,2,5,6)P4), D-myo-Inositol 1,4,5,6-Tetrakisphosphate (Ins(1,4,5,6)P4) and D-myo-Inositol 3,4,5,6-Tetrakisphosphate (Ins(3,4,5,6)P4). They are mainly found in barley aleurone tissue, pea flour and duckweed etc. The main metabolic pathways of different tetrakisphosphates are partial dephosphorylation of phytic acid with phytase, by phosphorylation of D()-2,6-di-O-benzyl-myo-inositol with dibenzyl N,N0–diisopropylphosphoramidite or by enzymatic phosphorylation of D-Ins(1,4,5)P3 with Ins(1,4,5)P3 kinase (Frank, 2013).

2.11.5 MYOINOSITOL PENTAKISPHOSPHATES

Myoinositol pentakisphosphates are found more in soybean seeds, mungbeans and aleurone tissues of barley. So far fifteen different myoinositol pentakisphosphates are reported. Various pathways for the synthesis of pentakisphosphates are enzymatic hydrolysis of phytic acid with wheat bran phytase or wheat germ phosphatise or by the reaction between N-

benzoylphosphoramidic acid with DL-4-O-benzyl-myo-inositol and DL-3-O-benzyl-myo-inositol (Frank, 2013).

2.12 LOW PHYTIC ACID MUTANTS

Phytic acid has some negative impacts on plants and animals despite of its usefulness. Therefore, plants with low phytic acid are a desirable goal for genetic improvement by plant breeders. Guttieri *et al.* (2004) identified a non-lethal mutant from 562 ethyl-methanesulfonate (EMS) mutagenized lines of wheat (*Triticum aestivum* L.) which expressed a great reduction in phytic acid and the inorganic phosphate amount was increased without no drastic change in total seed phosphorus. Phytic acid content was greatly reduced from 74.7 per cent of wild type or non-mutant to 48.2 per cent in seeds of mutated wheat.

Larson *et al.* (2000) identified the inheritance, isolation and genetic mapping of first *ipa1* genes and characterized mutants of rice that may interrupt the functions of *myo inositol 1-phosphate synthase (MIPS) gene.* Two mutants, one lethal and the other one a non-lethal were identified in rice. The phytic acid content in seeds of *ipa1-1* was reduced from 71 to 39 per cent and the inorganic phosphate was increased from 5 to 32 per cent of total phosphorus content. Shi *et al.* (2005) reported a mutant in maize *ipa 3*, Mu- insertion mutant and it was observed that phytic acid was less produced in this mutants with no intermediate myoinositol derivatives but with high myoinositol accumulation. *Ipa3* encoded for Inositol kinase protein which belongs to pfkb carbohydrate kinase family, revealing the direct involvement of myoinositol and MIK in phytic acid biosynthesis in developing seeds.

2.13 PAGE

Earlier the amount of phytic aicd was determined by nonspecific methods such as extraction of the food/feed by hydrochloric acid (HCl) and subsequent precipitation of ferric iron phytate in dilute acid solution. Later Association of Analytical Chemists (AOAC), the extraction with hydrochloric acid (HCl) and anion exchange chromatography on AGI resin for purification and stepwise

elution with NaCl was put forward. The disadvantage of these methods is that these methods show a lack of specificity. With the advancement of ion-exchange high-performance liquid chromatography (IEX-HPLC), quantitative determination of phytate and some of its degradation products (InsP6–InsP3) has now become possible. After that isomeric-specific high-performance ion-exchange liquid chromatography (HILC) methods using gradient elution and post column reaction with $Fe3^{+}$ ions and UV detection was introduced for the determination of phytic acid specifically. These HILC methods have got wide range of applications in food and nutrition research. Estimation of phytic acid can also be accomplished by HPIC-mass spectrometry (MS), inductively coupled plasma-MS (ICP-MS), or ICP-atomic emission spectrometry (ICP-AES) (Sandberg and Scheers, 2016).

High performance liquid chromatography (HPLC) method is used to detect accurate quantity of phytate content in plants, but this method is complicated. A simple, reliable and cost effective method for the quantification of InsP6 in plants was recently developed using polyacrylamide gel electrophoresis (PAGE) (Alimohammadi *et al.*, 2013; Losito *et al.*, 2009; Giridhari *et al.*, 2017). Tiselius (1938) was the first to propose electrophoretic method for detection of proteins. It was known as moving boundary electorphoresis. The non-denaturing polyacrylamide gel electrophoresis was later proposed by Davis and Ornstein, (1959), which later on became a widely spread method for protein quantification.

Chrambach *et al.* (1971) developed a new general strategy, Polyacrylamide Gel Electrophoresis (PAGE), for isolation of macromolecules and macromolecule mapping. Loening (1967) reported that PAGE is an efficient technique with greater resolution for the fractionation of RNA and separated 4s and 5s RNA in 5 per cent and 7.5 per cent gels respectively.

Materials and Methods

3. MATERIALS AND METHODS

The present investigation on "Allelic difference in the putative gene *ipk1* sequence and phytic acid (InsP6) content in black pepper (*Piper nigrum* L.)" was carried out at the Department of Plant Biotechnology, College of Agriculture, Padannakkad, during 2017-2019. Details regarding the materials used and methodologies followed for various experiments in the study are given below.

3.1 MATERIALS

3.1.1 Plant materials

The plant materials used for the study included Panniyur 1 for whole genome amplification and directional genome walking. The validation of the sequence and the phytic acid estimation were done using the following 10 different cultivars, (in addition to Panniyur 1), which show polymorphism at molecular and morphological level based on previous reports (Table 1).

Table 1: List of black pepper genotypes selected for *ipk1* gene sequence analysis

Sl. No.	Variety
1	Panniyur 5
2	Panniyur 7
3	Chettanvally
4	Kottanadan
5	Karimunda 7
6	PRS 160
7	Chumala
8	Thotamundy
9	Karimunda kuttyatur
10	Payyanganam 2

Young fresh leaves and berries of variety Panniyur 1 for DNA isolation phytic acid estimation, respectively, were collected from the 'Hi-Tech Pepper Nursery' maintained under the Department of Plant Biotechnology, College of Agriculture, Padannakkad. The leaves and berries from remaining 10 varieties were collected from Pepper Research Station, Panniyur and taken to the laboratory in icebox and used for isolation of genomic DNA and estimation of phytic acid.

3.1.2 Laboratory chemicals

All standard chemicals and the chemicals for DNA isolation, agarose gel electrophoresis and phytic acid extraction and PAGE were of analytical/ molecular biology grade and were purchased from Merck India Ltd, Bengaluru and SRL Laboratories, Mumbai.

The specific chemicals used for PCR reaction such as 3U Taq DNA polymerase, 10x PCR buffer (A) with $MgCl_2$ were from Genie, Bengaluru. For RCA amplification, the chemicals used include Phi 29 DNA polymerase and the corresponding buffer, Bovine serum albumin (New England Biolabs, Bengaluru), dNTPs (10mM, Thermofischer, Bengaluru). All primers were synthesised from Integrated DNA Technologies, U.S. and Merck India Ltd., Bengaluru and Gene JET gel extraction kit (Thermofischer Scientific, Bengaluru) was used for gel elution.

Orange G gel loading dye (Genei, Bengaluru), toluidine blue and phytate dipotassium salt (Sigma Aldrich, Bengaluru) were used for estimation of phytic acid using PAGE.

3.1.3 Softwares and databases used

The following software and databases were used in this study

- Oligocalc (www.basic.northwestern.edu/biotools/OligoCalc.html)
- ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

- DNA Baser
- National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/)
- BLAST (https://www.ncbi.nlm.nih.gov/blast/)
- NCBI conserved domains database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)
- ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/)
- GelQuant.NET

3.1.4 Equipments and machineries used

The equipments available at Department of Plant Biotechnology, College of Agriculture, Padannakad were used. The concentration and quality of DNA were checked with the help of Biospectrophotometer (Eppendorf, Germany). The polymerase chain reaction was done in Master Cycler (Eppendorf, Germany) and the visualisation of both agarose and polyacrylamide gel was done with the help of Gel documentation system (BioRad, USA).

3.2 METHODS

3.2.1 DNA isolation

Genomic DNA was isolated from tender leaves of Panniyur 1 and different black pepper genotypes which show polymorphism at molecular and morphological level based on previous reports with the protocol of modified CTAB method (Doyle and Doyle, 1987). Tender leaves of Panniyur 1 variety were used freshly and leaves of other 10 black pepper genotypes were collected from Pepper Research Station, Panniyur; freeze dried; transferred in ice box and used later for the DNA isolation. The black pepper genotypes collected were Panniyur 5, Panniyur 7, Chumala, Chettanvally, Kottanadan, PRS 160, Karimunda Kuttyatur, Karimunda 7, Thotamundy and Payyanganam 2.

Chemicals: Liquid nitrogen, PVP, CTAB extraction buffer (10% CTAB, 5M NaCl, 0.5 M EDTA, 1 M Tris), β-mercapto ethanol, choloroform:isoamyl alcohol (24:1), isopropanol, 5 M NaCl, 70% ethanol, distilled water.

Procedure

- Collected leaves were wiped clean with 70 % ethanol and leaf midribs were removed.
- Weighed 1 g of leaf sample and placed in a pestle and mortar for grinding with liquid nitrogen.
- While grinding with liquid nitrogen great care was taken to prevent shearing of the genomic DNA.
- Immediately a pinch of PVP was added, without allowing it to sweat, mixed it and then immediately it was added to a 10 ml pre-warmed (65°C) CTAB extraction buffer and 40µl β-mercapto ethanol. The solution was mixed thoroughly and gently.
- The solution was kept in water bath at 65°C and incubated for 30-60 min. While incubating, the solution was mixed in every 15 min interval gently taking care that no agitation would degrade the DNA.
- After incubation, the lysate was cooled to room temperature and half volume equivalent of chloroform:isoamyl alcohol (24:1) mixture was added to the lysate and gently mixed.
- After that the mix was centrifuged for 15 min at 10000 rpm at 4°C. Then the supernatant was pipetted out into a fresh centrifuge tube and the organic phase was discarded.
- Half volume equivalent of choloroform:isoamyl alcohol (24:1) mixture was added to the collected supernatant and gently mixed.
- Again the mix was centrifuged at 10000 rpm for 10 minutes at 4°C.
- After centrifugation, the supernatant was collected into a fresh centrifuge tube and half volume 5 M NaCl and 1.5 times the volume chilled isopropanol were added into the centrifuge tube containing supernatant.

40

- The mix was incubated for 30 min in -20°C. After incubation, the centrifugation step was again repeated at 10000 rpm for 15 min at 4°C.
- The supernatant obtained after centrifugation was discarded carefully without disturbing the DNA pellet found in the centrifuge tube. Then washed the DNA pellet with chilled 70 per cent ethanol.
- The DNA pellet was kept for air dry and dissolved the pellet in 100 µL sterile water and stored it in -20°C.

3.2.1.1 Quantity and quality of genomic DNA

Chemicals: Agarose, 6X loading dye, ethidium bromide (0.5µg/ml), DNA ladder of various sizes, 1X TBE buffer of pH-8.0 (Tris base, boric acid, 0.5 M EDTA).

Procedure:

One gramme of agarose powder was weighed for 100 ml 1X TBE buffer. Weighed agarose was added to the 1X TBE buffer and boiled in a microwave oven till the agarose was completely dissolved in the buffer. The gel was then allowed for cooling. The gel casting tray and comb were rinsed with distilled water and then wiped with 70 % ethanol. Open ends of the gel casting tray were completely sealed with cello tape and comb was placed appropriately making sure that gel tray was kept on level surface.

When the solution was lukewarm, 2 µL ethidium bromide was added and mixed well. Solution was then poured into the gel casting tray making sure that formation of air bubbles were prevented and was then allowed to solidify. After solidification of gel, the comb was removed gently and cello tapes from both the open ends were removed. The gel casting tray was then slowly placed into the gel tank with running buffer 1X TBE and the respective electrodes are connected with the power pack.

3.2.2 PRIMER DESIGNING

Primers were designed for the directional genome walking towards 5' end of *ipk1* gene sequence in black pepper, for the validation of newly assembled sequence of *ipk1* gene and for the detection of allelic variation in newly assembled *ipk1* gene sequence among different black pepper genotypes. Four different walker adaptors (WA1, WA2, WA3 and WA4) for whole genome amplification and walker primers (WP1 and WP2) for locus specific and nested locus specific amplifications were the same as reported by Reddy *et al.* (2008). Two sets of primers were designed for directional genome walking which was the preliminary step in the study. Two locus specific and two nested locus specific primers were designed based on the assembled sequence of *ipk1* gene (*pnipk1*, 1072 bp) reported by Giridhari (2017). The primers, one forward and one reverse, for confirmation of newly assembled *ipk1* gene after genome walking towards unknown 5' flanking region of *pnipk1* gene (1072 bp) sequence (Giridhari, 2017) and for detection of allelic variation if any in black pepper genotypes for *ipk1* gene were designed based on the newly assembled *ipk1* gene fragment using BLAST software (https://blast.ncbi.nlm.nih.gov/).

3.2.2.1 Primer designing for rolling circle amplification (RCA)

Four different walker adaptors (WA1, WA2, WA3 and WA4) suggested by Reddy *et al.* (2008) were used for whole genome amplification. RCA results in producing long overlapping fragments with walker adaptor sequences attached at the 5' end of each separate fragment. This was to facilitate the attachment of walker primers (WP1) and nested walker primers (WP2) during directional genome walking. Walker primers (WP1 and WP2) for locus and nested locus amplifications were also the same as reported by Reddy *et al.* (2008).

For directional genome walking towards 5' flanking unknown region of *ipk1* sequence, two locus specific and corresponding two nested locus specific primers were designed based on the *ipk1* (*pnipk1*, 1072 bp) gene sequence (Giridhari, 2017). LSPF3 was designed from the 3' end and LSPR3 was designed

from the 5' end. Similarly NLSPF3 and NLSPR3 were also designed from the 3' and 5' regions of previously reported sequence *pnipk1* (1072 bp) of Giridhari (2017).

3.2.2.2 Primer designing for confirmation of assembled sequence (1535 bp) and allelic variation detection in *ipk1* gene.

For the amplification and confirmation of newly assembled *ipk1* gene sequence in black pepper variety Panniyur 1 and for detection of any allelic variation in *ipk1* gene of black pepper genotypes, one forward and reverse primer set was designed from the 5' and 3' ends of newly assembled *ipk1* gene sequence (1535 bp) with the help of BLAST software. The forward primer was designed 118 bp away from the upstream end and reverse primer was designed 255 bp from downstream end of assembled sequence (1535 bp).

3.2.3 ROLLING CIRCLE AMPLIFICATION

Rolling circle mode of amplification by Phi 29 DNA polymerase as suggested by Reddy *et al.* (2008) was employed to introduce walker primer binding sites at random areas in the genomic DNA after the denatured genomic DNA was annealed by degenerate primers. It later resulted in the synthesis of large number of overlapping fragments of the genomic DNA of Panniyur 1 with the walker adaptor sequence attached to 5' end of all fragments.

RCA reaction was set up in a total volume of 20 µL. The constituents were 10x buffer (2µL), 10 mM dNTP (2 µL), 150 ng WA primers (1.5 µL), 100 ng genomic DNA (1 µL), 10 U phi 29 polymerase (1 µL), 100 ng / µL bovine serum albumin (BSA) (0.25 µL), 13.25 µL double distilled water. Two methods of RCA were followed using the four different walker adapter primers (WA1, WA2, WA3 and WA4).

Procedure

Method 1: A total reaction volume of 20 µL was prepared. To each of the four separate PCR tubes containing 1X buffer, template DNA (100 ng) and double

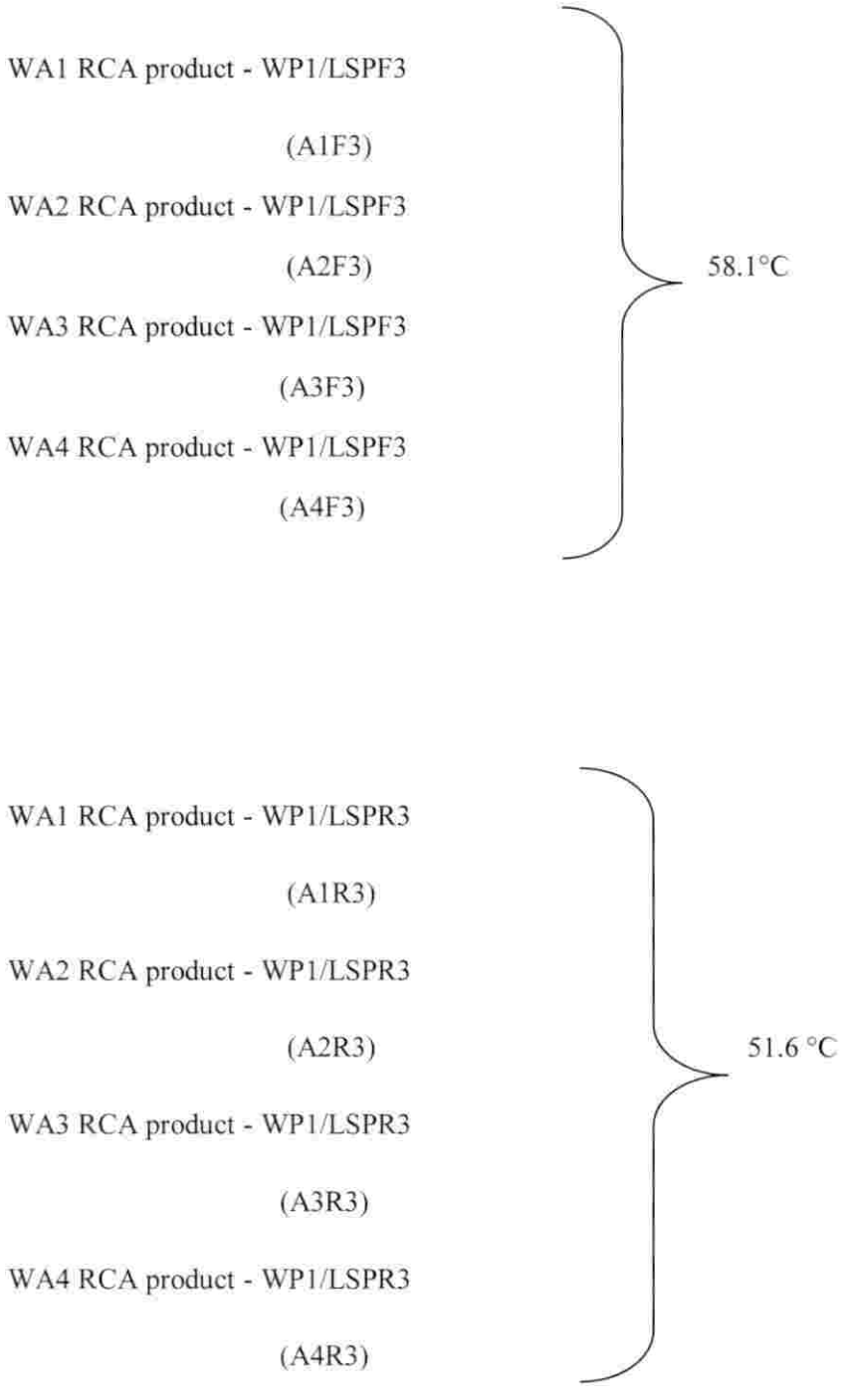
distilled water, four different walker adapters (150 ng each) were added respectively. Initially, the denaturation step of genomic DNA was performed at 94°C for 5 min. After that the four tubes containing walker adaptors were placed in ice and kept in -20°C deep freezer. Ten mM dNTPs, Phi 29 polymerase and BSA were added to the PCR tubes after taking it out from -20°C deep freezer. After adding the remaining constituents, the tubes were incubated at 30°C for a time period of 1 h and 30 min and termination reaction was carried out at 65°C for 10 min. The product after RCA was stored at -20°C deep freezer.

Method 2: Steps as such in method 1 from denaturation to incubation at 30°C for 1 h and 30 min were followed and instead of incubating at 65°C for 10 min, the tubes were incubated overnight at 30°C. Both the methods of RCA were carried out. On comparing both the methods of RCA, RCA done in overnight gave better results for further amplifications.

3.2.4 GENOME WALKING USING LOCUS SPECIFIC AMPLIFICATION

Locus specific amplification of RCA products as reported by Reddy *et al.* (2008) were done to amplify the flanking regions using the primers designed. Each RCA products were amplified with locus specific forward primer LSPF3 and a locus specific reverse primer LSPR3 at 56.8°C. At 51.6°C, reverse locus specific primer alone was utilized for PCR amplification of RCA product. Similarly locus specific forward primer was only used in PCR amplification at temperature 58.1°C (Table 3). Therefore a total of 16 reactions were carried out.

WA1 RCA product -	WP1/LSPF3 (A1F3)	WP1/LSPR3 (A1R3)	56.8 °C
WA2 RCA product -	WP1/LSPF3 (A2F3)	WP1/LSPR3 (A2R3)	
WA3 RCA product -	WP1/LSPF3 (A3F3)	WP1/LSPR3 (A3R3)	
WA4 RCA product -	WP1/LSPF3 (A4F3)	WP1/LSPR3 (A4R3)	


WA1 RCA product - WP1/LSPF3
(A1F3)
WA2 RCA product - WP1/LSPF3
(A2F3)
WA3 RCA product - WP1/LSPF3
(A3F3)
WA4 RCA product - WP1/LSPF3
(A4F3)
58.1°C
WA1 RCA product - WP1/LSPR3
(A1R3)
WA2 RCA product - WP1/LSPR3
(A2R3)
WA3 RCA product - WP1/LSPR3
(A3R3)
WA4 RCA product - WP1/LSPR3
(A4R3)
51.6 °C

The PCR reactions were carried out in a 0.2 ml flat cap PCR tubes and total reaction mix was made upto 50 µL. Details of reaction components of PCR reaction is given below (Table 2)

Table 2: Details of PCR components for locus specific amplification

Components	Volume (µL)
3U Taq DNA polymerase	0.6
Template DNA	0.5
10 mM dNTPs	1
10 x buffer	5
WP1	1
LSPF3 & LSPR3	1
Water	40.9
Total	50 µL

Table 3: Details of PCR conditions for locus specific PCR amplification

Sl. No.	Steps	Temperature (°C)	Time (min)
1	Initial denaturation	94	2
2	Denaturation	92	1
3	Annealing	51.6, 56.8 and 58.1	1
4	Extension	72	2
Repeat steps from 2 to 4 for 35 cycles			
5	Final extension	72	10
6	Hold	4	

3.2.5 Nested locus specific amplification

The protocol followed for nested locus specific amplification was same as reported by Reddy *et al.* (2008). Locus specific amplified products were diluted

at 1:50 ratio and the diluted amplified products were used as template for nested locus specific amplification using walker primer 2 (WP 2) and nested locus specific primers. The reaction was set upto a final volume of 50 µL and the details of reaction components are given below in Table 4.

Table 4. Details of PCR components for nested locus specific PCR amplification

Sl. No.	Components	Volume (µL)
1	3U Taq DNA polymerase	0.6
2	Template DNA	5
3	10 mM dNTPs	1
4	10 x buffer	5
5	WP2	1
6	NLSPF3 & NLSPR3	1
7	Water	35.9
Total		50 µL

Reactions were carried out at three different temperatures at 51.6°C, 56.8°C, 58.1°C and resulted in a total of 16 reactions similar as of locus specific amplifications. The thermal profile is same as that of locus specific amplification (Table 3). The 16 different reactions are given below.

At 56.8°C

WA1	WA2	WA3	WA4
WP2NLSPF3	WP2NLSPF3	WP2NLSPF3	WP2NLSPF3
WP2NLSPR3	WP2NLSPR3	WP2NLSPR3	WP2NLSPR3

At 58.1°C

WA1	WA2	WA3	WA4
WP2NLSPF3	WP2NLSPF3	WP2NLSPF3	WP2NLSPF3

At 51.6°C

WA1	WA2	WA3	WA4
WP2NLSPR3	WP2NLSPR3	WP2NLSPR3	WP2NLSPR3

3.2.6 Elution of amplified products from gel after genome walking

Four PCR products were selected for sequencing (Table 5). The size of each band was determined with the help of DNA ladder. The required band was excised out from the agarose gel using a new sterilized razor blade. The gel slice was then weighed in a pre-weighed 1.5 ml microfuge tube. After that to the obtained gel slice, an equivalent volume of binding buffer was added and incubated at 50-60°C in water bath for nearly 15 min until the gel slice was completely dissolved in it. The tube was intermittently mixed often to make sure that the gel slice was completely dissolved in it.

The tube containing binding buffer with dissolved gel slice was vortexed thoroughly. A volume of 800 µL gel containing binding buffer was then transferred to a purification column. The column was centrifuged for 1 min. The flow through was discarded. With the remaining gel solution, the centrifugation step was repeated. Again the flow through was discarded. Column was kept back to the collection tube. Wash buffer diluted with absolute alcohol was added to purification column. Column was centrifuged for 1 min. Flow through was again discarded. Centrifuged again for 1 min to remove the residual wash buffer. The purification column was now transferred to a new micro centrifuge tube. Thirty µL elution buffer was added through the centre of column. The tube along with column was centrifuged and the purification column was discarded. Eluted DNA was collected in collection tube and stored at -20°C.

48

Table 5: Details of eluted products

Sl. No.	PCR products	Approximate Size (kb)	Temperature (°C)
1.	A1R3	0.75	51.6
2.	A1R3	0.75	56.8
3.	A4R3	1	51.6
4.	A4R3	1	56.8

3.2.7 Sequencing

Four products after genome walking were selected and eluted from gel for obtaining sequence information. The information of sequences obtained were analysed for its similarity with *ipk1* gene in other crops using BLAST. All the four PCR amplified products exhibited similarity with *ipk1* gene in other crops. The amplicons A1R3 (51.6°C), A4R3 (51.6°C) and A4R3 (56.8°C) sequenced with WP2 showed no similarity with *ipk1* gene of other crops whereas sequence information obtained by sequencing A1R3 (51.6°C), A4R3 (51.6°C) and A4R3 (56.8°C) with NLSPR3 exhibited similarity with *ipk1* gene of other crops. The sequence information obtained by sequencing the amplicon A1R3 (56.8°C) both with WP2 and NLSPR3 exhibited similarity with *ipk1* gene of other crop species. Later all the sequences which exhibited similarity with *ipk1* gene were selected and assembled together in software DNA Baser. The contig obtained after assembling was found to exhibit overlapping fragment at the 5' region of previously reported *ipk1* gene fragment (*pnipk1*-1072 bp) reported by Giridhari (2017) and both the sequences were assembled together in DNA Baser software to form a newly assembled gene sequence. The newly assembled *ipk1* gene sequence (*pnipk1*-1535 bp) was subjected in ORF Finder to find out the coding region in the assembled gene sequence. The conserved domains were also checked by analyzing the amino acid sequence in NCBI conserved domains database (CDD) (https://www.ncbi.nlm.nih.gov/ Structure/cdd/wrpsb.cgi).

3.2.8 Validation of newly assembled sequence

3.2.8.1 Primer designing for confirmation of newly assembled *ipk1* gene sequence (*pnipk1*- 1535 bp)

For the amplification and confirmation of newly assembled *ipk1* gene sequence in black pepper variety Panniyur 1, forward and reverse primers were designed from the 5' and 3' ends of newly assembled sequence (*pnipk1*-1535 bp) with the help of BLAST software. The forward primer is designed 118 bp away from the upstream end and reverse primer is designed 255 bp from downstream end of assembled *ipk1* gene sequence (*pnipk1*-1535 bp).

3.2.8.2 PCR amplification for validation of newly assembled sequence

For confirmation of newly assembled sequence of *ipk1* fragment in black pepper variety Panniyur 1, PCR amplification was carried out with a forward primer F11 and a reverse primer R11 (Table 7). Reactions were carried into a total volume of 25µL. Details of reaction components are given below in Table 6.

Table 6. Constituents of PCR mix for validation of *ipk1* gene (*pnipk1*-1535 bp) in black pepper

Sl. No.	Components	Volume (µL)
1	3U Taq DNA polymerase	0.6
2	Template DNA	0.5
3	10 mM dNTPs	1
4	10 x buffer	2.5
5	F11	1
6	R11	1
7	Water	18.4
Total		25 µL

Table 7. Details of thermal profile for validation of assembled sequence, *ipk1* gene (*pnipk1*-1535 bp) in black pepper

Sl. No.	Steps	Temperature (°C)	Time (min)
1	Initial denaturation	94	5
2	Denaturation	92	1
3	Annealing	61-66	1
4	Extension	72	2
Repeat steps from 2 to 4 for 40 cycles			
5	Final extension	72	10
6	Hold	4	

After PCR amplification, the products were analysed on a 1% agarose gel in which 5 µL of sample was loaded with 1 µL 6X gel loading dye along with DNA ladders of various sizes.

3.2.9 PHYLOGENETIC ANALYSIS

The newly assembled sequence and the amino acid sequence obtained by analysis in ORF finder were subjected to homology search in BLAST. Similarity was found with *ipk1* gene and translated amino acid sequences reported in other crop species and were downloaded for phylogenetic analysis. Phylogenetic analysis was also done in BLAST.

3.2.10 PCR amplification for allelic variation in *ipk1* gene sequence

For detecting the allelic variations in *ipk1* gene in the genomic DNA of Panniyur 1, the genomic DNA of black pepper genotypes were subjected to PCR amplifications with primers mentioned in 3.2.8.1. PCR reaction was set up to a total volume of 25 µL and the details of reaction mix is same as of Table 6.

Table 8: Details of thermal profile for allelic difference analysis in assembled sequence of *ipk1* gene (*pnipk1*-1535 bp) in black pepper

Sl. No	Steps	Temperature (°C)	Time (min)
1	Initial denaturation	94	5
2	Denaturation	92	1
3	Annealing	61.5	1
4	Extension	72	2
Repeat steps from 2 to 4 for 40 cycles			
5	Final extension	72	10
6	Hold	4	

After PCR amplification, the PCR products were loaded at 5 µL with 1 µL 6X gel loading dye and separated on agarose gel electrophoresis.

3.2.11 PHYTIC ACID DETECTION WITH POLYACRYLAMIDE GEL ELECTROPHORESIS

Chemicals : Liquid nitrogen, 0.4M HCl, 40 % acrylamide / bisacrylamide, 10X TBE pH 8.0, 5X TBE pH 6.0, double distilled sterilized water, 10% ammoniumpersulphate (APS), tetramethylethylenediamine (TEMED), 6X orange G gel loading dye, toluidine blue, phytate dipotassium salt, methanol, glycerol.

Phytic acid content of 10 genotypes of black pepper were determined according to the protocol suggested by Alimohammadi *et al.* (2013). The phytic content can be quantitavely estimated and visualized in crop samples with this method. All the 10 black pepper genotypes, from which phytic acid was extracted, were collected from Pepper Research Station, Panniyur. Details of black pepper varieties are given below (Table 9).

Table 9. List of crops selected for phytic acid estimation

Sl. No.	Variety
1	Panniyur 5
2	Panniyur 7
3	Chettanvally
4	Kottanadan
5	Karimunda 7
6	PRS 160
7	Chumala
8	Thotamundy
9	Karimunda Kuttyatur
10	Payyanganam 2

3.2.11.1 PHYTIC ACID EXTRACTION

Phytic acid is primarily found in storage parts of plants like seeds. Therefore for the estimation of phytic acid, berries were used for extraction purposes. The procedure for extraction is given below

Steps

1. The outer covering of berries were removed and weighed 1.2 g berries.
2. The weighed berries were placed in a mortar and pestle.
3. The berries were grinded thoroughly with 3 ml of 0.4 M HCl and liquid nitrogen and this was collected in a microfuge tube.
4. The homogenised tissue was then allowed to boil for 5 min.
5. Later it was centrifuged at 12000 rpm for 10 min.
6. Supernatant was collected into a fresh 1.5 ml centrifuge tube.
7. The tubes containing supernatant were snap frozen in liquid nitrogen.
8. Afterwards it was melted by keeping it in room temperature.

9. The samples were again subjected for centrifugation at 12000 rpm for 5 min
10. After centrifugation, the supernatant was collected in a fresh microfuge tube and was stored in -20°C freezer.

3.2.11.2 Polyacrylamide Gel Electrophoresis (PAGE), staining and visualisation

The polyacrylamide gel electrophoresis (PAGE) analysis of 10 genotypes of black pepper was done. The polyacrlyamide gel was composed of 33.3% resolving gel in TBE. The components in resolving gel is given below in Table 10.

Table 10. Constituents of resolving gel

Sl. No.	Components	Volume
1	40 % acrylamide/bisacrylamide(19:1)	3.7 ml
2	10 x TBE (pH- 8)	0.5 ml
3	Double distilled water	0.3 ml
4	10% APS	32 µl
5	TEMED	3.5 µl

Immediately after pouring resolving gel, a layer of butanol was poured over the gel for uniformity of gel formation. After the resolving gel got solidified, stacking gel was topped over resolving gel. The components of stacking gel are given below in Table 11.

Table 11. Constituents of stacking gel

Sl. No.	Components	Volume
1	16 % acrylamide/ bisacrylamide (19:1)	1.0 ml
2	5x TBE (pH- 6)	120 µl
3	Double distilled water	69.5µl
4	10% APS	8.5 µl
5	TEMED	1 µl

Immediately after pouring stacking gel, the comb was placed for well formation. While placing the well, it was made sure that no bubbles were trapped inside the gel. After the gel has been solidified, the comb was carefully removed and the gel was preruned for 20 min at 100V/10mA in 1X TBE buffer. After that 10 µL of phytic acid samples from black pepper genotypes were mixed with 5 µL of 6X orange gel loading dye and phytic acid standards were made out from commercially available standard phytic acid potassium salts. Both samples and standards were made upto total volume of 25 µL. The extracts and standards were loaded to the gel and were run at 100V/mA in 1X TBE buffer. Till the dye front reached above 2 cm from the bottom of the gel, the gel were allowed to run. After the gel running was stopped the gel was stained with staining solution constituted of 20% methanol, 2% glycerol and 0.05 % toluidine blue for 20 min. After staining, the gel was destained overnight with destaining solution which constituted of all components of staining solution except toluidine blue. Destaining of gel was done for overnight in shaker with many changes of solution without dye. With the help of gel imaging system, gel images were visualised and analyzed.

3.2.11.3 Data Analysis

Estimation of the phytic acid concentration in samples was done by quantification of gel images in a software called GelQuant.NET. The standard curve was made by plotting a graph by taking quantity of standard phytic acid (nanomoles) along X-axis and volume (intensity) along Y axis. With the standard curve obtained from the values of standard phytic acid concentration, the phytic acid concentration in samples were determined.

Results

4. RESULTS

The results of the study entitled "Allelic difference in the putative gene *ipk1* sequence and phytic acid (InsP6) content in black pepper (*Piper nigrum* L.)" carried out during 2017-19 at College of Agriculture, Padanakkad are presented in this chapter. A unigene (*pnc135*-995 bp) showing similarity with *ipk1* gene coding for inositol pentakisphosphate-2- kinase enzyme was reported in an earlier work (Sujatha, Unpublished data). Later this unigene was partially sequenced (*pnipk1*-1072 bp) by Giridhari (2017). In the present investigation, genome walking method reported by Reddy *et al.* (2008) was used for finding out the unknown sequence towards 5' end of this putative gene fragment, *pnipk1*.

The experiment was conducted in two phases. The first part included performing genome walking in the genomic DNA isolated from the leaves of Panniyur 1 for the unknown flanking region towards 5' end of the putative *ipk1* gene (*pnipk1*-1072 bp) reported by Giridhari (2017) and thereafter screening 10 black pepper genotypes for detecting the allelic differences in the *ipk1* gene sequence among these black pepper genotypes. The newly assembled *ipk1* gene sequence was further validated in Panniyur 1 and other 10 diverse black pepper genotypes for detecting the allelic differences in the gene sequence, if any.

The second phase included estimation of phytic acid in the selected black pepper genotypes utilizing PAGE. In this study the quantitative estimation of phytic acid was carried out in the above mentioned 10 genotypes of black pepper for detecting similarity between the allelic difference and phytic acid content, if any. The phytic acid content of plant samples were worked out by the standard curve obtained by readings of commercially available phytic acid standards.

4.1 GENOME WALKING

4.1.1 DNA isolation

Genomic DNA was extracted from Panniyur 1 variety of black pepper using modified CTAB method (Plate 1) and the DNA obtained was immediately dissolved in nuclease free sterile water and stored in -20°C.

4.1.2 Quality and quantity of genomic DNA

Quantification of genomic DNA was done with the help of spectrophotometer. DNA was diluted at a ratio of 1:50 and concentration of genomic DNA was 3.26 µg/µL. The isolated DNA of Panniyur 1 showed an absorbance ratio of 1.87 at $A_{260/280}$ nm. A good quality DNA will have a ratio of 1.8 and 2.0 at $A_{260/280}$ nm, this indicate that the isolated genomic DNA is of good quality.

Agarose gel electrophoresis was also performed for analysing the DNA quality. Five µL of genomic DNA was loaded to check the DNA quality. Bands with no shearing or contamination were seen in the gel, revealing a good quality of DNA. This DNA was later used in gene amplifications.

4.1.3 Primers for rolling circle amplification (RCA) and genome walking

Four different walker adaptors were used for whole genome amplification using the method of RCA as reported by Reddy *et al.* (2008). These are degenerate primers with bases near to 5' end being identical and four bases at its 3' end makes the four adaptor walker primers different (Table 12).

For directional genome walking, primers for locus and nested locus amplification were designed based on the assembled *ipk1* gene sequence (*pnipk1*, 1072 bp) reported by Giridhari (2017). Two locus specific primers, *i.e.* one forward locus specific and one reverse locus specific primers were designed (Fig 1). The forward locus specific primer was designed from the downstream position towards the 3' end of *ipk1* gene whereas the reverse locus specific was

designed from the upstream part of assembled sequence towards 5' end. Similarly the nested locus specific forward and reverse primers were designed corresponding to the forward and reverse locus specific primers. The details of primers are given below and Table 12.

pnipk1 gene (1072 bp) sequence assembly

5'GTCTTTTCGGGTCTCAAATTTCATAGCTGAGATAACTTCATCAAAAA
GTGTGTGTCTCGTTTTAAAATGCACCAACCCTTGAAATTTCACAAGAA
AGAGGTAATTTTCACCTGTGCTTATATATTCCTTTGTTTAAAAGAAAAA
ACTTAGATTACACTAATTGTTGAGGATGCTTTTCTTCATCCTTTCTGTA
CACATGGATTTGGCTATCATTTTCTAATTATTTTTCACTTCTCTCTCAGA
TAGGGGAAATAAGTGAGTATGATCCAGTCGATCTGTTTTCAGGTTCTA
AAGATAGAATAAATCGTGCTATGAAATCTCTCTATGCCATCCCGCAAA
ACAATTTTCGTATCTTTTTCAATGGCTCTTTGTTATTTGGTGGATTGGG
GGGAGCCATGGATAATACGAAATCTAAATCCATGGAAGCTATTGATGA
TGAATTAGGTGTGTTAATTGATGCAGCACCTGGACTACGACTATCTAG
ATTTCTGGAACTTGTTTCATTGACAATTTTACAGTCAGATGTCCTGGTT
GGTCTTCTTAATACGCAAATGTTTGACCTTCTTGATATAGAAGGGGCA
ATTCATGCCTATTACAACATTATTTCTCAACCTAACATGGTCGGCAAGG
TCCTAGGCGGCCTTTCTAAGAAGTACTTTTCCCTACATTATCTTTCTTTG
GAAGCAAGTGTTAAGATTGTTCGCGATTTTTTGATCGCTGCAACTGCA
AAGGACTGTAGCTTGATGATCAGTTTCAGACTGTTATCAGGGGAGATG
ATATCTGAAATTGATGTTGTGCACTTAGAGACAACTGGCCAAAGTTTT
TTGCTATAAGGTACTTGATGTGCTCATCTCCTTGGTTTGTTTTTGTGATG
ATATTGCCCTTGAACCATTTTCATTACTCCTATGATCTGAATTTTGCAA
TGCTGCCTATTAAGAATTATGTAGATGAAGAAAACATGTGTTATTAAT
AAATTTAACATGCATCAATGAAGTTCTGAAATTGCATGAATTAATTTA
TTTTTATGTCTATTATATTTTGTGAAACCATTTTAAGGTTAGTGTTAAA
CACC 3'

Fig 1. The *pnipk1* gene fragment (1072 bp) with primers for genome walking in black pepper DNA

Table 12. Primers used for rolling circle amplification and genome walking in black pepper DNA for *ipk1* gene sequencing

Sl. No	Primer	F/ R	Sequence 5'-3'	Primer length (bp)	GC %	Tm (°C)
1	WA1		GTGAGCGCGCGTAATACGACT CACTATAGGGNNNNATGC	39	53.8	67
2	WA2		GTGAGCGCGCGTAATACGACT CACTATAGGGNNNNGATC	39	53.8	67
3	WA3		GTGAGCGCGCGTAATACGACT CACTATAGGGNNNNTAGC	39	53.8	67
4	WA4		GTGAGCGCGCGTAATACGACT CACTATAGGGNNNNCTAG	39	53.8	67
5	WP1		GTGAGCGCGCGTAATACGA	19	57.9	58
6	WP2		GTAATACGACTCACTATAGGG	21	42.9	49
7	LSP F3	F	TTGCCCTTGAACCATTTTCAT	21	38	55
8	LSP R3	R	CCTCAACAATTAGTGTAATCT AAG	24	33	58
9	NLSP F3	F	ACATGCATCAATGAAGTTCTG	21	38	55
10	NLSP R3	R	GTGAAATTTCAAGGGTTGGTG	21	43	57

4.1.4 RCA for attachment of 5' walker adapter sequences

Whole genome amplification using the method of rolling circle amplification as reported by Reddy *et al.* (2008) was carried out using walker adapter primers. These walker adaptors with the help of Phi 29 DNA polymerase would generate overlapping strands that would cover the whole genomic DNA

eliminating the necessity of restriction enzymes and ligation processes. Four different amplification products were obtained using the four walker adaptors.

4.1.5 PCR amplifications using locus specific and nested locus specific primers

Four different RCA products after rolling circle amplification were used as template for the directional genome walking with the locus and nested locus specific primers. Reactions were carried out at different temperatures *viz.*, 51.6°C, 56.8°C, 58.1°C in locus specific amplification with a forward locus specific primer and a reverse locus specific primer. Therefore a total of 16 reactions were performed.

After locus specific amplification, the amplified PCR products were subjected for a nested PCR using nested locus specific primers. This two round PCR amplifications with locus and nested locus specific PCR will assure specific amplification with less off-targets. The locus specific amplified products were diluted at 1:50 before nested specific PCR amplification. To analyze the nested PCR products, 5 µl of sample was loaded with 1 µl 6x gel loading dye and gel electrophoresis was done in 1% TBE agarose gel with DNA ladders of sizes 1Kb and 100 bp (Plate 2).

After visualisation, four nested products were chosen for sequencing towards the 5' flanking region of *pnipk1*(1072 bp) gene sequence assembly, *viz.*, A4R3 and A1R3 generated at temperatures of 51.6°C and 56.8°C (Plate 2). Bands showing smaller size were not taken for sequencing purpose. Multiple bands of different sizes were shown by PCR products. The selected bands for sequencing were eluted and purified from agarose gel (Plate 3).

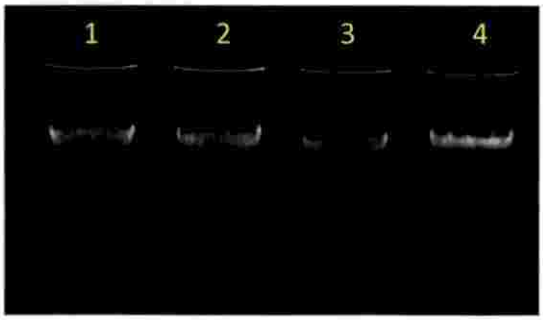


Plate1. Gel image of isolated genomic DNA from Panniyur 1 variety samples (Lanes 1,2,3,4 – Replicates of DNA samples from Panniyur 1)

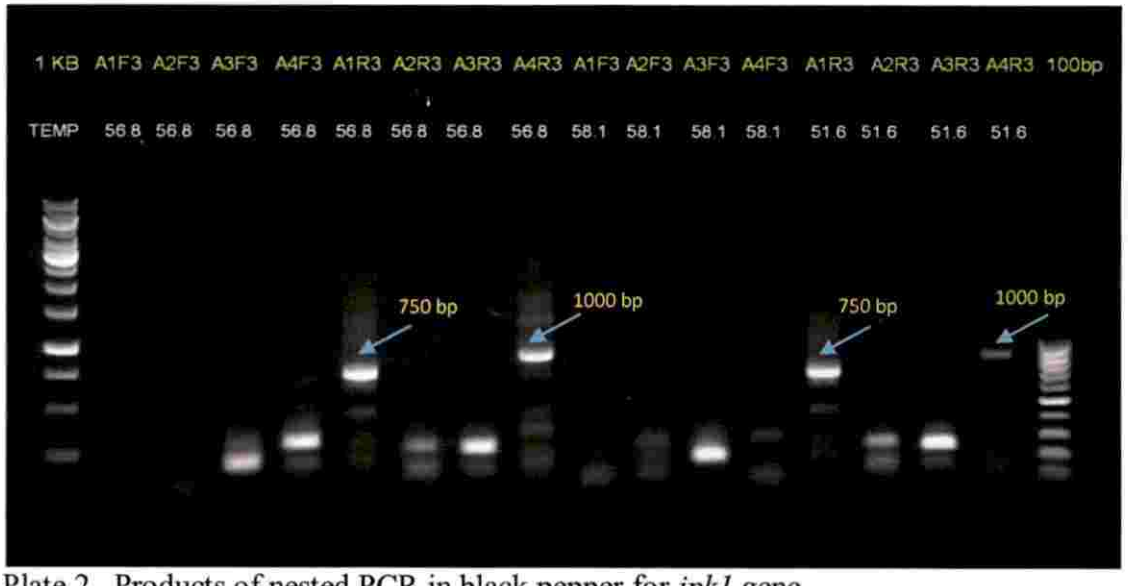


Plate 2. Products of nested PCR in black pepper for *ipk1* gene

Lane 1- 1 Kb, lane 2- WA1:NLSPF3, lane 3- WA2:NLSPF3, lane 4- WA3: NLSPF3, lane 5- WA4: NLSPF3, lane 6- WA1:NLSPR3, lane 7- WA2:NLSPR3, lane 8- WA3:NLSPR3, lane 9- WA4:NLSPR3, lane 10- WA1:NLSPF3, lane 11- WA2:NLSPF3, lane 12- WA3:NLSPF3, lane 13- WA4:NLSPF3, lane 14- WA1:NLSPR3, lane 15- WA2:NLSPR3, lane 16- WA3:NLSPR3, lane 17- WA4:NLSPR3, lane 18- 100 bp

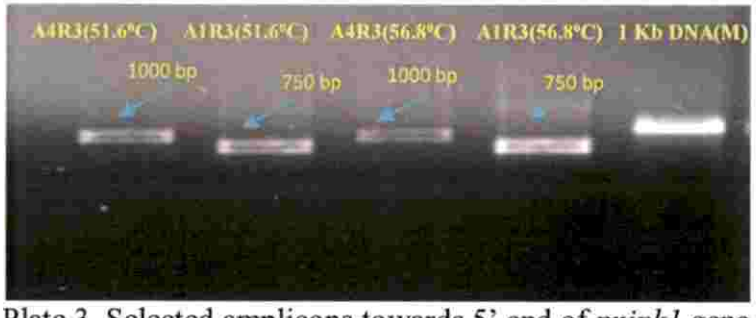


Plate 3. Selected amplicons towards 5' end of *pnipk1* gene fragment (1072 bp) in black pepper used for sequencing

4.1.6 Sequence analysis of amplicons obtained by genome walking sequence

Four PCR products were selected for sequencing which was obtained when locus specific products were amplified with WP2 walker primer and NLSPR3 primer at temperatures 51.6°C and 56.8°C. All the four products selected had an approximate size range between 750 bp and 1.0 Kb (Plate 3).

4.1.6.1 A4R3 (51.6°C)

The 5' end of product A4R3 was sequenced with the primer WP2. It produced a sequence length of 606 bp. When the A4R3 sequenced with WP2 was analysed in blast for homology with *ipk1* gene, no similarity was observed in any other crops. The details of sequenced product and electrograph are given below (Fig 2a).

63

>0719_445_007_PCR_A4R3_51_C_WP2-C05.ab1

AGCATGTATACCAGTATGTCATTCTATTTATAAACTGTTTATATATAAT
AATATAGTAAATGAAAAGAGAACCACCTCTCTTATGCAAATTCTTTCC
CATTATTGTTGGTAACATTATTCTTTTTTTTTTTTTTTAAACCCCAAGGC
GGGTTTTTTAAAATCCCCCCCCCCCTTTAAAAAAAGGGGGTGGGGGAA
AAAAATTTTTGGGAATTTTTGGAAAAAAAATTTTAAAACTTGCCCCCC
CTTGGGGGGGGTGGGGGGGGAAAGGGTAAGGTTCCCCGGGATCCCCC
CCCTTTTTTTTCAAAACTTTCCCTTTTTTCCCGGGGAAAAACCTTAATTT
TTAACCCCCCACCAAAAAAAAGTTTTTTTACACGGGTTTTTTGCTTTTT
ATTGGGGCCGTTTTTTGAAAAACATTTAATTGCTTCTTCAGGGTTTTTA
CAAAAAAAACGCCCGCATGACGACTAGCTAGAGTTTCTCGTATGACTT
TTCTTTTAGATCATGTCGTTCATCGATCGCTGCTCAGGTCAGCTGCTGT
CGTCTCATCGTCTGAGCAGAGTGAGGCTTTATTTATTCGATACCAAAG
CTCGCGACTCTGGTCCTAACTGG

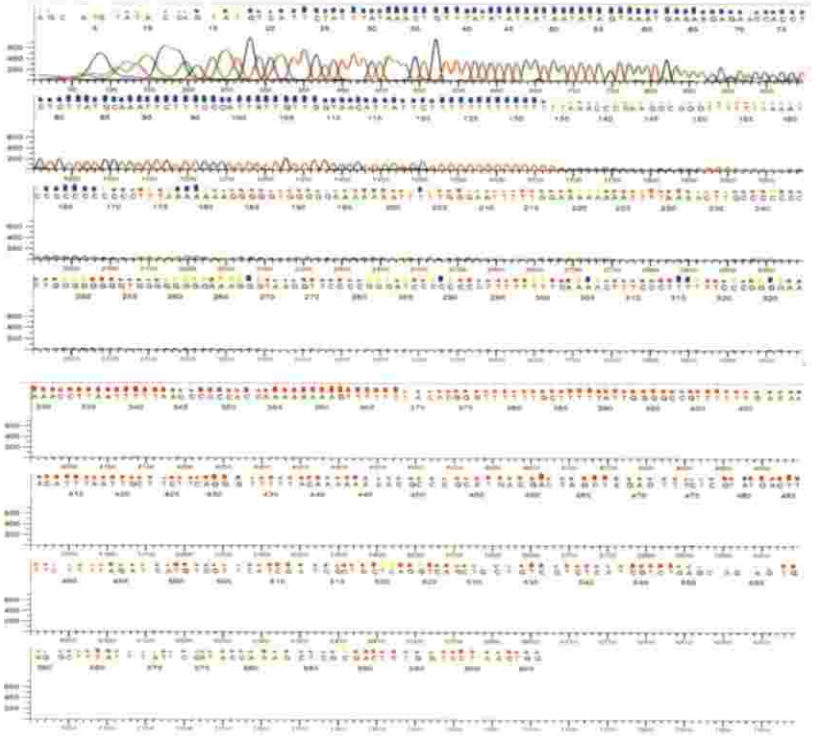


Fig 2a. Sequence of nested product A4R3(51.6ºC) sequenced with WP2

64

The 3' end of amplified nested product at 51.6°C was sequenced with NLSPR3. The product A4R3 had a total length of 517 bp (Fig 2b). When homology search was done, A4R3 at 51.6°C sequenced with NLSPR3 showed similarity with *ipk1* gene in other crops (Table 13).

>0719_445_008_PCR_A4R3_51_C_NLSP_R3-D05.ab1

CCTAAGGAGACCACTTTTTGATGAGTTATCTTCAGCTATAGAATTTTG
AGACTGGAAGAAAACCACACTTTGGCTGCTTCCTCAATCCAAAAAAA
ACAAAGAGCGAGTAATTGAGCATTGAGTACATGAATTGATTAAAAAA
CTAAGAAGTCATAACATCAAGGGAAATGCCACCTTTATTTCCACTGCT
ATGCACCGATCCTCTCTAACACTTCCTGGAAAGACAATAAAAGAGTA
TAACAAAAAACTGACCAATTAAAAAGCAAGAACCATGTGTGAAACA
AACTTTTTCTCTGCTGCGAGTCCAACGCGCGAGTACCCCCTCTGGGCC
GATGAGGGGCCATACACCCTTACCAACCACGTACCTGCGGGAAACTG
AACACTTGCCAGCACGTCAAGATGTATTTTGCCCATCCGTTAAGTCCA
CCTGCTTCCCCGCTGAACTATATGCTCCAAGCCCCCACCCGGTTTGGT
GATAAACATCCCCCCCAAAACACTGCTCCTCTTTTTTGTCATA

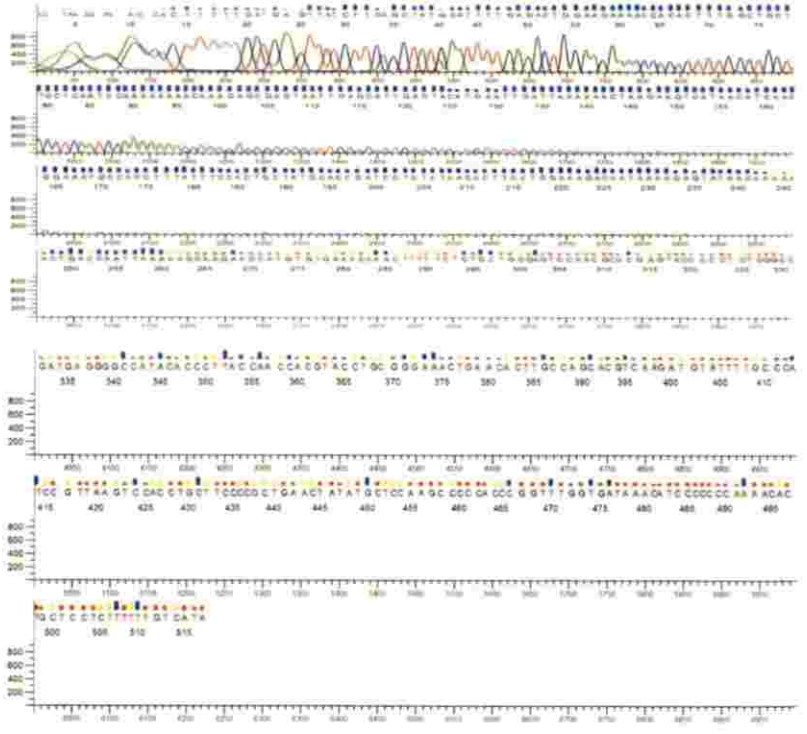


Fig 2b. Sequence of nested PCR product A4R3(51.6ºC) sequenced with NLSPR3

Table 13.Homology of 3' end of A4R3 (51.6°C, sequenced with NLSPR3) with *ipk1* gene in other crops

Description	Accession	Max score	Query cover	E value	Identity (%)
PREDICTED: *Ziziphus jujuba* inositol-pentakisphosphate 2-kinase-like (LOC107403694), transcript variant X10, mRNA	XM_025068353.1	52.7	11 %	0.029	81.67
PREDICTED: *Camellia sinensis* inositol-pentakisphosphate 2-kinase-like (LOC114288632), transcript variant X9, mRNA	XM_028232158.1	50	10%	0.10	81.48
PREDICTED: *Dendrobium catenatum* inositol-pentakisphosphate 2-kinase *IPK1* (LOC110094659), transcript variant X2, mRNA	XM_020819930.2	48.2	7%	0.35	90.48

4.1.6.2 A1R3 (51.6°C)

The second nested PCR product was A1R3 amplified at 51.6°C. It was sequenced with walker primer WP2 and a product with length of 371 bp was obtained. No similarity was shown when blast homology search was done for *ipk1*

gene. Details of sequence of nested product A1R3 at 51.6°C along with electrograph are given below in Fig 3a.

>0719_445_003_PCR_A1R3_51_C_WP2-G04.ab1

GGGGTGGGCCATTATGTGCTAATGTTATGAGTCCACTGCCTAGGCT

CAAAGCATGTCGATGCTGGGGTACTTTCCTATAAGGAGTTCTTCAA

TTTTTTTCCTTGGTAACAATTTCACTTTTAAAGGACTGAGATTGTTC

TTTTTTGTTCAATGCTAATGTAATAACCCAGTAATGTCAATTCTATT

TATAAACTGTTTATATATAATAATATAGTAAATGAAAAGAGAACCA

CCTCTCTTATGCAAATTCTTTCCCATTATTGTTGGTAACATTATTCTT

TTTTTTTTTTTTTTAACCCCCACCCTTTTTTTTTTCTATTCCCCCCTTCT

TTTAAAAAACTGGCCAGGTTTTAAAAAATTTTTGGAAATCC

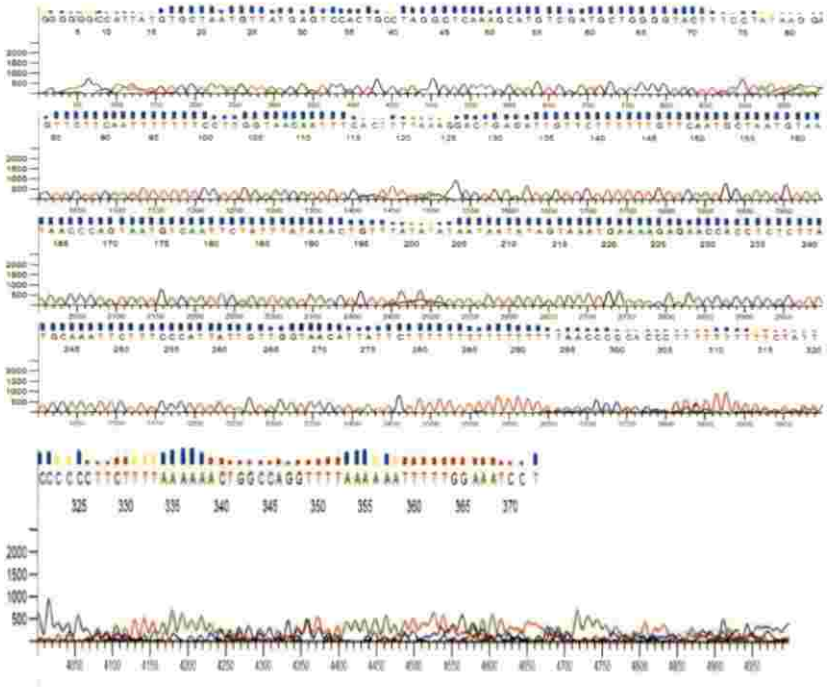


Fig 3a. Sequence of 5' end of nested PCR product A1R3 at 51.6°C sequenced with WP2

Similarly sequencing of A1R3 amplified at 51.6°C was done with NLSPR3 (Fig 3b). It showed a length of 315 bp and similarity with *ipk1* gene of other crops when searched for homology in blast. It showed similarity with many crops *viz.*, *Cicer arietinum, Camellia sinensis, Ziziphus jujuba, Dendrobium catenatum* etc. (Table 14).

>0719_445_004_PCR_A1R3_51_C_NLSP_R3-H04.ab1 315

TGTTACGAGACCACTTTTTGATAGAGTTATCTTCAGCTATAGAATTTT
GAGACTGGAAGAAAACCACACTTTGGCTGCTTCCTCAATCCAAAAAA
AACAAAGAGCGAGTAATTGAGCATTGAGTACATGAATTGATTAAAAA
ACTAAGAAGTCCTAACATCAAGGGAAATGCCCCCTTTATTTCCACTGC
TCTGCACCGATCCTCCCTACCAATTCCCGGAAACACAAACAGCTAGT
GCTTTTTCATGTTTCTCATTGGCGAGTAAGCCAATTGCGCCCACAATT
GTTTCTCAACCACAATTAACAACAAATGCA

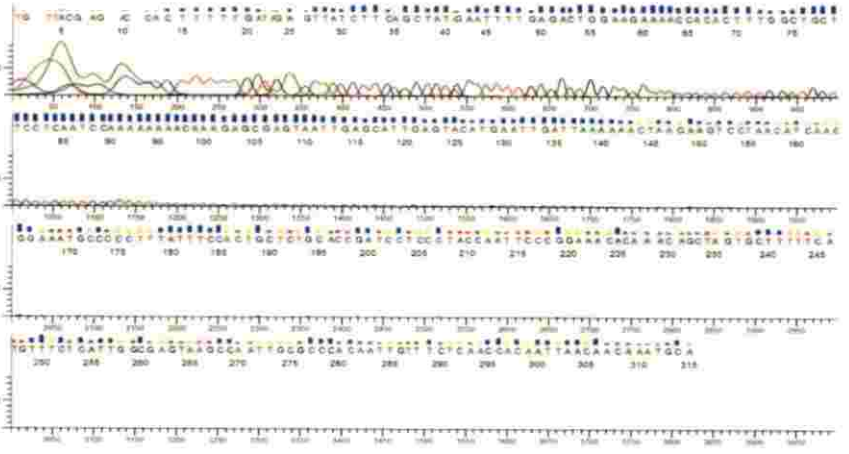


Fig 3b. Sequence of 3' end of nested PCR product at 51.6 °C A1R3 sequenced with NLSPR3

Table 14. Homology of 3' end of A1R3 (51.6°C, sequenced with NLSPR3) with *ipk1* gene in other crops

Description	Accession	Max score	Query cover	E value	Identity (%)
PREDICTED: *Cicer arietinum* inositol-pentakisphosphate 2-kinase-like (LOC101495995), transcript variant X4, Mrna	XM_004500934.3	57.2	20 %	4e-04	80.95
PREDICTED: *Camellia sinensis* inositol-pentakisphosphate 2-kinase-like (LOC114288632), transcript variant X9, mRNA	XM_028232158.1	55.4	21%	0.001	79.10
PREDICTED: *Ziziphus jujuba* inositol-pentakisphosphate 2-kinase-like, transcript variant X10, mRNA	XM_025068353.1	51.8	19%	0.017	80.00
PREDICTED: *Dendrobium catenatum* IPK1 mRNA	XM_020819930.2	48.2	13%	0.2	90.48

4.1.6.3 A4R3 (56.8°C)

Sequencing of 5' end of nested PCR product A4R3 amplified at temperature 56.8°C was done with WP2. Sequencing of product A4R3 at 56.8°C obtained a sequence length of 593 bp. Blast homology search was done for the product A4R3 at 56.8°C and no similarity was observed for A4R3 at 56.8°C with *ipk1* gene. Details of sequence length and electrograph of nested PCR product A4R3 at 56.8°C are given below (Fig 4a).

>0719_445_005_PCR_A4R3_56_C_WP2-A05.ab1

CCGGCTATGTATACCAGTATGTCATTCTATTTATAAACTGTTTATATAT
AATAATATAGTAAATGAAAAGAGAACCACCTCTCTTATGCAAATTCTT
TCCCATTATTGTTGGTAACATTATTCTTTTTTTTTTTTTTTAAACCCAAA
GGCTTTTTTTTTAAAATCCCCCCCCCCCTTTAAAAAAAGGGGGTTTTTT
AAAAAAATTTTTGGGAAATTTTGGAAAAAAAATTTTAAACCTTGCCCC
CCTTTTGGGGGGTTTAAGGGGGAAAGGGTAAGTTTCCCGGGGATTCCC
CCCTTTTTTTTTTAAAACTTTCCCTTTTTTCCCGGGGGAAAAAATTTTTT
TTTTACCTCTTACCAAAAAAAAATTTTTCCCACCGGGGTTGGTCTTTTT
ATGGGGAGTTTTTTTTTTACCCTTTTATTGTTTTTCCCGAGGTTGTTAGG
GGGAGGGGTCTATCAAGGGGGAAAAAGGGGTTTCCTTCTTTTTTAACC
TTTCTTTTTATAACATTCGAGCCAGTCAATTATATCGCGTATTTTTGGA
GCAGAATTCTCTAGTGTTTCTTCCTACCAAACTTAAGCAGTAAATACTC
CAACGG

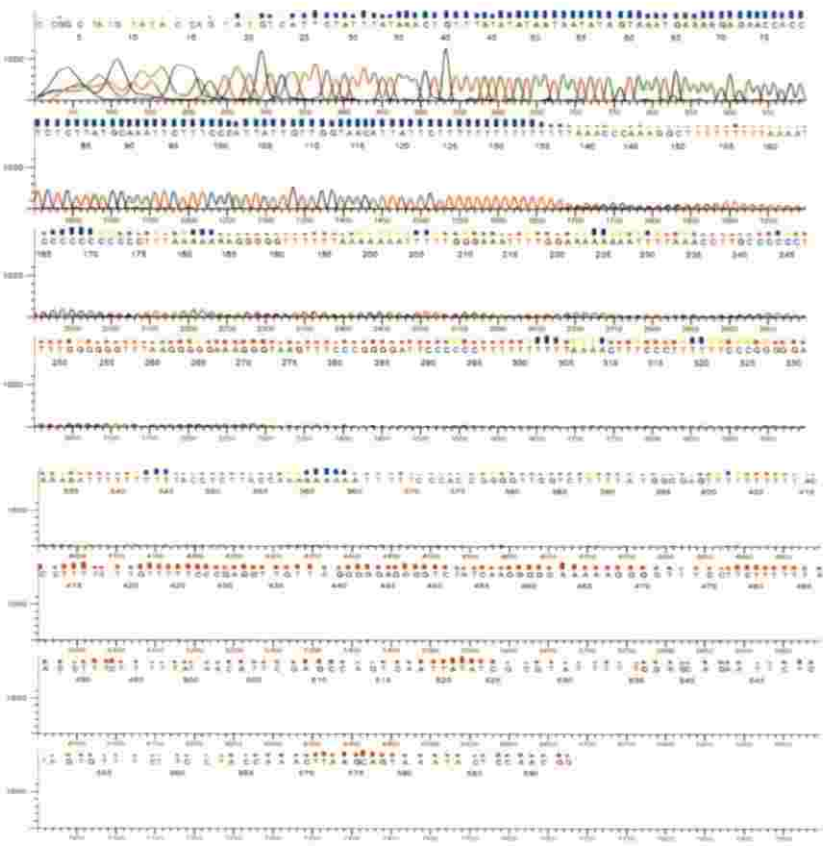


Fig 4a. Sequence of nested PCR product A4R3 at 56.8°C sequenced with WP2

Sequencing of nested PCR product A4R3 amplified at 56.8°C with NLSPR3 was also done and the sequence had a length of 423 bp (Fig 4b). When blast homology of A4R3 at 56.8°C sequenced with NLSPR3 was performed, similarity was found with *ipk1* gene in other crop plants (Table 15).

>0719_445_006_PCR_A4R3_56_C_NLSP_R3-B05.ab1

GCCTAAGAGAAACACTTTTTTGATGAAGTTATCTTCAGCTATGAATTT
TGAGACTGGAAGAAAACCACACTTTGGCTGCTTCCTCAATCCAAAAA
AAACAAAGAGCGAGTAATTGAGCATTGAGTACATGAATTGATTAAAA
AACTAAGAAGTCATAACATCAAGGGAAATGCCACCTTTATTTCCACT
GCTATGCACCGATCCTCTCTAACACTTCCTGGAAAGACAATAAAAGA
GTATAACAAAAAACTGACCAATTAAAAAGCAAGAACCATGTGTGAA
ACAAACTTTCTTCTGCTAAGAGTTCAAAAGTTAAGTCTTTACCATGGA
TAAATAGTGAATGATCTGAAATAACAAGAGCTGAATCACAGAGAACA
TTAACCTTTGCCTCATCAACACACCGAGATGGGCGCAAGCTATGAA

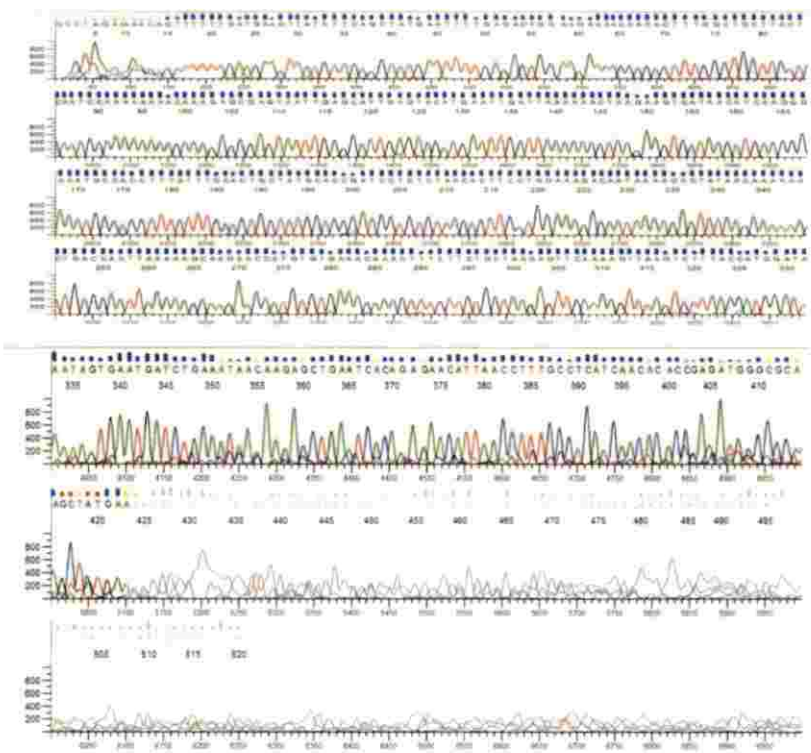


Fig 4b. Sequence of nested PCR product A4R3 at 56.8°C sequenced with NLSPR3

Table 15. Homology of A4R3 (56.8°C, sequenced with NLSPR3) with *ipk1* gene in other crops

Description	Accession no	Max. Score	Query cover	E value	Identity (%)
PREDICTED: *Nelumbo nucifera* inositol-penta-kisphosphate 2-kinase-like,transcript variant X8, mRNA	XM_010256463.2	59	35 %	2e-04	73.91
PREDICTED: *Ziziphus jujuba* inositol-pentakisphosphate 2-kinase-like, transcript variant X10, mRNA	XM_025068353.1	58.1	13%	5e-04	81.36
PREDICTED: *Ananas comosus* inositol-penta-kisphosphate 2-kinase IPK1, mRNA	XM_020242250.1	56.3	11%	0.002	85.42
PREDICTED: *Citrus sinensis* inositol-pentakisphosphate 2-kinase-like, transcript variant X3, misc_RNA	XR_001507770.2	55.4	14 %	0.002	80.00
PREDICTED: *Vitis vinifera* inositol-pentakisphosphate 2-kinase , mRNA	XM_010666413.2	55.4	10 %	0.002	86.67

4.1.6.4 A1R3 (56.8°C)

The second product of A1R3 amplified at 56.8°C was sequenced with WP2 (Fig 5a). It showed a total length of 498 bp and blast homology analysis showed similarity of sequence with *ipk1* gene of other crops. It showed similarity with various crops like *Daucus carota*, *Papaver somniferum*, *Populus trichocarpa* etc. (Table 16).

>0719_445_001_PCR_A1R3_56_C_WP2-E04.ab1

AACGGGGCCCCGCATTTCTTTTTTTACTGGCGATTATGTGCTAATGTT
ATGAGTCACTGCTAGGCTCAAAGCATGTCGATGCTGGGGTACTTTTCT
ATTTTTAGTTCTTCAATTTTTTTCCTTGGTAACAATTTCAATTTTTGTG
ACTGAGATTGTTCTTTTTTGTTCAATGCTAATGTAATAACCCAGTAAT
GTCAATTCTATTTATAAACTGTTTATATATAATAATATAGTAAATGAA
AAGAGAACCACCTCTCTTATGCAAATTCTTTCCCATTATTGTTGGTAA
CATTATTCTTTTTTTTTTTTTTTAAACCCAAAGGCTTTTTTTTTTAAATC
CCCCCCCCCTTTTAAAAAATTTGGCTTGGTTAAAAAAATTTTTGGGAA
ATTTTGAAAAAAAAATTTCAAAACTTGCCCCCCTTTTGGGGGTTTTAA
AAGGAAAAGGTTAATTTTCCCGGGAATTCCCCCCTTTTTTTTTAAAAA
CTTTCCCTTTTTTCC

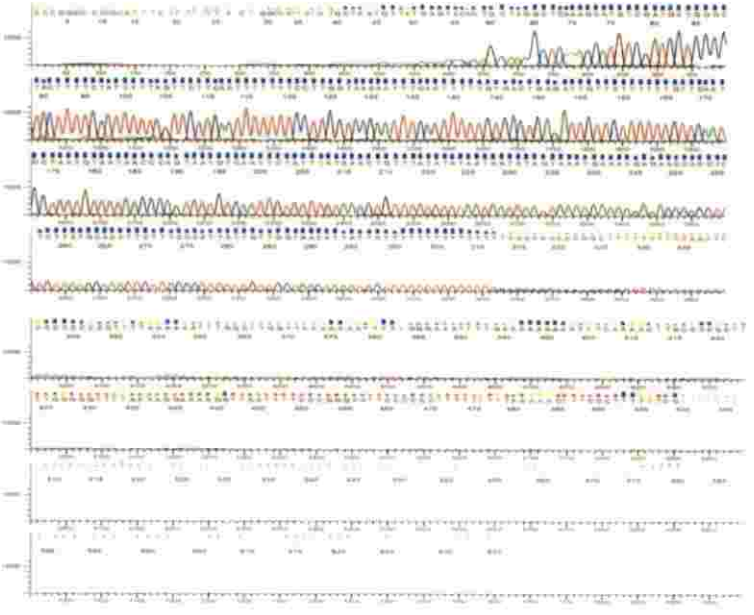


Fig 5a. Sequence of A1R3 at 56.8^{0}C sequenced with WP2

Table 16. Homology of A1R3 (56.8°C, sequenced with WP2) with *ipk1* gene in other crops

Description	Accession no	Max Score	Query cover	E value	Identity (%)
PREDICTED: *Daucus carota* subsp. sativus inositol-pentakisphosphate 2-kinase-like (LOC108224031), Mrna	XM_017398551.1	54.5	9 %	0.008	85.11
PREDICTED: *Papaver somniferum* inositol-pentakisphosphate 2-kinase-like (LOC113288681), mRNA	XM_026537798.1	50	12 %	0.097	78.12
PREDICTED: *Populus trichocarpa* inositol-pentakisphosphate 2-kinase (LOC7471939), transcript variant X3, mRNA	XM_002301984.3	50	8 %	0.097	85.71
PREDICTED: *Arachis ipaensis* inositol-pentakisphosphate 2-kinase (LOC107645264), transcript variant X2, mRNA	XM_021103646.1	48.2	9 %	0.34	82.61

Similarly sequencing of nested PCR product A1R3 amplified at 56.8°C was done with NLSPR3 (Fig 5b) and read a length of 533 bp. Blast homology search was done and found similarity with *ipk1* gene of other crops like *Camellia sinensis*, *Nelumbo nucifera*, *Solanum tuberosum*, *Erythranthe guttatus* etc. (Table 17)

>0719_445_002_PCR_A1R3_56_C_NLSP_R3-F04.ab1

TAAAGAACACTTTTTTGATGAAGTTATCTTCAGCTATGAATTTTGAGAC
TGGAAGAAAACCACACTTTGGCTGCTTCCTCAATCCAAAAAAAACAAA
GAGCGAGTAATTGAGCATTGAGTACATGAATTGATTAAAAAACTAAG
AAGTCATAACATCAAGGGAAATGCCACCTTTATTTCCACTGCTATGCA
CCGATCCTCTCTAACACTTCCTGGAAAGACAATAAAAGAGTATAACAA
AAAACTGACCAATTAAAAAGCAAGAACCATGTGTGAAACAAACTTTCT
TCTGCTAAGAGTTCAAAAGTTAAGTCTTTACCATGGATAAATAGTGAA
TGATCTGAAATAAGAAGAGCTGAATCACAGAGAACATTAACCTTTGCC
TCATCAACACGCCAAGATGGGCGCAAGCTCTGAACTTGTTCTTCCAAT
GATTCCAGAAAGTCTCTTGACACATGCACATGTTTCTAAATGACGAGA
GGAATATAATAAAACAAGCATATGGATTCAAAAAAAAAATAAGTATG
AGTGAT

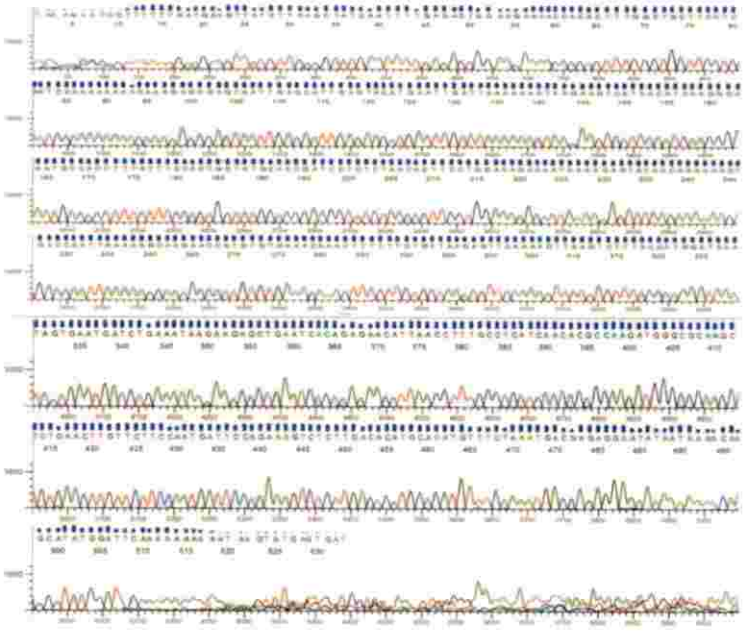


Fig 5b. Sequence of A1R3 at 56.8°C sequenced with NLSPR3

Table 17. Homology of A1R3 (56.8°C, sequenced with NLSPR3) with *ipk1* gene in other crops

Descriptions	Accession no	Max Score	Query cover	E value	Identity (%)
PREDICTED: *Camellia sinensis* inositol-pentakisphosphate 2-kinase-like, transcript variant X3, Mrna	XM_028247579.1	90.6	27 %	1e-13	73.47
PREDICTED: *Nelumbo nucifera* inositol-pentakisphosphate 2-kinase-like, transcript variant X8, mRNA	XM_010256463.2	77.9	37 %	7e-10	72.14
PREDICTED: *Solanum tuberosum* inositol-pentakis-phosphate 2-kinase-like, transcript variant X3, mRNA	XM_015306405.1	68	24 %	4e-07	72.52
PREDICTED: *Erythranthe guttatus* inositol-pentakis-phosphate 2-kinase-like, transcript variant X4, mRNA	XM_012987351.1	67.11	35 %	1e-06	71.97
PREDICTED: *Nicotiana attenuata* inositol-pentakis-phosphate 2-kinase-like, transcript variant X3, mRNA	XM_019400262.1	66.2	14%	1e-06	78.95

17

Table 17 continued

PREDICTED: *Asparagus officinalis* inositol-pentakis-phosphate 2-kinase IPK1-like, transcript variant X3, mRNA	XM_020397897.1	63.5	15 %	2e-05	77.85
PREDICTED: *Capsicum annuum* inositol-pentakisphosphate 2-kinase-like, transcript variant X3, mRNA	XM_016713042.1	61.7	24 %	6e-05	71.54
PREDICTED: *Nicotiana tabacum* inositol-pentakisphosphate 2-kinase-like, transcript variant X7, mRNA	XM_016582667.1	61.7	14%	6e-05	77.63
PREDICTED: *Cajanus cajan* inositol-pentakisphosphate 2-kinase, transcript variant X8, mRNA	XM_020362924.2	55.4	15 %	0.002	74.12

18

4.1.7 Assembly of overlapping regions and sequence analysis

A contig of length 523 bp was obtained by assembly of PCR products which showed similarity with *ipk1* gene in other crops with the help of DNA Baser software. This contig was assembled with *pnipk1* (1072 bp) in DNA Baser and got a sequence assembly with a length of 1535 bp (Fig 7).

Blast homology search was done with newly assembled *ipk1* gene sequence (*pnipk1*-1535 bp) and found similarity with *ipk1* gene of other crops (Table 18). Analysis was done in ORF finder for the coding regions of assembled sequence (Fig 6a). It showed 20 different open reading frames (ORFs) and ORF 2 was the longest with 645 bp, coding for 214 amino acids (Fig 6b). The ORF is included in the 5'-3' frame-1 with the start end at 652 bp and stop end at 1296 bp. When searched for conserved domains, one superfamily was detected named InsP5 kinase superfamily (cl05564) belonging to accession pfam06090. The amino acid sequence obtained by analysis in ORF Finder were subjected for BLASTp homology search (Table 19).

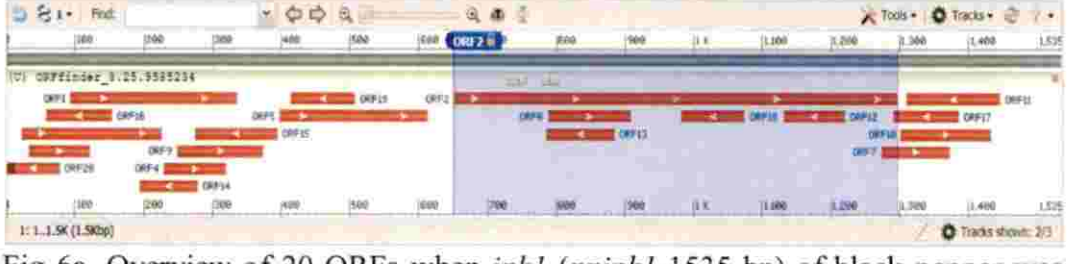


Fig 6a. Overview of 20 ORFs when *ipk1* (*pnipk1*-1535 bp) of black pepper was analysed in ORF Finder

>lcl|ORF2

MYTWIWLSFSNYFSLLSQIGEISEYDPVDLFSGSKDRINRAMKSLYAIPQ
NNFRIFFNGSLLFGGLGGAMDNTKSKSMEAIDDELGVLIDAAPGLRLSR
FLELVSLTILQSDVLVGLLNTQMFDLLDIEGAIHAYYNIISQPNMVGKVL
GGLSKKYFSLHYLSLEASVKIVRDFLIAATAKDCSLMISFRLLSGEMISEI
DVVHLETTGQSFLL

Fig 6b. Amino acid sequence of ORF2

Newly Assembled sequence, *pnipk1*(1535 bp)

5'ATCACTCATACTTATTTTTTTTTTGAATCCATATGCTTGTTTTATTATATTCCTCTCGTCATTTAGAAACATGTGCATGTGTCAAG
AGACTTTCTGGAATCATTGGAAGAACAAGTTCATAGCTTGCGCCCATCTTGGTGTGTTGATGAGGCAAAGGTTAATGTTCTCTGT
GATTCAGCTCTTGTTATTTCAGATCATTCACTATTTATCCATGGTAAAGACTTAACTTTTGAACTCTTGCAGAGAAGTTGTTTCAAC
ATTTCTTGCTTTTTATTCAGTTTTTTGTTATACTCTTTATTGTCTTTCCAGGAAGTGTTAGAGAGGATCGGTGCATAGCAGTGGAAA
TAAAGGTGGCATTTCCCTTGATGTTATGACTTCTTAGTTTTTTAATCAATTCATGTACTCAATGCTCAATTACTCGCTCTTTGTTTTT
TTTGGATTGAGGAAGCAGCCAAAGTGTGGTTTTCTTCCAGTCTCAAAATTCATAGCTGAAGATAACTTCATCAAAAAGTGTGTGT
CTCGTTTTAAAATGCACCAACCCTTGAAATTTCACAAGAAAGAGGTAATTTTCACCTGTGCTTATATATTCCTTTGTTTAAAAGAA
AAAACTTAGATTACACTAATTGTTGAGGATGCTTTTCTTCATCCTTTCTGTACACATGGATTTGGCTATCATTTTCTAATTATTTTT
CACTTCTCTCTCAGATAGGGGAAATAAGTGAGTATGATCCAGTCGATCTGTTTTCAGGTTCTAAAGATAGAATAAATCGTGCTAT
GAAATCTCTCTATGCCATCCCGCAAAACAATTTTCGTATCTTTTTCAATGGCTCTTTGTTATTTGGTGGATTGGGGGGAGCCATGG
ATAATACGAAATCTAAATCCATGGAAGCTATTGATGATGAATTAGGTGTGTTAATTGATGCAGCACCTGGACTACGACTATCTAG
ATTTCTGGAACTTGTTTCATTGACAATTTTACAGTCAGATGTCCTGGTTGGTCTTCTTAATACGCAAATGTTTGACCTTCTTGATAT
AGAAGGGGCAATTCATGCCTATTACAACATTATTTCTCAACCTAACATGGTCGGCAAGGTCCTAGGCGGCCTTTCTAAGAAGTAC
TTTTCCCTACATTATCTTTCTTTGGAAGCAAGTGTTAAGATTGTTCGCGATTTTTTGATCGCTGCAACTGCAAAGGACTGTAGCTTG
ATGATCAGTTTCAGACTGTTATCAGGGGAGATGATATCTGAAATTGATGTTGTGCACTTAGAGACAACTGGCCAAAGTTTTTTGC
TATAAGGTACTTGATGTGCTCATCTCCTTGGTTTGTTTTTGTGATGATATTGCCCTTGAACCATTTTCATTACTCCTATGATCTGAA
TTTTGCAATGCTGCCTATTAAGAATTATGTAGATGAAGAAAACATGTGTTATTAATAAATTTAACATGCATCAATGAAGTTCTGA
AATTGCATGAATTAATTTATTTTTATGTCTATTATATTTTGTGAAACCATTTTAAGGTTAGTGTTAAACACC 3'

Fig 7. Gene sequence of inositol pentakisphosphate-2-kinase gene (*ipk1*) sequence (*pnipk1*-1535 bp) in black pepper

Table 18. Crops showing homology with newly assembled *ipk1* gene sequence, (*pnipk1*-1535bp) in black pepper

Descriptions	Max score	Query cover	E value	Identity %	Accession
PREDICTED: *Vitis vinifera ipk1*, mRNA	155	41%	1e-32	67.05	XM_010666413.2
PREDICTED: *Nelumbo nucifera ipk1* like, mRNA	142	48%	7e-29	66.99	XM_010256463.2
PREDICTED: *Papaver somniferum ipk1* like, mRNA	141	39%	7e-29	66.54	XM_026537798.1
PREDICTED: *Morus notabilis ipk1*, mRNA	132	33%	1e-25	66.86	XM_024174507.1
PREDICTED: *Dendrobium catenatum* IPK1, mRNA	125	26%	5e-24	70.10	XM_020819929.2
PREDICTED: *Musa acuminata* subsp. malaccensis *IPK1*, mRNA	122	40%	6e-23	65.52	XM_009391905.2
PREDICTED: *Phalaenopsis equestris* IPK1-like, mRNA	116	25%	3e-21	69.49	XM_020718170.1
PREDICTED: *Ricinus communis ipk1*, mRNA	115	39%	9e-21	65.63	XM_015720256.2
PREDICTED: *Ipomoea nil ipkl* like, mRNA	113	33%	3e-20	66.47	XM_019296805.1
PREDICTED: *Camellia sinensis ipk1* like, mRNA	110	41%	4e-19	65.98	XM_028232158.1
PREDICTED: *Populus trichocarpa ipk1*, mRNA	106	40%	5e-18	65.54	XM_002301984.3
PREDICTED: *Cicer arietinum ipk1* like, mRNA	105	19%	5e-18	83.49	XM_004500934.3
PREDICTED: *Camelina sativa ipk1*, mRNA	102	19%	6e-17	74.83	XM_010479163.2
PREDICTED: *Eucalyptus grandis ipk1*, mRNA	102	33%	6e-17	67.03	XM_010062987.2

Table 19. Homology of amino acid sequence of *ipk1* (*pnipk1*-1535 bp) gene in black pepper with that of other crops.

Descriptions	Max score	Query cover	E value	Identity (%)	Accession
Phoenix dactylifera, IPK1	214	91 %	7e-65	60.20	XP_026662195.1
Durio zibethinus, ipk1-like	214	91 %	5e-64	57.14	XP_022769023.1
PREDICTED:*Nicotiana tabacum, ipk1* like	211	92 %	2e-63	56.06	XP_016435252.1
Low quality protein: *Malus domestica ipk1* like	213	91 %	2e-63	56.06	XP_017184491.2
Cephalotus follicularis Ins_P5_2-kin domain-containing protein	213	90 %	2e-63	56.35	GAV88707.1
PREDICTED: *Populus euphratica, ipk1*-like	211	91 %	7e-63	56.06	XP_011048791.1
Brassica napus, ipk1	209	91 %	5e-62	53.54	XP_013744383.1
PREDICTED:*Nicotiana tomentosiformis, ipk1*-like	208	91 %	1e-61	53.54	XP_009621351.1
Camellia sinensis, Ipk1 like	206	91 %	1e-61	55.05	XP_028087959.1
Phalaenopsis equestris IPK1-like, partial	200	90 %	3e-61	55.84	XP_020598130.1
Macleaya cordata, ipk 1	220	92 %	4e-66	57.50	OVA00539.1
PREDICTED: ipk1 *Raphanus sativus, ipk1*	204	91 %	4e-60	52.28	XP_018452190.1
IPK1 *Elaeis guineensis, IPK1*	204	91%	5e-60	57.71	XP_010940107.1
PREDICTED:*Gossypium arboretum, ipk1* like	204	91 %	5e-60	53.54	XP_017611662.1
PREDICTED: *Ipomoea nil, ipk1* like	204	91 %	5e-60	53.81	XP_019152350.1
PREDICTED: *Prunus mume, ipk1*	204	91 %	6e-60	52.02	XP_008220840.1
Herrania umbratica ipk1 like	202	80 %	3e-58	58.86	XP_021283697.1
Prunus persica ipk1	204	91 %	2e-59	52.23	XP_020425159.1

4.1.8 Primer designing for amplification and validation of assembled *ipk1* gene (*pnipk1*-1535 bp)

Primers were designed for the confirmation of assembled *ipk1* (*pnipk1*-1535 bp) gene sequence in Panniyur 1. Primers were designed with the help of BLAST. Forward primer and reverse primer were designed from the 5' and 3' ends of assembled sequence for amplification of *ipk1* gene (*pnipk1*-1535 bp) in genomic DNA of Panniyur 1(Fig 8). Details of primers are given below in Table 20.

Table 20. Primers for validation of newly assembled *ipk1* gene fragment (*pnipk1*-1535 bp) in black pepper

Primer	Sequence (5'---3')	Length (bp)	Tm (°C)	Expected amplicon
F11	ATAGCTTGCGCCCATCTTGG	20	68	1162 bp
R11	TGGCCAGTTGTCTCTAAGTGC	21	64	

4.1.9 PCR amplification of assembled *ipk1* gene (*pnipk1*-1535 bp)

PCR reactions were performed in order to amplify the assembled *ipk1* sequence, (*pnipk1*-1535 bp) using forward primer F11 and reverse primer R11. A total reaction volume upto 25 µl was set up. Gradient PCR was run at temperatures between 61 - 66°C. Amplification was found at all temperature tested and all the amplicons were in expected size region (Plate 4).

Newly Assembled sequence, *pnipk1* (1535 bp)

5'ATCACTCATACTTATTTTTTTTTTGAATCCATATGCTTGTTTTATTATATTCCTCTCGTCATTTAGAAACATGTGCATGTGTCAAGAGAC
TTTCTGGAATCATTGGAAGAACAAGTTCATAGCTTGCGCCCATCTTGGTGTGTTGATGAGGCAAAGGTTAATGTTCTCTGTGATTCAGCT
CTTGTTATTTCAGATCATTCACTATTTATCCATGGTAAAGACTTAACTTTTGAACTCTTGCAGAGAAGTTGTTTCAACATTTCTTGCTTTT
TATTCAGTTTTTTGTTATACTCTTTATTGTCTTTCCAGGAAGTGTTAGAGAGGATCGGTGCATAGCAGTGGAAATAAAGGTGGCATTTCC
CTTGATGTTATGACTTCTTAGTTTTTTAATCAATTCATGTACTCAATGCTCAATTACTCGCTCTTTGTTTTTTTTGGATTGAGGAAGCAGC
CAAAGTGTGGTTTTCTTCCAGTCTCAAAATTCATAGCTGAAGATAACTTCATCAAAAAGTGTGTGTCTCGTTTTAAAATGCACCAACCCT
TGAAATTTCACAAGAAAGAGGTAATTTTCACCTGTGCTTATATATTCCTTTGTTTAAAAGAAAAAACTTAGATTACACTAATTGTTGAG
GATGCTTTTCTTCATCCTTTCTGTACACATGGATTTGGCTATCATTTTCTAATTATTTTTCACTTCTCTCTCAGATAGGGGAAATAAGTGA
GTATGATCCAGTCGATCTGTTTTCAGGTTCTAAAGATAGAATAAATCGTGCTATGAAATCTCTCTATGCCATCCCGCAAAACAATTTTCG
TATCTTTTTCAATGGCTCTTTGTTATTTGGTGGATTGGGGGGAGCCATGGATAATACGAAATCTAAATCCATGGAAGCTATTGATGATG
AATTAGGTGTGTTAATTGATGCAGCACCTGGACTACGACTATCTAGATTTCTGGAACTTGTTTCATTGACAATTTTACAGTCAGATGTCC
TGGTTGGTCTTCTTAATACGCAAATGTTTGACCTTCTTGATATAGAAGGGGCAATTCATGCCTATTACAACATTATTTCTCAACCTAACA
TGGTCGGCAAGGTCCTAGGCGGCCTTTCTAAGAAGTACTTTTCCCTACATTATCTTTCTTTGGAAGCAAGTGTTAAGATTGTTCGCGATT
TTTTGATCGCTGCAACTGCAAAGGACTGTAGCTTGATGATCAGTTTCAGACTGTTATCAGGGGAGATGATATCTGAAATTGATGTTGTG
CACTTAGAGACAACTGGCCAAAGTTTTTTGCTATAAGGTACTTGATGTGCTCATCTCCTTGGTTTGTTTTTGTGATGATATTGCCCTTGA
ACCATTTTCATTACTCCTATGATCTGAATTTTGCAATGCTGCCTATTAAGAATTATGTAGATGAAGAAAACATGTGTTATTAATAAATTT
AACATGCATCAATGAAGTTCTGAAATTGCATGAATTAATTTATTTTTATGTCTATTATATTTTGTGAAACCATTTTAAGGTTAGTGTTAA
ACACC 3'

Fig 8. Assembled *ipk1* gene sequence (*pnpik1*-1535 bp) with primers for validation of gene

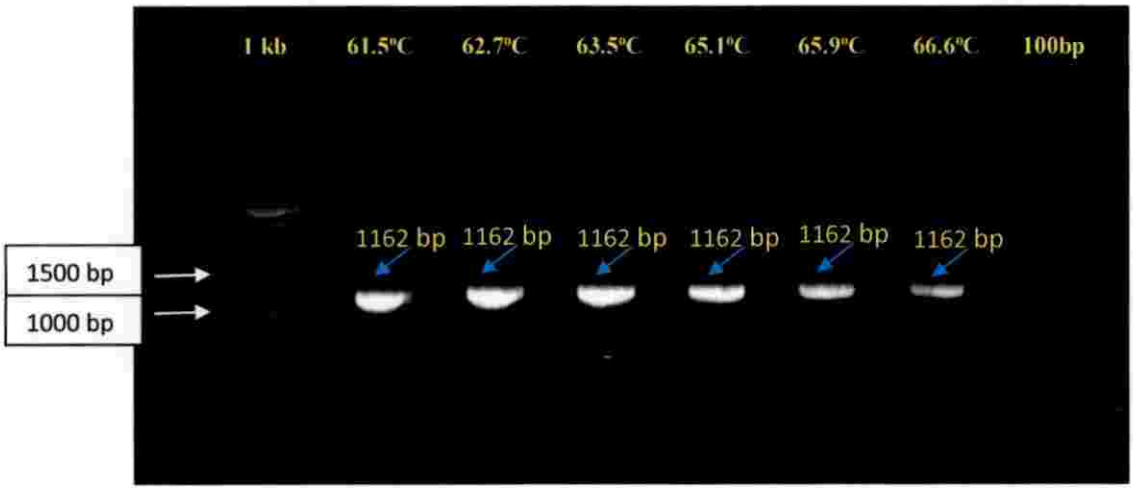


Plate 4. Gel image of products of PCR amplification of assembled *ipk1* gene sequence (*pnipk1*-1535 bp)

4.1.10 Phylogenetic analysis

The BLAST homology search of assembled *ipk1* gene sequence (*pnipk1*-1535 bp) revealed that it had similarity with *ipk1* gene sequence in other crop species (26 crop species) with an identity value between 65 and 83 % and with a query coverage of 12 to 48 % in range. Phylogenetic analysis was performed with the assembled sequence along with crop species showing identity greater than 65 % along with a query cover greater than 25 per cent (Table 21).

Table 21. List of crop species used for phylogeny tree construction.

Sl. No.	Plants	Accession No.
1	*Vitis vinifera*	XM_010666413.2
2	*Nelumbo nucifera*	XM_010256463.2
3	*Papaver somniferum*	XM_026537798.1
4	*Morus notabilis*	XM_024174507.1
5	*Dendrobium catenatum*	XM_020819929.2
6	*Ricinus communis*	XM_015720256.2
7	*Ipomoea nil*	XM_019296805.1
8	*Phalaenopsis equestris*	XM_020718170.1
9	*Durio zibethinus*	XM_022913292.1
10	*Populus trichocarpa*	XM_002301984.3
11	*Camellia sinensis*	XM_028232158.1

The phylogeny tree mainly exhibited 4 branches (Fig 9). One branch had only *Papaver somniferum*, second branch with a single crop species *Nelumbo nucifera*, third branch consisted of a group containing *Piper nigrum* and *Dendrobium catenatum* and *Phalaenopsis equestris* and fourth branch consisted of *Vitis vinifera*, *Morus notabilis*, *Ricinus communis*, *Ipomoea nil*, *Durio zibethinus*, *Populus trichocarpa*, *Camellia sinensis*.

Similarly, phylogenic tree based on amino acid sequence translated from assembled sequence of *ipk1* gene (*pnipk1*-1535 bp) was also constructed. When homology search in BLASTp was done, the translated amino acid sequence showed similarity with *ipk1* of about 50 different crop plants along with a query cover ranging between 90-92% and identity between 50-58%. For constructing phylogenetic tree, sequences having more than 50 percent identity with the amino acid sequence translated from *ipk1* gene sequence (*pnipk1*-1535 bp) was selected (Table 22).

Table 22. List of amino acid sequences of different crop species phylogeny tree construction

Sl. No.	Organism	Accession No.
1	*Phoenix dactylifera*	XP_026662195.1
2	*Durio zibethinus*	XP_022769023.1
3	*Nicotiana tabacum*	XP_016435252.1
4	*Malus domestica*	XP_017184491.2
5	*Cephalotus follicularis*	GAV88707.1
6	*Populus euphratica*	XP_011048791.1
7	*Brassica napus*	XP_013744383.1
8	*Nicotiana tomentosiformis*	XP_009621351.1
9	*Camellia sinensis*	XP_028087959.1
10	*Phalaenopsis equestris*	XP_020598130.1
11	*Macleaya cordata*	OVA00539.1

The phylogeny tree constructed in BLAST had 4 main branches (Fig 10). *Brassica napus* was singly occupied. Similarly, *Durio zibethinus* was also grouped into a separate group. The third branch clustered with *Populus euphratica, Macleaya cordata, Piper nigrum, Phoenix dactylifera* and *Phalaenopsis equestris*. In the third main group, *Piper nigrum* was subgrouped into a group containing *Phoenix dactylifera* and *Phalaenopsis equestris* indicating

the evolutionary relationship of *Piper nigrum* with *Phoenix dactylifera* and *Phalaenopsis equestris*. The fourth group contained *Camellia sinensis, Cephalotus follicularis, Malus domestica, Nicotiana tabacum* and *Nicotiana tomentosiformis*.

In both the phylogeny tree constructed based on the nucleotide sequence of *ipk1* gene sequence (*pnipk1*-1535 bp) and translated amino acid sequence obtained from *ipk1* gene (*pnipk1*-1535 bp), *Piper nigrum* was found to exhibit a revolutionary relationship with *Phalaenopsis equestris*.

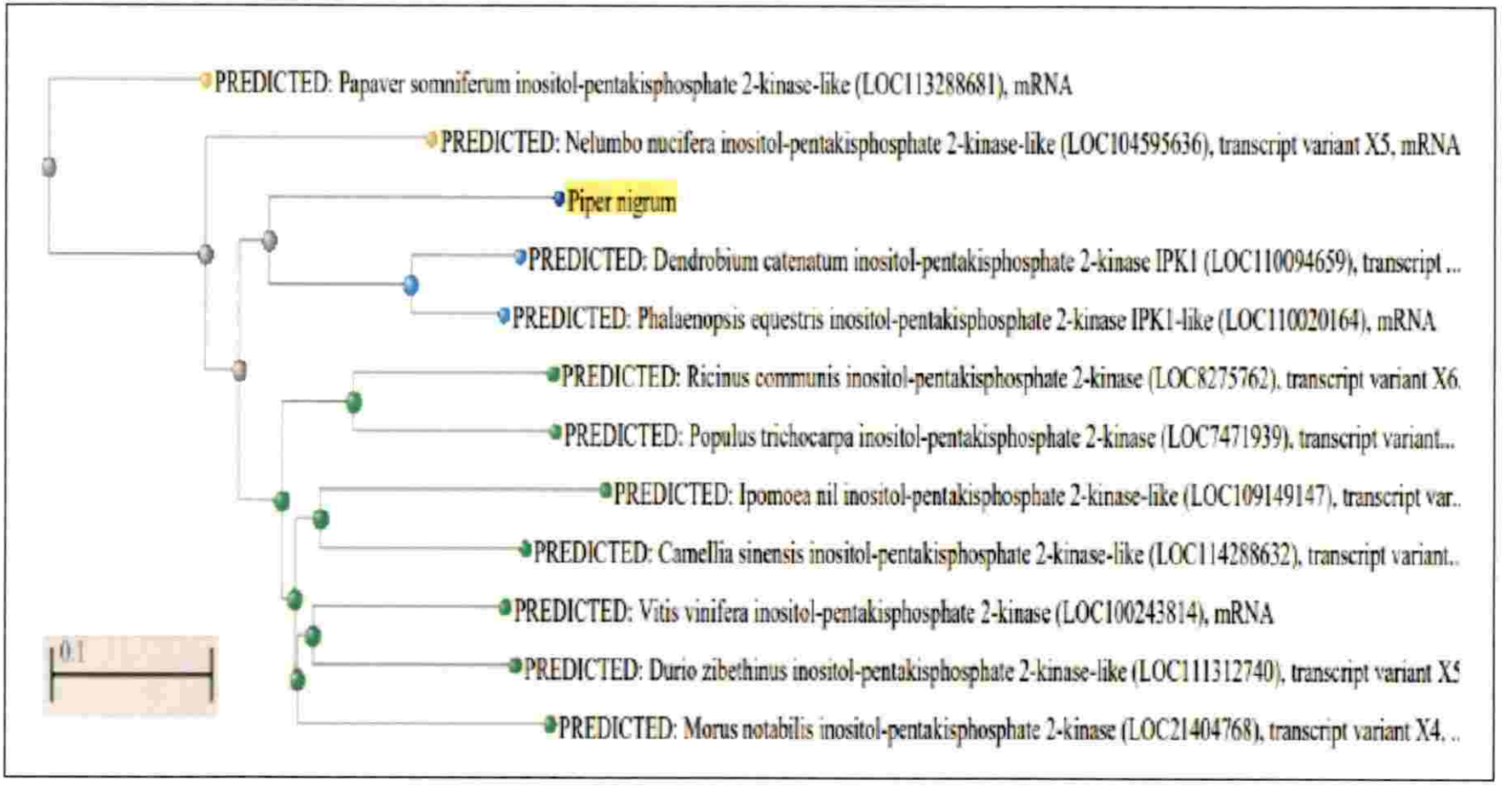


Fig 9. Phylogeny tree constructed with crop species showing similarity with assembled sequence of *ipk1* gene (*pnipk1*-1535 bp)

Evolutionary relationship was analysed with the help of BLAST software using neighbourhood joining method

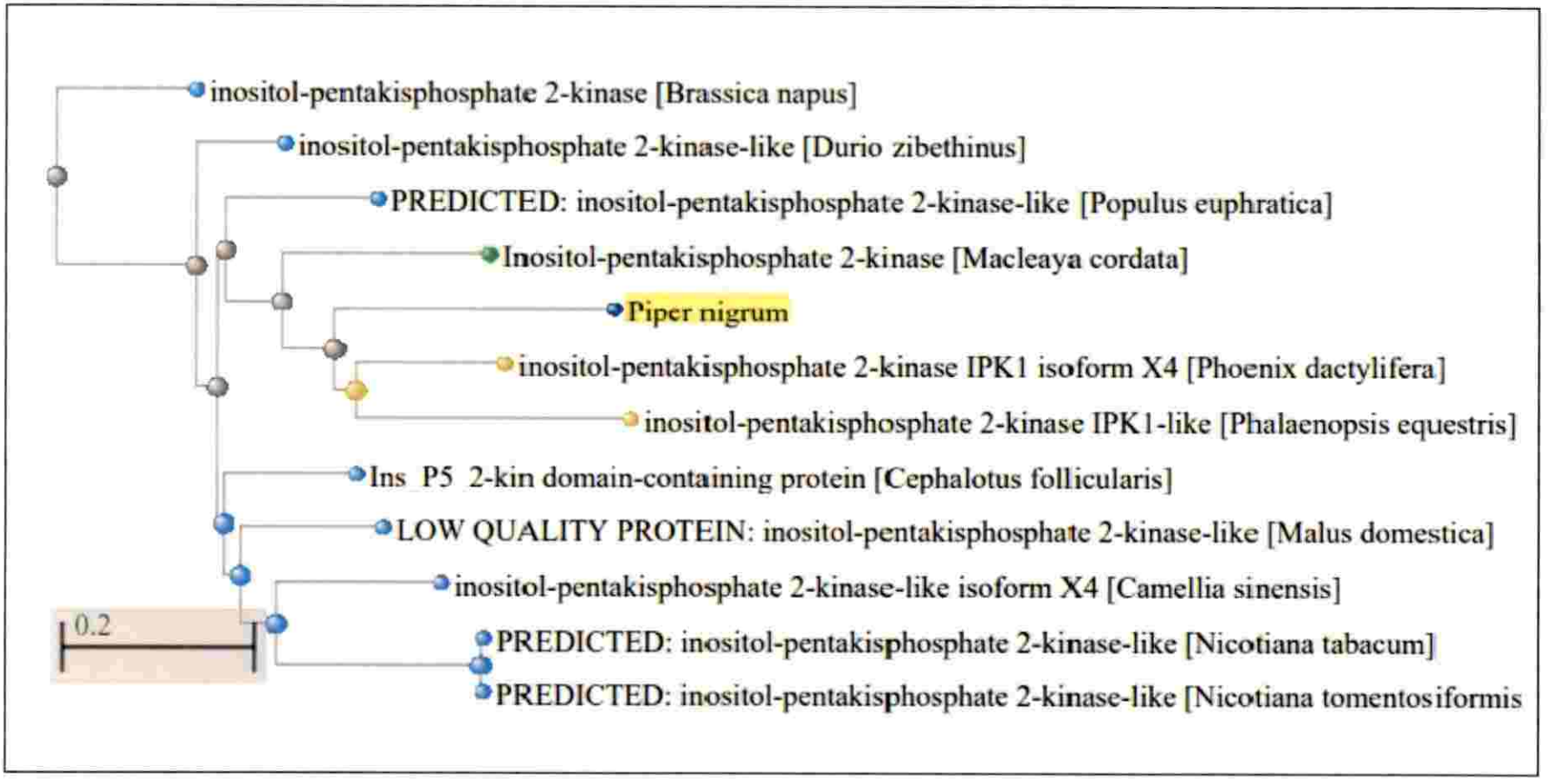


Fig 10. Phylogeny tree constructed based on the translated amino acid sequence of *ipk1* gene (*pnipk1*-1535 bp) along with crops showing similarity with it.

BLAST software was used to analyse the evolutionary relationship of amino acid sequence with the other crops using neighbourhood joining method.

4.2 Allelic difference analysis in black pepper genotypes

4.2.1 Genomic DNA isolation of ten black pepper genotypes for validation of assembled *ipk1* gene sequence

For finding out the allelic difference in *ipk1* gene (*pnipk1*-1535 bp) among different black pepper genotypes, genomic DNA was isolated from Panniyur 1 and 10 other black pepper genotypes using modified CTAB extraction protocol (Plate 5). The obtained DNA pellets were dissolved in nuclease free sterile water and kept in - 20°C.

4.2.2 Spectrophotometric analysis

The quantity and quality of the isolated DNA was determined with the help of spectrophotometer. DNA was diluted at 1:50 ratio for the spectrophotometric analysis. The details of readings taken for the 10 black pepper genotypes are given below (Table 23)

Table 23. Details of quantity and quality of genomic DNA of 10 black pepper genotypes

Sl no	Black pepper genotypes	$A_{260/280}$	DNA yield (µg/µL)
1	Panniyur 5	1.92	4.87
2	Panniyur 7	1.85	3.69
3	Chettanvally	2.09	4.21
4	Kottanadan	1.84	4.65
5	Karimunda 7	1.78	4.93
6	PRS 160	1.98	3.67
7	Chumala	2.03	4.05
8	Thotamundy	2.01	3.63
9	Karimunda kuttyatur	1.96	3.28
10	Payyanganam 2	1.88	3.84

4.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to check the quality and integrity of DNA isolated from the black pepper genotypes. Five µL DNA sample mixed with 1 µL 6X gel loading dye was loaded in 1 % agarose gel. A clear band was observed when agarose gel was visualised in gel documentation system (BioRad image reader).

4.2.4 PCR Amplification for determining allelic difference in *ipk1* gene (*pnipk1*-1535 bp) of black pepper

The genomic DNA isolated from 10 black pepper genotypes along with genomic DNA of Panniyur 1 was amplified with a forward primer F11 and a reverse primer R11. The PCR reaction mix was set up with a total volume of 25 µL and amplified at an annealing temperature of 61.5°C. The obtained PCR products were separated on 1% agarose gel in 1X TBE and after running gel electrophoresis, gel was visualised with gel documentation system. Amplification was obtained in the black pepper genotypes Panniyur 7, Panniyur 5, Karimunda 7 and Panniyur 1 (Plate 6). The amplified bands obtained from genomic DNA of Panniyur 7, Panniyur 5, Karimunda 7 were of same size as that obtained in the genomic DNA of Panniyur 1. No amplification was obtained in the genomic DNA of other black pepper genotypes (Chumala, Thotamundy, Chettanvally, Kottanadan, PRS 160, Karimunda kuttyatur and Payyanganam 2). The amplification in Panniyur 5, Karimunda 7, Panniyur 7 suggested that there was no allelic variation with the *ipk1* gene sequence (*pnipk1*-1535 bp) assembled in the Panniyur 1 variety of black pepper.

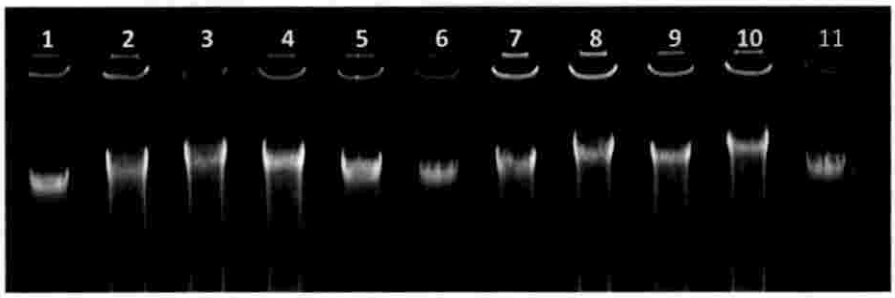


Plate 5. Gel image of genomic DNA from 10 black pepper genotypes along with Panniyur 1

Lane 1- Panniyur 1, lane 2-Panniyur 5, lane 3-Panniyur 7, lane 4-Chettanvally, lane 5- Kottanadan, lane 6- Karimunda 7, lane 7- PRS 160, lane 8- Chumala, lane 9- Thotamundy, lane 10- Karimunda kuttyatur, lane 11- Payyanganam 2

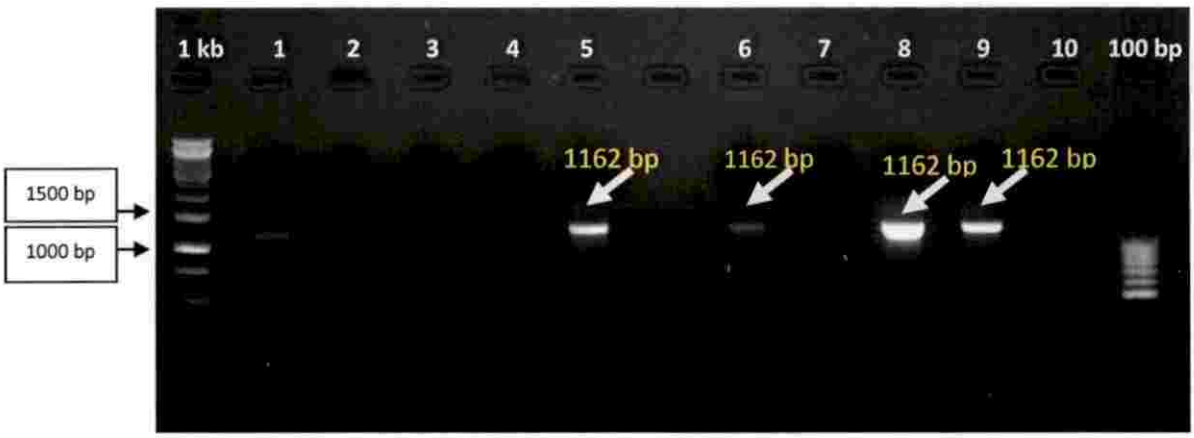


Plate 6. Gel image of PCR products amplified with F11 and R11 primers designed for *ipk1* (*pnipk1*-1535 bp) gene in black pepper genotypes

Lane 1 - Panniyur 5, lane 2 - Chumala, lane 3- Kottanadan, lane 4 - Chettanvally, Lane 5 – Panniyur 1, lane 6 - Panniyur 5, lane 7 – Karimunda kuttyatur, lane 8 - Karimunda 7, lane 9 - Panniyur 7, lane 10 – Payyanganam 2

4.3 Quantification of phyitc acid using PAGE

Estimation of phytic acid in 10 different black pepper genotypes along with Panniyur 1 and standard phytates was performed according to a protocol suggested by Alimohammadi *et al.* (2013). Phytic acid was detected with the help of polyacrylamide gel electrophoresis (PAGE) analysis (Plate 7a and Plate 7b) and estimated by comparing the values of standard phytates prepared from standard phytic dipotassium salt (Table 24). A standard curve was plotted with quantity of standard phytates (nanomoles) along X-axis and volume (intensity) along Y-axis (Fig 10).

Table 24. Band intensities of concentration of different phytate standards

Quantity (nM)	Volume (intensity)
5	3531804
10	3784502
15	4216485
20	4326182
25	4623900

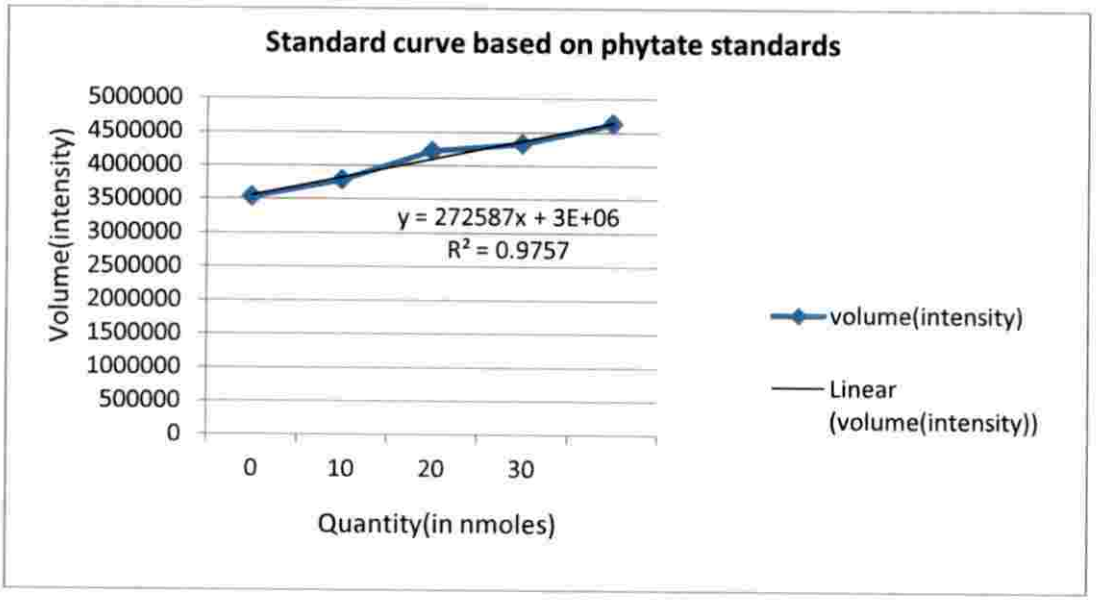


Fig 10. Standard curve obtained based on band intensities of different concentrations of standard phytates.
X-axis- Quantity of phytate standard (nM) loaded in gel.
Y-axis- Volume/band intensities corresponding to the standards loaded on gel.

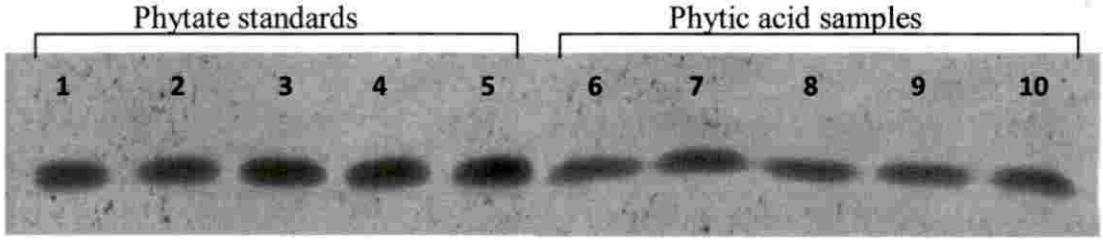


Plate 7a. Image of PAGE gel loaded with phytate standards along with black pepper varieties
Lane 1-5 nM phytate, lane 2 -10 nM phytate, lane 3-15 nM phytate, lane 4 - 20 nM phytate, lane 5 - 25 nM phytate, lane 6 – PRS 160, lane 7 – Karimunda Kuttyatur, lane 8 - Kottanadan, lane 9 - Chettanvally, lane 10 – Panniyur 5

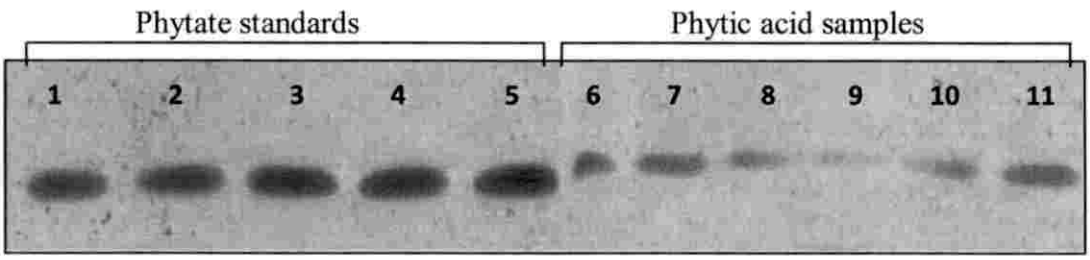


Plate 7b. Image of PAGE gel loaded with phytate standards along with black pepper varieties
Lane 1-5 nM phytate, lane 2-10 nM phytate, lane 3-15 nM phytate, lane 4 - 20 nM phytate, lane 5 - 25 nM phytate, lane 6 - Panniyur 1, lane 7-Thotamundy, lane 8 - Panniyur 7, lane 9 - Chumala, lane 10 - Payyanganam 2, lane 11 – Karimunda 7

Table 25. Quantity of phytic acid of 10 black pepper genotypes

Sl. No.	Black pepper genotypes	Phytate content (nM / g)
1	Panniyur 5	502.5
2	Panniyur 7	367.5
3	Chettanvally	511.5
4	Kottanadan	463.5
5	Karimunda 7	387.0
6	PRS 160	697.5
7	Chumala	201.0
8	Thotamundy	198.0
9	Karimunda kuttyatur	637.5
10	Payyanganam 2	196.5
11	Panniyur 1	275.0

The phytic acid contents of samples of black pepper variety was worked out using the equation obtained by standard curve plotted (Volume = 272587× Quantity of InsP6+ 3E+06) (Table 25). The values were obtained in nM InsP6 per 10 µL of plant sample. Thereafter the value was converted to nM / g. The highest phytic acid content among black pepper genotypes was observed in PRS 160 (697.5 nM / g) and lowest phytic acid content was observed in Payyanganam 2 (196.5 nM / g).

Discussion

5. DISCUSSION

Black pepper (*Piper nigrum* L.), due to its wide demand in international trade, has been titled as the 'black gold' proving its huge significance in various fields. It is also called as 'King of Spices' establishing its importance among other spices. Since ages, black pepper has been used in various traditional medicines and also for daily requirements. Among its family Piperaceae, black pepper is the most cultivated species owing to its huge pharmacological potential and spicy qualities. This potentiality is solely due to the different alkaloids (major one is piperine) and volatile oils present in it. It has got versatile pharmacological roles like anti-tumour, anti-obesity, anti-fungal, anti-thyroid, insecticidal, hepato-protective, anti-inflammetry, anti-diabetic, anti-fertility, blood purifier, immune-modulator and anticancer etc. (Joshi *et al.*, 2018). Moreover black pepper being native of India, the indigenous genetic resources for favourable genes are still waiting to be exploited for crop improvement programmes, thus making it a perfect area for genetic studies and programmes for scientists.

The execution of high throughput sequencing methods is highly effective for molecular study and exploration of gene sequences and their expression. Our understanding about chromosomes and unravelled genome sequences of organisms has undoubtedly increased with the high throughput genome sequencing technologies. These next generation sequencing methods like genome walking along with the discovery of PCR techniques and *de novo* sequencing has tremendously empowered us to explore the involvement of gene in various metabolic pathways and discovery of unknown gene sequences.

With the help of Ion Torrent technology, a unigene *viz.*, *pnc135* was able to be sequenced by assembling several contigs generated from expressed sequence tags (ESTs) from developing berries of black pepper. The unigene was reported to have a length of 995 bp with exon size ranging from 1 to 941 bp. Gene ontology analysis revealed that the obtained unigene showed similarity with a putative gene *ipk1* gene encoding for the enzyme insoitol pentakisphosphate-2-kinase, which

play an important role in the metabolic pathway of inositol hexakisphosphate, otherwise commonly called phytic acid (Sujatha, R., Unpublished data). The role and functions of this compound are discussed in chapter 2 'Review of Literature.

Later this unigene, '*pnc135*' was partially sequenced towards 3' end of the gene and the assembled sequence 'F6R5-NLSPF1' had a length of 1072 bp which was designated as *pnipk1* (Giridhari, 2017). However the sequencing towards 5' end of the newly assembled sequence was not completed.

The present study was therefore conducted to sequence the *ipk1* gene in black pepper as no previous data containing the information about the gene sequence was available in black pepper and related crops. The complete sequence will help in acquiring genetic information for researches in various crop improvement programmes in future.

A high throughput genome walking method reported by Reddy *et al.* (2008) was employed for deciphering the gene sequence of *ipk1* gene and this method relied upon RCA of the whole genome utilising the enzyme Phi 29 DNA polymerase along with locus specific and nested locus specific amplification. With the help of sequence specific primers, it was possible to identify the unknown flanking regions, if a known part of gene sequence is present. Various limitations like lack of specificity, requirement of restriction maps, finite walk range and failure rates of other genome walking techniques such as ligation mediated PCR, inverse PCR, randomly primed PCR etc. are surpassed by this protocol of genome walking reported by Reddy *et al.* (2008). The advantage of this high throughput method is that universal primers and sequence specific primers are used for characterizing the unknown flanking regions of a gene even if the known region of target gene constitute a smaller region, thus lowering the dependence on ligation and restriction methods.

For the detection and quantification of phytic acid in black pepper varieties, polyacrylamide gel electrophoresis (PAGE) was performed and the

analysis and values were obtained based on the standard curve obtained with standard phytic acid available commercially.

5.1 Genome Walking

Genome walking is a systematic strategy for finding out an unknown flanking region from a known region of gene. The convenience of a genome walking method is that it allows the identification of an unknown gene sequence in a species, if there is a known region of the target gene with stable conserved regions in the characterised species. The gene *ipk1* is solely responsible for the production of inositol pentakisphosphate-2-kinase enzyme in higher plants. The final step in the biosynthesis pathway of phytic acid is the phosphorylation of myoinositol 1,3,4,5,6 pentakisphosphate to the phytic acid and this step is being catalysed by the inositol pentakisphosphate-2-kinase enzyme (Matrynov and Dhorkov, 2014). Two pathways have been identified for biosynthesis of phytic acid, one is lipid independent and the other is lipid dependent pathway (Raboy and Bowen, 2006).

In the present investigation, genome walking was done by rolling circle amplification method using Phi 29 DNA polymerase for identification of *ipk1* gene sequence in black pepper variety Panniyur 1.

Good quality DNA was isolated from fresh leaves of Panniyur 1 for whole genome amplification and other PCR amplifications. Genomic DNA was checked for its purity both quantitatively and qualitatively. DNA was isolated according to the protocol of modified CTAB method (Doyle and Doyle, 1987).

5.1.1 Rolling circle amplification (RCA) of genomic DNA

Rolling circle amplification is a whole genome amplification method in which multiple overlapping products are generated by flicking apart the 5' ends along downstream strand covering the whole genome. It is more advantageous than the existing genome walking protocols like inverse and randomly primed PCR which requires restriction and ligation processes; and often leads to off-

target amplification and generation of fragments of length 300 to 10000 bp products.

In the present study, four different walker adaptors with partial degeneracy at its 3' end reported by Reddy *et al.* (2008) were first annealed to the genomic DNA of Panniyur 1. These walker adapters will randomly bind to the genomic DNA and with the help of Phi 29 DNA polymerase enzyme, and causes multiple strand displacement leading to overlapping genomic strands. The high processivity and tight binding of Phi 29 DNA polymerase will result in a number of larger strands of average size ranging between 12-15 kb (Cheung and Nelson, 1996).

5.1.2 Directional genome walking towards 5' end

Genome walking method is found to be an efficient method of exploring the flanking unknown regions like promoter region from a known gene sequence (Shapter and Waters, 2014). The position and direction of genome walking will be decided by the locus specific primers. Genome walking with locus specific primers will provide selective amplification with lesser off-targets.

Locus and nested locus specific primers were designed based upon the partially sequenced *pnipk1* gene (1072 bp) assembled by Giridhari (2017) in black pepper variety of Panniyur 1. This was the only information regarding *ipk*1 gene of black pepper variety Panniyur 1, no other sequence information was available for reference in other black pepper varieties or in any related crops.

Four amplicons with an approximate length ranging between 750 bp and 1 kb were obtained after genome walking. The fragments obtained by sequencing A4R3(51.6°C), A4R3(56.8°C) and A1R3(51.6°C) with WP2 did not exhibited any similarity with the *ipk1* gene of other crops whereas the sequence information obtained by sequencing these amplicons towards NLSPR3 showed similarity with *ipk1* gene reported in other crop species. The fragments obtained by sequencing the amplicon A1R3(56.8°C) with WP2 and NLSPR3 showed similarity with *ipk1* gene reported in other crops.

5.1.3 Assembly and analysis of sequence based on overlapping regions

The present investigation was successful in amplifying the 5' end of previously known fragment of *ipk1* (*pnipk1*-1072 bp) reported by Giridhari (2017). A contig of 523 bp was obtained towards the 5' end flanking region. The contig thus obtained had similarity with *ipk1* gene in other plant crops when homology search was conducted with BLAST. The newly amplified region was assembled to the *pnipk1* gene fragment (*pnipk1*-1072 bp) by joining the overlapping regions of known region (*pnipk1*-1072 bp) with the 523 bp contig towards 5' end. An overlap of 90 bp upstream was found between previously known sequence (*pnipk1*-1072 bp) and the 523 bp contig and were manually assembled to produce a total length of 1535 bp.

Josefsen *et al.* (2007) identified and cloned *ipk1* gene from rice (*OsIpk1*) by the search in rice genome with the help of NCBI Blast database using the available inositol triphosphate sequences of *Arabidospsis* and humans (*Homo sapiens*). They amplified the *ipk1* gene in rice genome with an open reading frame of 1029 bp which encodes a protein of 342 amino acids. Sun *et al.* (2007) had isolated a cDNA named *ZmIPK1* from maize (*Zea mays*) and it had a size of 2012 bp with an open reading frame encoding for 352 amino acids.

When homology analysis was done for checking similarity with *ipk1* in other crop species, it showed a query cover between 12 and 48 % with an identity ranging from 65 to 83 %. It showed identity with nearly 26 crops and exhibit 67.05 % identity with *Vitis vinifera* (Query cover – 41%, Accession no: XM_010666413.2), 66.99 % identity with *Nelumbo nucifera* (Query cover – 48 %, Accession no: XM_010256463.2) and 65.98 % identity with *Camellia sinensis* (Query cover-41%, Accession No: XM_028232158.1).

Even though region towards 5' end was sequenced, sequence information about the full length of gene was not obtained. This might be due to the number of non-specific amplifications occurred during PCR amplification. Partially degenerate primers have the ability to create non-specific amplifications in

genomic DNA. Partially degenerate primers may anneal and hybridise in a non-target region or mispriming of locus specific and walker primer on an undesired gene sequence may lead to non-specific amplifications (Guo and Xiong, 2006).

In the present study, multiple bands were obtained in the PCR amplifications. This may be due to the potentiality of walker primer to get attached to multiple sites of genomic DNA sequences. The flanking regions both at 5' and 3' end could be find out in future by a specific primer designed from the newly assembled *ipk1* sequence (*pnipk1*-1535 bp) hoping to amplify and sequence the whole target gene *ipk1* in black pepper variety Panniyur 1.

Chang *et al.* (2018) has reported a protocol based upon the stepwise partially overlapping primer (SPOP-primers) for finding out the flanking regions of known DNA sequences and claims to inhibit all types of non-specific amplification including that of walker primer. They have successfully amplified the flanking unknown regions of glutamate decarboxylase gene (*gadA*) in *Lactobacillus brevis* and hygromycin gene (*hyg*) of rice.

Leoni *et al.* (2008) has reported a protocol based upon the classical Rapid amplification of cDNA ends (RACE) with some improvements in it and have successfully amplified the flanking regions of cDNA coding for *Lhcb1.1* gene of spinach which encodes for a light harvesting protein Lhcb1.

5.1.4 Validation of gene

The confirmation and amplification of newly assembled sequence of *ipk1* gene (*pnipk1*-1535 bp) in the genomic DNA of Panniyur 1 was performed with a forward primer F11 and a reverse primer R11. Both the primers were designed from the 5' and 3' ends of newly assembled *ipk1* gene sequence (*pnipk1*-1535 bp). The amplicon was of the expected size (1162 bp). Fillepi *et al.* (2010) has isolated and characterized various genes *viz.*, *PvMIPSs* and *PvMIPSv*, *PvIMP*, *PvMIK*, *PvIPK2*, *PvIPK1*, *PvITPKα* and *PvITPKβ* encoding for various enzymes involved in the biosynthetic pathway of phytic acid in common bean (*Phaseolus vulgaris* L.) and amplified gene *PvIPK1* of length 2,258 bp with an ORF

encoding for 352 amino acids. Abreu and Aragao (2006) has isolated and characterized a full length cDNA from yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) of size 1951 bp with an open reading frame of 1533 bp encoding 510 amino acids. Similarly many genes related to phytic acid synthesis have been reported in several crops like *Arabidopsis thaliana*, maize, rice, barley, several legumes etc.

5.1.5 Phylogenetic analysis

The coding region of newly assembled sequence was taken with the help of ORF Finder. The Open Reading Frame with 645 bp encodes for 214 amino acids and similarity was found with *ipk1* gene of other crops when homology search was done with BLAST. Search for conserved domains revealed the presence of a superfamily pfam06090 *i.e.*, the Ins_P5_2-kin family. The role of inositol pentakisphosphate-2-kinase in *Saccharomyces cerevisiae*, encoded by gene *Scipk1* in production of inositol 1,2,3,4,5,6 hexakisphosphate(IP6) or phytic acid was proved by Ives *et al.* (2000). They reported that mutant cells lacking *Scipk1* gene resulted in a reduction or no production of IP6. They have also proposed that a conserved family of IP5 2-kinase enzymes is present in all eukaryotes. Thus proving the fact that the translated amino acid sequence from *ipk1* gene sequence (*pnipk1*-1535 bp) found in black pepper is a part involved in phytic acid biosynthesis.

Phylogenetic analysis of newly assembled *ipk1* gene sequence (*pnipk1*-1535 bp) was done with other known sequences of 11 crops which showed an identity above 65 % and query cover greater than 25 % with *pnipk1* gene (1535 bp). The phylogenetic analysis revealed that *Dendrobium catenatum* and *Phalaenopsis equestris* exhibited a closer evolutionary relationship with nucleotide sequence *pnipk1*(1535 bp) in *Piper nigrum* from the rest 9 crop species. Despite of showing more similarity of *Vitis vinifera*, *Nelumbo nucifera* and *Papaver somniferum* with *pnipk1*(1535 bp) gene sequence, it was found that they were clustered into different clades. The evolutionary tree was reliable because a close proximity between *Dendrobium catenatum* and *Phalaenopsis*

equestris can be observed from evolutionary tree. Similarly an evolutionary tree was constructed for the amino acid sequence obtained from the newly assembled sequence by using ORF finder. The analysis showed that the amino acid sequence translated from *pnipk1*(1535 bp) in *Piper nigrum* shared similarity with *Phoenix dactylifera* and *Phalaenopsis equestris* indicating the closer evolutionary relationship of *Piper nigrum* with these crops. Close proximity of *Nicotiana tabacum* and *Nicotiana tomentosiformis* in the evolutionary tree made it clear that the evolutionary tree generated was accurate.

Josefsen *et al.* (2007) has conducted the phylogeny analysis of *OsIpk1* gene in rice (*Oryza sativa*) and *HvIpk1* gene in barley (*Hordeum vulgare*). The phylogeny analysis plant ipks were grouped into a different clade from that of animal ipks like of human, frog etc. and an identity between *OsIpk1*, *HvIpk1* and maize *ZmIpk1* was found to be 77 % and 54 % with *AtIpk1* gene of *Arabidopsis thaliana.* An evolutionary study using MIPS gene was conducted by Majumder *et al.* (2003) and the phylogenic analysis revealed that the amino acid sequences in plants are largely conserved and the eukaryotic and prokaryotic sequences are clustered separately. Phylogenetic analysis and evolutionary relationships of *IPK1* gene in *Ricinus communis* exists with that of many organisms like humans (*Homo sapiens*), *Arabidopsis thaliana* and *Saccharomyces cerevisiae* (Yu *et al.*, 2014). Similar studies were conducted by Ajay *et al.* (2016) to analyse the phylogenetic relationships between two genes, *AhITPK1* gene encoding for inositol tetraphosphate 1-kinase and *AhIPK2* encoding for inositol 1,4,5 trisphosphate kinase in peanut and found out that in evolutionary analysis, *AhITPK1* and *AhIPK2* genes were genetically different from plant genera. All these results confirm that the biosynthesis pathway of IP6 or else called phytic acid production share a similar evolutionary pathway and conserved among species.

5.2 Allelic variation in *ipk 1* gene in black pepper genotypes

The genomic DNA of Panniyur 1 and 10 black pepper genotypes were PCR amplified with forward primer F11 and reverse primer R11 for the amplification of *ipk1* gene fragment (*pnipk1*-1535 bp). The primers were

designed based on the assembled *ipk1* gene fragment, (*pnipk1*-1535 bp). The obtained PCR products were separated on 1% agarose gel electrophoresis and amplifications were observed in Panniyur 1 along with other 3 black pepper genotypes Panniyur 5, Panniyur 7 and Karimunda 7. The amplifications were of same range indicating that no allelic variation can be detected in the *ipk1* gene fragment (*pnipk1*-1535 bp) among Panniyur 1, Panniyur 5, Panniyur 7 and Karimunda 7. However, the other black pepper genotypes Chumala, Thotamundy, Chettanvally, Kottanadan, PRS 160, Karimunda Kuttyatur and Payyanganam 2 showed no amplification when amplified with the primers F11 and R11. This may be due to variation in the primer binding sites in these genotypes indicating some allelic variations in the *ipk1* gene (*pnipk1*-1535 bp) sequenced in the Panniyur 1 variety with that of other black pepper genotypes. Saha and Reddy, (2015) has detected allelic variation in an SSR marker *NCPGR90* located in the 5' UTR in promoter region of *CaIMP* gene in chickpea (*Cicer arietinum* L.). Allele of *NCPGR90*, NCPGR90_170 was carried by drought tolerant accessions and NCPGR90_190 and NCPGR90_200 were carried by drought susceptible accessions of chickpea.

5.3 Detection of Phytic acid using PAGE

In the present study, the crude plant sample containing phytic acid content in various black pepper varieties was extracted and estimated using a standard curve obtained by plotting a standard curve taking volume(intensity) and quantity of standard phytates. Phytic acid contents in berries of black pepper varieties varied among varieties ranging from lowest in Payyanganam 2 having 196.5 nM/g to highest being in the hybrid PRS 160 with phyitc acid content of 697.5 nM/g. Phytic acid contents in rest of the varieties were, 502.5 nM/g in Panniyur 5, 7367.5 nM/g in Panniyur 7, 511.5 nM/g in Chettanvally, 463.5 nM/g in Kottanadan, 387 nM/g in Karimunda 7, 201 nM/g in Chumala, 637.5 nM/g Karimunda Kuttyatur, 198 nM/g in Thotamundy and 275 nM/g in Panniyur 1. Based on the phytic acid content in the genotypes, they can be classified into low (<210 nM/g), medium (210-510 nM/g) and high (>510 nM/g). Thottamundi and

Payyanganam 2 were included in the low phytic acid content and Panniyur 1,Panniyur 5, Panniyur 7, Karimunda 7, Kottanadan fall under medium phytic acid content and Chettanvally, Karimunda Kuttyatur, PRS 160 fall under high phytic acid content.

In a previous study using leaves of black pepper variety Panniyur 1 by Giridhari *et al.* (2017), the amount of phytic acid was reported to be 620 nM/g. This is found to be greater than the quantity of phytic acid extracted from berries of Panniyur 1 with 275 nM/g in the present study indicating the possibility of variation of phytic acid content in the plant tissues. However, the low phytic acid content in the present study may be due to the half matured berries selected for present study. Alimohammadi *et al.* (2013) conducted estimation of phytic acid in a similar way in tomato, tobacco and rice. The values of phytic acid were different in each crop and detected 100 nM/g in tomato, 200 nM/g in tobacco and 450 nM/g in rice respectively.

Earlier methods of detection of phytic acid and lower inositols were High Performance Liquid Chromatography (HPLC), Anion Exchange Chromatography (AEC), Colorimetric methods etc. Later, Losito *et al.* (2009) devised the method of PAGE detection of phytic acid and phosphoinositols. They reported PAGE method can be utilized to analyze the higher phosphoinositols either staining by 4', 6- diamidino-2-phenylindole (DAPI) or by toluidine blue for the detection of phytic acid. Kwanyuen and Burton, (2005) estimated the phytic acid content in soybeans and soy products at various developmental stages and the phytic acid content varied in each developing stages and varieties of soybean with a range between 14.5 to 25.4 mg per gram dry weight in mature soybean seeds and their products. These works indicate that phytic acid differs in crop species and differ in wide range, thereby making clarity in the cause of varying results of phytic acid content in varieties of a single crop black pepper as in the present study.

In HPLC method, on a macroporous polymer for analysis of inositol phosphates like IP4, IP5 and IP6 (phytic acid) in various cereal and legume crops,

phytic acid quantity of crop species varied in a wide range (Lehrfeld, 1989). Philippy *et al.* (2015) has also estimated phytic acid in various crop plants belonging to Malvaceae family, and various other unrelated crops. The phytic acid was found to be highest in crops belonging to Malvaceae family. Phytic acid content in rice was 9-49 µM, *Arabidopsis thaliana* 29-89 µM and tobacco with 11-24 µM respectively, whereas crops in Malvaceae family like cotton had greater values 11-169 µM and okra with 90-336 µM.

Compared to PAGE detection of phosphoinositols, methods like HPLC, and AEC are more labour intensive and non-economical. However HPLC estimation of phytic acid content in plants is more reliable and accurate because there exists a chance of underestimation of phytate content in plants with the use of PAGE. So in the present study, there was a wide range of values for phytic acid contents across different varieties confirming that quantity of phytate is independent of crops and developmental stages of single crop. So further evaluation of phytic acid in plant samples must be performed at different developmental stages of plant in future.

5.3.1 Allelic differences and phytic acid variation among black pepper genotypes

Despite of no allelic variation between the *ipk1* gene fragment (*pnipk1*-1535 bp) among Panniyur 1, Panniyur 5, Panniyur 7 and Karimunda 7 genotypes of black pepper, the phytic acid content among these genotypes exhibited variations. This might be due to the reason that phytic acid content variation depends upon the developmental stages, seasons, tissues even within the same plant species. Ali *et al.* (2013) has reported that RNAi mediated silencing of *ipk1* gene has decreased the production of phytic acid in rice proving the role of *ipk1* gene in the synthesis of phytic acid and involvement of *ipk1* gene encoded inositol 1,3,4,5,6 pentakisphosphate-2-kinase enzyme in the last step of biosynthetic pathway of phytic acid in rice. Saxena *et al.* (2013) has reported that the expression of *CaIMP* gene in chickpea is differentially expressed in different

organs and stress conditions in plant. Liu *et al.* (2005) has reported that environmental effects are more predominant than genetic factors over phytic acid synthesis in japonica variety of rice. He studied 24 cultivars and observed that there was substantial difference in the phytic acid content in these rice cultivars and the variation in phytic acid content depended upon the effects of cultivars, environment and interaction between them. Similarly, Feil and Fossati, (1997) have proved that the phytic acid levels can be greatly varied among 10 hexaploid winter triticale cultivars by cropping year. Similar findings have also been reported that environment has effect on phytic acid variation.

The present study has given a preliminary data about the sequence information and confirmation of *ipk1* gene in different varieties of black pepper (*Piper nigrum* L.). The quantitative estimation of phytic acid using PAGE in black pepper varieties has shown variation ranging from 196.5 nM/g to 697.5 nM/g. The study has also helped in understanding that allelic variations may occur in *ipk1* gene as the varieties Chumala, Chettanvally, Payyanganam 2, Karimunda Kuttyatur, PRS 160, Thotamundy, Kottanadan did not show any amplification of the gene. The primer designed was specifically from the hybrid variety Panniyur 1 which failed to amplify the corresponding regions of *ipk1* gene in these varieties. However the phytic acid content in some of these varieties were even higher than Panniyur 1. This shows that there may be other genes involved in the pathway (Fillepi *et al.*, 2010; Bhati *et al.*, 2014; Hegeman *et al.*, 2001)

However, variations in phytic acid content may be due to genetic and environmental factors. Identification and isolation of genes like *ipk1* can help in revealing the biosynthesis pathway of phytic acid. Information about gene sequence helps in studying gene expression and for development of mutants for conducting various genetic studies. More studies in genes involved in phytic acid and inositolphosphates biosysntheis will help to decipher the signalling pathways of inositolphosphates to tackle various problems like lesser intake of nutrients in animal and plant bodies.

Summary

6. SUMMARY

Black pepper (*Piper nigrum* L.) belonging to the family '*Piperacea*' grown for its fruit, known for peppercorns, is an important spice crop with high therapeutical and medicinal values with culinary qualities. The significant benefits include antioxidant properties, anti-inflammatory potential, antibacterial properties, antidepressant, and also acts as an effective deterrent to insects etc. However genetic information regarding black pepper is still needs to be explored. So the crop provides a suitable platform for genetic studies and crop improvement programmes.

In a previous study, genes expressed during fruit development were sequenced using new generation sequencing technologies and generated expressed sequence tags. A unigene *pnc135* (995 bp) showing homology with *ipk1* was generated by assembling the expressed sequence tags. The *ipk1* gene is responsible for encoding inositol pentakisphosphate -2- kinase enzyme which is involved in the biosynthesis of phytic acid. Later this unigene was extended (1072 bp) towards 3' end flanking region by genome walking (Giridhari, 2017) and 5' region was yet to be identified.

In this connection, the study entitled "Allelic difference in the putative gene *ipk1* sequence and phytic acid (Insp6) content in black pepper (P*iper nigrum* L.)", the objective of the study was to decipher the unknown sequence towards 5' flanking region of *pnipk1* gene sequence (1072 bp) and to analyse the allelic difference in the *ipk1* gene among black pepper genotypes and to detect the presence and quantify the phytic acid content in these black pepper genotypes with the help of Polyacrylamide gel electrophoresis (PAGE).

1. Isolation of DNA from Panniyur 1 and other 10 black pepper genotypes

- DNA was isolated by utilising modified CTAB method (Doyle and Doyle, 1987). Modification was done by adding one and half volumes of chilled isopropanol and half volume of 5M NaCl for DNA precipitation.
- The absorbance ratio of genomic DNA of Panniyur 1 at $A_{260/280}$ was found to be 1.87 and a concentration of 3.26 µg/µL.
- The absorbance ratio of genomic DNA of 10 black pepper varieties ranged between Karimunda 7 with 1.78 to Chettanvally with 2.09.
- The quantity of genomic DNA was also checked, ranging from Karimunda Kuttyatur – 3.28 µg/µL to Karimunda 7 – 4.93 µg/µL.
- The genomic DNA of Panniyur 1 and other 10 black pepper genotypes were of good quality.

2. Genome walking for finding out the 5' region towards *pnipk1* (1072 bp) gene

- Primers were designed based upon the previously known gene sequence, *pnipk1* gene, 1072 bp (Giridhari, 2017). A locus specific forward primer, LSPF3 and a locus specific reverse primer, LSPR3 was designed based upon *pnipk1* gene fragment (1072 bp). Similarly a nested locus specific forward primer NLSPF3 and a nested locus specific reverse primer NLSPR3 was also designed corresponding to locus specific primers.
- Four walker adaptors (WA1, WA2, WA3 and WA4) same as reported by Reddy *et al.* (2008) were utilised for whole genome amplification of genomic DNA of black pepper variety Panniyur 1.
- Two walker primers, WP1 and WP2 for locus and nested locus specific amplifications same as reported by Reddy *et al.* (2008) were used for locus specific and nested locus specific amplifications. The RCA products are used as template DNA for genome walking.
- Two separate RCA products amplified by NLSPR3 and WP2 at two different temperatures 51.6°C (A1R3 and A4R3) and 56.8°C (A1R3 and

A4R3) were selected for sequencing towards 5' region of *pnipk1* gene fragment (1072 bp).

- Amplicons A1R3(51.6ºC), A4R3(51.6ºC) and A4R3(56.8ºC) sequenced with NLSPR3 exhibited similarity with *ipk1* gene reported in other crops whereas the sequence fragment A1R3 (56.8ºC) amplified with both WP2 and NLSPR3 were similar to *ipk1* gene of other crops.
- The selected sequences showing similarity to *ipk1* gene were assembled together to form a contig of 523 bp which shared an overlapping region of 90 bp in the 5' region with *ipk1* gene fragment (*pnipk1*-1072 bp) of Giridhari (2017). Assembly of 523 bp contig and *pnipk1* gene sequence give an assembled sequence of total length 1535 bp.
- The assembled *ipk1* gene sequence (*pnipk1*-1535 bp) in black pepper showed a sequence identity of 67% with *ipk1* gene of *Vitis vinifera* and 66% with that of *Nelumbo nucifera* and *Papaver somniferum*.

3. Validation of assembled *ipk1* gene, (*pnipk1* -1535 bp)

- Confirmation and validation of assembled *ipk1* gene sequence (*pnipk1*-1535 bp) in Panniyur 1 black pepper variety was performed with two primers F11 and R11.
- Both the primers were based upon the assembled *ipk1* gene sequence (*pnipk1*-1535 bp)
- The expected amplicon size was 1162 bp and clear band of PCR products amplified between temperature 61-66ºC was obtained and all the bands were in the expected amplicon size region confirming the presence of *ipk1* gene fragment (*pnipk1*- 1535 bp) in Panniyur 1.

4. Analysis in ORF Finder for Open Reading Frames (ORFs)

- The *ipk1* gene fragment (*pnipk1*-1535 bp) was subjected in ORF finder to analyse the coding region (Open Reading Frame) and the ORF was found to be 645 bp encoding for 214 amino acids.
- The ORF contained a single conserved domain – Ins_P5_2-kin family.

5. Phylogenetic study for analysing the evolutionary relationships

- A phylogenetic tree constructed revealed that *ipk1* gene sequence (*pnipk1*-1535 bp) in *Piper nigrum* showed closer evolutionary relationship with *Dendrobium catenatum* and *Phalaenopsis equestris.*
- A close evolutionary relationship of *Piper nigrum* with *Phoenix dactylifera* and *Phalaenopsis equestris* was observed when phylogenetic tree constructed based on the amino acid sequence of *pnipk1* (1535 bp) gene.

6. Allelic variation in *pnipk1* (1535 bp) gene among black pepper genotypes

- Genomic DNA isolated from 10 black pepper genotypes Panniyur 5, Panniyur 7, Chettanvally, Chumala, Kottanadan, Thotamundy, Karimunda 7, Karimunda kuttyatur, Payyanganam 2 and PRS 160 along with Panniyur 1 were amplified with primers F11 and R11.
- Amplicons of expected and same size were obtained in genotypes Panniyur 1, Panniyur 5, Panniyur 7 and Karimunda 7 revealing no allelic variation among these genotypes.
- No amplification was observed in Chettanvally, Chumala, Kottanadan, Thotamundy, Karimunda kuttyatur, Payyanganam 2 and PRS 160.

7. Phytic acid content in black pepper genotypes

- Bands were obtained corresponding to the standard phytates in the PAGE gel analysis.
- Standard curve was made by plotting a graph between quantity of standard phytic acid (nanomoles) along X-axis and volume (intensity) along Y axis.
- The quantity of phytic acid in samples were Panniyur 5 with 502.5nmoles/g, Panniyur 7 with 367.5nmoles/g, Chettanvally- 511.5 nmoles/g, Kottanadan- 463.5 nmoles/g, Karimunda7- 387 nmoles/g, Chumala- 201 nmoles/g, Karimunda kuttyatur- 637.5 nmoles/g, Payyanganam 2- 196.5 nmoles/g and Panniyur 1- 275 nmoles/g, Thottamundi- 198 nmoles/g, PRS 160- 697.5 nmoles/g.
- Phytic acid content in varieties Panniyur 1, Panniyur 5, Panniyur 7 and Karimunda 7 varied despite of no allelic variation in the *ipk1* gene fragment (*pnipk1*-1535 bp).

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Abstract

ALLELIC DIFFERENCE IN THE PUTATIVE GENE *ipk1* SEQUENCE AND PHYTIC ACID (INSP6) CONTENT IN BLACK PEPPER (*Piper nigrum* L.)

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ABSTRACT

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ABSTRACT

Black pepper (*Piper nigrum* L.) is one of the world's highly demanded and most traded spices with high medicinal and therapeutic values. A unigene *pnc135* (995 bp) was developed by the Expressed sequence tags data obtained by next generation sequencing. This unigene was found to show similarity with *ipk1* gene reported in other crop species which encodes for inositol pentakisphosphate-2 kinase enzyme (Unpublished data, Sujatha,R.). This enzyme is involved in the phosphorylation of inositol pentakisphosphate to inositol hexakisphosphate or phytic acid (InsP6), last step in the biosynthetic pathway of phytic acid. This unigene was later partially sequenced (1072 bp) towards the 3' end by directional genome walking by Giridhari (2017). Phytic acid functions as the major storage form of phosphorus in seeds, cereals and legumes possessing significant benefits including signalling, plant communication, messenger RNA transport etc. However, phytic acid also acts as an anti-nutrient in animals as its chelating property will cause malnutrition in organisms and also leads to environmental pollution due to phosphorus excretion by non-ruminant animals. Therefore researchers are finding ways to create *ipk1* mutants for either to decrease or increase the phytic acid content in organisms. However the genetic information about the black pepper crop remains very limited and the metabolic pathways and the genes related to it are also poorly understood.

So in this study entitled "Allelic difference in the putative gene *ipk1* sequence and phytic acid (InsP6) content in black pepper (*Piper nigrum* L.)", the objective was to find out the flanking region towards the 5' region of *pnipk1* gene fragment (1072 bp) reported earlier by Giridhari (2017), to identify the allelic differences in *pnipk1* gene in 10 black pepper genotypes and to estimate and quantify the phytic acid content in these 10 black pepper genotypes using polyacrylamide gel electrophoresis (PAGE).

Genomic DNA was isolated from Panniyur 1 variety of black pepper and used it for the whole genome amplification by Rolling Circle Amplification method using Phi 29 DNA polymerase and walker adaptors (WA1, WA2, WA3

and WA4) reported by Reddy *et al.* (2008). After whole genome amplification, genome walking using primer combinations of walker primers, locus specific primers and nested locus specific primers were performed to find out the flanking region towards the 5' region of *pnipk1*(1072 bp) gene fragment of black pepper. The walker primers (WP1 and WP2) used for genome walking were same as that of reported in Reddy *et al.* (2008) and the locus and nested locus specific primers were designed on the basis of *pnipk1* gene fragment (1072 bp) sequenced by Giridhari (2017).

From the nested PCR amplification four products, two amplicons, A1R3 and A4R3, each at two different temperatures *viz.*, 51.6°C and 56.8°C were obtained and sequenced. On assembling the sequences a contig of length 523 bp was obtained towards the 5' region of *pnipk1* gene fragment and this showed similarity to *ipk1* gene in other crops. This 523 bp contig was assembled with *ipk1* gene fragment (*pnipk1*-1072 bp) to get a total length of 1535 bp.

The newly assembled *ipk1* gene sequence (*pnipk1*-1535 bp) was analysed in ORF finder for the coding region and found an Open Reading Frame (ORF) with 645 bp encoding for 214 amino acids. Phylogenetic analysis of the sequence and translated amino acid sequence showed closer evolutionary relationship with that of *Dendrobium catenatum.*

Primers were designed based upon the *pnipk1* gene sequence (1535 bp) to amplify the genomic DNA of Panniyur 1 and other 10 black pepper genotypes. The selected 10 genotypes were Panniyur 5, Panniyur 7, Chettanvally, Kottanadan, Karimunda 7, Thottamundy, Karimunda kuttyatur, Payyanganam 2, PRS 160 and Chumala. Amplification of *pnipk1* gene (1535 bp) was obtained from genomic DNA of Panniyur 1, Panniyur 5, Panniyur 7 and Karimunda 7 with the expected of amplicon size indicating a similar sequence among these genotypes. Whereas amplification was not obtained in genomic DNA in rest of the genotypes showing allelic variation is present for *ipk1* gene in these genotypes.

To estimate and quantify the phytic acid content in Panniyur 1 and 10 black pepper genotypes, polyacrylamide gel electrophoresis (PAGE) was

performed. Phytic acid was extracted from black pepper varieties same as that of selected for allelic difference analysis in *ipk1* gene (*pnipk1*-1535 bp). The samples were loaded with phytate standards and band intensities of each concentration were determined with Gelquant.NET. The values of phytic acid in black pepper genotypes were estimated by the standard curve. The quantity of phytic acid in samples are: Panniyur 5 with 502.5nmoles/g, Panniyur 7 with 367.5nmoles/g, Chettanvally-511.5nmoles/g, Kottanadan-463.5nmoles/g, Karimunda7-387nmoles/g, Chumala-201nmoles/g, Karimunda kuttyatur-637.5nmoles/g, Payyanganam 2-196.5nmoles/g and Panniyur 1- 275 nmoles/g, Thotamundy-198nmoles/g, PRS 160-697.5nmoles/g. Based on the phytic acid content in the black pepper genotypes, they can be classified into low (<210 nmoles/g), medium (210-510 nmoles/g) and high (>510 nmoles/g) phytic acid content. The *ipk1* gene fragment (*pnipk1*-1535 bp) was amplified in the genotypes Panniyur 1, Panniyur 5, Panniyur 7 and Karimunda 7. These genotypes all came under the category of medium phytic acid content group.

The study resulted in sequencing a total of 1535 bp long segment of *ipk*1 gene black pepper variety Panniyur 1 and analysing the presence of allelic variation in *ipk1* gene and phytic acid content in selected black pepper genotypes.