



Identification of resistance gene analogs involved in *Phytophthora capsici* recognition in black pepper (*Piper nigrum* L.)

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Received: 10 November 2019 / Accepted: 1 June 2020 / Published online: 3 July 2020
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Abstract

Black pepper (*Piper nigrum* L.) is an important spice crop with high economic value. However, its production is severely hampered by the oomycete pathogen, *Phytophthora capsici*. Integrative disease management strategies have been developed to control the pathogen, but the pathogen is in the phase of evolving its virulence. Absolute resistance against *Phytophthora* rot was not reported in black pepper germplasm. However, *Piper colubrinum*, a wild species is reported as resistant. Resistance proteins are involved in continuous surveillance of pathogen entry and activation of plant defense signalling pathways for an effective hypersensitive response to prevent pathogen invasion. In this study, a sequence-based homology approach using the conserved nucleotide-binding site (NBS) of known plant resistance genes was used to isolate Resistance Gene Analogs (RGA) and assess their transcript level during *Phytophthora* infection. The RGA transcript level was evaluated in resistant wild species (*P. colubrinum*), two moderately resistant black pepper genotypes (IISR Sakthi and 04-P24-1), and one susceptible genotype (Subhakara). The identified RGAs of black pepper were found to be of non-TIR R gene class with NBS motifs. The expressions of six PnRGAs were assessed employing qRT-PCR at different time points after challenging with highly aggressive *Phytophthora* isolate. The kinetics of differential expression post-infection with *P. capsici* indicates the differential timing and magnitude of pathogen recognition in resistant *P. colubrinum* and moderately resistant black pepper genotypes compared to susceptible genotype. In silico analysis revealed that differentially expressed *P. nigrum* RGAs function through ADP phosphorylation, which is a key process in pathogen recognition. The identification of *P. nigrum* RGAs induced by *P. capsici* should provide valuable information for cloning and characterization of resistance genes.

Keywords Resistance gene analogs · *Phytophthora* foot rot · Black pepper · Differential expression profiling · Host resistance

Introduction

Black pepper (*Piper nigrum* L.) is an important spice crop grown for its berries, commercially traded both in fresh and dried form and used as a spice as well as medicine. Being native to the humid, tropical evergreen forests of the Western Ghats of the Indian Peninsula (Ravindran et al. 2000), black pepper are also cultivated in tropical and subtropical regions around the globe. Vietnam, Indonesia, Bulgaria, India, Brazil are the top five black pepper producing nations (FAOSTAT 2017). Among the diseases that threaten black pepper cultivation, the *Phytophthora* foot rot caused by *Phytophthora capsici* is the most devastating. The application of chemicals mainly based on contact and systemic fungicide practiced by pepper growers could protect the crop only to some extent but may lead to the emergence of resistant pathogen strains. Moreover in India, *Phytophthora* disease is monsoon bound, and the mixed cropping system practiced by pepper growers along

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s42161-020-00586-3>) contains supplementary material, which is available to authorized users.

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with coconut or arecanut gives a conducive microclimatic condition for the pathogen spread and possibility of cross-infection. *P. capsici* can infect all parts of the black pepper through an aerial phase in which foliar infection in leaves, stem, and spike occurs or as soil phase in which root and collar will be infected (Anandaraj and Sarma 1995; Anandaraj 2000).

Complete resistance against *P. capsici* was not reported among the cultivated black pepper genotypes. However, resistance screening to Phytophthora foot rot disease has resulted in the identification of resistant *P. colubrinum* (Purseglove et al. 1981), and a few black pepper genotypes viz., IISR Sakthi (Sarma et al. 1994) and O4-P24-1 (Bhai et al. 2010). Defense mechanism in IISR Sakthi was correlated to membrane integrity of the host, accumulation of Phenylalanine ammonia lyase (PAL), and induction of pathogenesis-related proteins (Shamina 1997; Jebakumar et al. 2001). There are also reports on specific biochemical responses that lead to moderate resistance in O4-P24-1, an open-pollinated progeny of black pepper (Vandana et al. 2014). The compatible and incompatible interaction of black pepper and *P. colubrinum* with *P. capsici* involves transcriptional activation or repression of a large number of genes. A fraction of these pathogenesis-related genes has been characterized at the molecular level, including expression changes upon infection with *P. capsici* (Vandana and Bhai 2018; Malik and George 2018). However, recognition factors for pathogen recognition were not elucidated yet.

Resistance genes are involved in pathogen recognition either by direct interaction with the pathogen effectors or with pathogen modified host protein during the invasion and subsequent activation of signaling pathways leading to defense against the pathogen. R proteins were categorized into five classes based on the combination of conserved protein signatures possessed such as serine/threonine protein kinases (PKs), leucine-rich repeats (LRRs), nucleotide-binding sites (NBS), and transmembrane domains (TMs) (Gururani et al. 2012).

The majority of the R gene analogs (RGA) belong to NBS-LRR superfamily and the presence of such structural motifs makes them potential regions for providing disease resistance (Li et al. 2016). Earlier, the technique for identification of resistance gene analogs capitalizes on conserved regions for designing degenerate primers and isolation using the polymerase chain reaction (PCR) (Leister et al. 1996; Kanazin et al. 1996). Genome-wide investigations revealed that RGAs accounts for approximately 0.2–1.3% of genes predicted in plant genomes where they occur in clusters, and however RGA content varies with different plant genotypes (Ameline-Torregrosa et al. 2008; Yang et al. 2008; Bayer et al. 2019). Thus NBS-LRR genes are often polymorphic between individuals of a host population, and the complete set of these genes defines the repertoire for the detection of polymorphic pathogen effectors (Bakker et al. 2006; Zhang et al. 2009; Maekawa et al. 2011). More than 4500 NBS-LRR-type RGAs have been amplified via PCR from a wide range of

plant species, and they have been arranged in clusters similar to R genes in plant genomes (Marone et al. 2013).

The main objective of the study was to identify RGAs in black pepper using degenerate primers designed to amplify conserved NBS domain between kinase-1a and GLPL motif and assess the RGA transcript level in response to *P. capsici* infection. The differentially expressed RGAs in resistant and susceptible genotypes would help develop markers linked to disease resistance in black pepper.

Materials and methods

Identification of RGAs from black pepper

Plant material and genomic DNA isolation

Black pepper genotypes, i) IISR Sakthi, moderately resistant to *P. capsici*, ii) O4-P24-1, the open-pollinated progeny of IISR Sakthi, moderately resistant to *P. capsici*, iii) Subhakara, susceptible to *P. capsici* were used for the isolation of R gene analogs. Rooted plants of 3–4 leaf stage raised in sterile potting mixture and maintained under greenhouse conditions at ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India were used. Leaves of black pepper were harvested, flash-frozen in liquid nitrogen, and stored at -80°C until use. Genomic DNA was isolated using the protocol described by Doyle and Doyle (1987) with modifications.

Degenerate primers and PCR amplification

Four pairs of primers varying in degeneracy were used to amplify the conserved region between the P-loop and GLPL domain (Table 1) of plant R genes. PCR amplifications were conducted in a total volume of 25 μl containing 100 ng of template DNA, 1x PCR buffer with 2 mM MgCl_2 , 250 μM dNTPs, 2.5 U of Taq polymerase (Takara, Japan), 2.5 μM each of forward and reverse primer (Sigma, USA). The thermocycler profile was as follows: initial denaturation of 94°C for 5 min, followed by denaturation at 94°C for 30 s, annealing ($45\text{--}60^{\circ}\text{C}$) for 1 min, and extension of 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR products were separated by 1.5% agarose gel, and the amplicons were gel-purified by using Gen elute gel extraction kit (Sigma, USA). The purified DNA was ligated into pTZ57R/T vector (Insta clone T/A cloning kit, Thermo Scientific, USA) and transformed into *Escherichia coli* DH5 α cells according to the manufacturer's instructions.

Cloning and sequencing of PCR products

The purified plasmids DNA of twenty recombinant clones from each degenerate primer set were sequenced using ABI

Table 1 Degenerate primers used to identify resistance gene analogs from black pepper and *Piper* spp.

Primer code	Primer sequence (5'-3')	Conserved motif	R-Gene/RGA	Amplicon length (bp)	References
P2 G16	ATGGGTGGNGTNGGNAAC AGNGCAGNGGNAGTCC	NBS/PLOOP NBS/GLPL	RGA from <i>C. annuum</i> , <i>C. chinense</i> , <i>Nicotiana</i> <i>tabacum</i> , <i>Solanum</i> <i>demissum</i> and <i>S. tuberosum</i>	500	This study
LM638 LM637	GGGGIGTIGGIAAIACIAC ARIGCTARIGGIARICC	NBS/P-LOOP NBS/GLPL	Rps2, N and L6	450	Kanazin et al. 1996
P1F P1R	GGGGIRTIGGIAARACIAC WTIARIGGIARIGGIARICC	NBS/P-LOOP NBS/GLPL	RPM1, Rps2, N and L6	500	Joshi et al. 2012
P6F P6R	GGACCTGGTGGGGTTGGGAAGACAC CAACGCTAGTGGCAATCC	NBS/P-LOOP NBS/GLPL	Rps2 and N	500	Ohmori et al. 1998

RPM1 and RPS2 (*Arabidopsis thaliana*), N (*Nicotiana glutinosa*) and L6 (*Linum usitatissimum*)

prism (Xcelris Labs Ltd., Ahmedabad, India) with universal M13 forward and reverse primers. Contigs were assembled using DNA baser v.3.5.4 (Heracle Biosoft, Romania), and vector sequences were removed using Vecscreen (<http://www.ncbi.nlm.nih.gov/tools/vecscreen/>), and primers were trimmed using BioEdit software (Hall 1999). Sequence homology was compared by searching NCBI using BLASTX. Open reading frames were identified using the ORF finder module of NCBI (<http://www.ncbi.nlm.nih.gov/project/gorf>). Out of the 60 random clones sequenced, sequences that do not have sequence similarity with R genes or RGAs and those possess internal stop codons were excluded from further analysis. The selected clones were translated using Emboss transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq) followed by BLASTP searches and InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan5>) to analyze the identity of the sequences. Conserved motifs were predicted using the MEME Suite software package version 5.1.0 (Bailey et al. 2009). Sequence alignment of PnRGA sequences was carried out using Clustal omega (Sievers et al. 2011). Diversity analysis was performed with the deduced amino acid sequences of PnRGAs identified in this study and NB-ARC domain of known TIR and non-TIR R genes of *Arabidopsis thaliana*, *Capsicum annuum*, *Solanum lycopersicum* as well as NB-ARC domain of R gene transcripts of *P. nigrum* fruit and leaf transcriptome (Hu et al. 2015; Johnson et al. 2012) by using MEGA 6.0 (Tamura et al. 2011) using maximum likelihood method of amino acid substitution model, Jones-Taylor-Thornton (JTT) with 1000 bootstrap replications.

Gene expression studies of selected *Piper nigrum* RGA transcripts

Plant material and pathogen isolate

Three black pepper genotypes and a wild species known for its differential response to *P. capsici* infection were used: IISR Sakthi, 04-P24-1, Subhakara, and *P. colubrinum*. Rooted

plants of 3–4 leaf stage raised in sterile potting mixture maintained under greenhouse conditions were used for challenge inoculation. A highly aggressive isolate of *P. capsici* (05-06) isolated from an infected spike from Peruvannamuzhi, Kozhikode, Kerala, India was used for infection. The genotype ‘Subhakara’ is highly susceptible to *P. capsici* isolate 05-06 and ‘IISR Sakthi’ and ‘04-P24-1’ are moderately resistant and *P. colubrinum* exhibits hypersensitive responses.

P. capsici inoculum plugs of 3 mm were excised from a 72-h old culture on carrot agar medium and used to inoculate leaves of *P. colubrinum* and *P. nigrum* genotypes. For artificial inoculation, the mycelial plugs were placed on the abaxial surface of the third or fourth leaf from the apex. A moist cotton strip was placed over the inoculum plug and was secured using cellophane tape. Another set of plants mock inoculated only with carrot agar plugs without *P. capsici* inoculums was used as a control. Leaf samples at different time intervals (0.5, 1, 2, 4, 8, 12, 16, 24, 48 and 72 h post-inoculation (hpi) were used for RGA expression studies. The remaining plants were also observed for the lesion development to confirm the occurrence of a viable infection. The experimental design comprised of three biological replicates ($n = 3$), each of which contained three technical replicates.

RT-qPCR analysis for differential expression of *P. nigrum* RGA

Total RNA was isolated from leaves of inoculated and uninoculated *P. colubrinum* and *P. nigrum* genotypes using TRIZOL (Invitrogen, USA) according to the manufacturer’s instructions. Total RNA was quantified and was treated with DNase I to remove genomic DNA. For cDNA synthesis, 1 µg of RNA was primed with 18mer oligodT and 200 units of RevertAid reverse transcriptase (Thermo Scientific, USA) for 1 h at 42 °C and 10 min at 72 °C.

Six sets of primers (Table 2) were designed from *P. nigrum* RGAs using the Primer quest tool (<http://eu.idtdna.com/primerquest/home/index>) to study the expression pattern of PnRGAs following Phytophthora challenge in black pepper.

Table 2 qRT-PCR primers used to determine the expression profiles of cloned RGA using the corresponding RGA-specific primers

PnRGA	Primer sequence (5'-3')	Position of primers in PnRGA	Amplicon length (bp)
PnRGA1	F: GCGTTTCCAAGGACTTCACTA R: GCTTCCCATACATCATCTAGCA	87-108 225-247	160
PnRGA3	F: GATGTACACCCTCACAGTCGTG R: CAGGCTGCATCTGTGAGCATAC	154-176 331-353	199
PnRGA5	F: CCGGAGGAAACTAGCCTTACTT R: CTGCATCTGTGAGCATACATCTTC	184-206 308-332	148
PnRGA8	F: CCTCTAACTTCAAAGGCCTCTCTAC R: GTAAGGCTAGTTTCTCTGGCTTC	36-61 186-210	174
PnRGA11	F: GATTGACCAAAGAGGCTGTTGA R: CGATCGTAGTTCTCGTTCCAAAC	93-115 218-241	148
PnRGA24	F: GATGATTTGTGGGATCGTGATG R: CATGAATGCCAAGAGCTAAGAG	245-267 393-415	170

The endogenous control gene used for expression analysis in black pepper and *P. colubrinum* were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Umadevi et al. 2019) and actin (Malik and George 2018) respectively. The qRT-PCR was set in a volume of 20 µl, containing 1x Fast start Essential SYBR green master mix (Roche, Germany), 0.5 µM each primer (Sigma, USA), 10 ng of RNA equivalent cDNA. Real-time PCR was carried out in Rotor Gene Q real-time PCR (Qiagen, Germany). The reaction conditions were set as follows: 94 °C for 10 min, 35 cycles of 94 °C for 10 min, and 60 °C for 30 s and 72 °C for 30 s. A dissociation curve analysis was performed with a stepwise increase of 1 °C per cycle from 62 to 99 °C at the end of the reaction to confirm a single PCR product. A non-template control (NTC) was also tested for all primer pairs to detect any genomic DNA contamination.

The expression level of PnRGAs in each genotype at different time points after inoculation was compared with the expression in mock-inoculated samples. Relative quantification of RGA differential expression among genotypes and time points post-inoculation was performed by a comparative ($2^{-\Delta\Delta C_t}$) method (Livak and Schmittgen 2001). A heat map was generated based on the relative fold change in RGA expression compared to uninoculated control using Clustvis (Metsalu and Vilo 2015).

Results

Identification of RGAs from black pepper

The nucleotide-binding site-encoding DNA regions between Kinase-1a and GLPL were amplified using degenerate primers generated 450- 500 bp amplicon. Out of the 60 recombinant clones sequenced, after removing the repetitive sequences, 23 sequences with uninterrupted open reading frames (ORFs) having domains with high

homology to resistance R-genes of other plant species were named as PnRGAs and were deposited in the GenBank (Supplementary table).

Sequence analysis, classification and motif characterization of PnRGAs

Multiple sequence alignment of 23 PnRGAs isolated from the study with NBS regions of characterized TIR and non-TIR-R genes of *A. thaliana*, *C. annuum*, *S. lycopersicum* revealed the presence of conserved domains such as P loop / Kinase 1a, RNBS A non-TIR, Kinase-2a, RNBS-B, RNBS-C and GLPL within the PnRGAs. All of the 23 *Piper* RGAs possessed a conserved tryptophan (W) residue at the end of the kinase-2a domain, confirming their similarity to a non-TIR class of NBS-LRR R genes. Percentage of amino acid identity was derived through pairwise comparison among isolated PnRGAs, NB-ARC domain of R gene transcripts of *P. nigrum* fruit and leaf transcriptome, and the NBS motifs of both TIR and non-TIR R genes (Table 3).

Multiple Expectation Maximization for Motif Elicitation analysis (MEME) confirmed that six major conserved motifs that determine the structural characteristics of NBS domain in identified PnRGAs. The P-loop (Kinase-1a) motif showed highest conservation (E value: 1.5e-218) followed by RNBS-B (E value: 2.6e-219), RNBS C (E value: 3.0e-208), Kinase-2a motif (E value: 8.7e-195), GLPL motif (E value: 5.5e-183) and RNBS-A non-TIR (2.1e-174) (Fig. 1).

A phylogenetic tree based on the Maximum likelihood algorithm constructed with PnRGAs identified in the present study and known R-genes (TIR and non-TIR). The resulting tree was divided into non-TIR and TIR-NBS LRR clades and 23 PnRGAs clustered with non-TIR R gene clade which was subdivided into four major groups (Fig. 2).

Table 3 Homology matrix based on the deduced amino acid sequences of six PnRGAs and selected RGA from *P. nigrum* transcriptome with other known plant resistance genes

PnRGA1_ <i>P. nigrum</i> (%)	100																			
PnRGA3_ <i>P. nigrum</i> (%)	33.54	100																		
PnRGA5_ <i>P. nigrum</i> (%)	33.54	88.62	100																	
PnRGA8_ <i>P. nigrum</i> (%)	33.75	84.02	84.02	100																
PnRGA11_ <i>P. nigrum</i> (%)	29.87	24.68	24.05	25.62	100															
PnRGA24_ <i>P. nigrum</i> (%)	27.22	25.61	23.78	34.57	29.37	100														
Pn_Locus_10510_ <i>P. nigrum</i> (%)	82.91	34.38	33.75	50.9	47.93	25	100													
Pn_CL16983_ <i>P. nigrum</i> (%)	37.27	50.3	88.17	94.74	26.88	22.89	33.95	100												
Pn_Unigene144_ <i>P. nigrum</i> (%)	33.75	84.62	88.17	54.49	53.85	26.25	25.15	37.42	80.59	56.8	100									
Pn_CL234_ <i>P. nigrum</i> (%)	37.27	54.49	57.49	34.36	35.76	29.49	25.15	83.44	35.54	34.94	100									
AAS01763_ <i>A. thaliana</i> (%)	82.61	33.13	14.29	13.66	15.53	20.65	20.25	17.53	14.91	18.01	14.65	100								
X87851_RPML_ <i>A. thaliana</i> (%)	17.76	21.21	20	20.36	24.07	24.07	32.76	29.19	19.64	20.36	32.32	21.12	100							
AAR21295_Bs4_ <i>S. lycopersicum</i> (%)	32.08	20.65	20.92	20	26.85	20	28.76	24.83	24.52	20.65	30.32	22.52	38.04	25	100					
AEY76900_CaRGA17_ <i>C. annuum</i> (%)	25.34	21.47	23.31	20.61	35.8	36.97	27.85	24.24	21.21	24.24	26.71	22.5	31.74	25.97	100					
ABI96214_ <i>S. lycopersicum</i> (%)	29.49	21.47	24.54	21.82	33.95	27.22	24.24	23.03	24.24	24.24	27.33	23.12	32.93	80.84	100					
FI605108_CaRGA9_ <i>C. annuum</i> (%)	28.21	22.7	23.08	22.15	26.32	24.36	25	23.42	23.42	27.85	23.38	35.54	25.16	24.84	25.48	100				
	24.16	22.15	23.08	22.15	26.32	24.36	25	23.42	23.42	27.85	23.38	35.54	25.16	24.84	25.48	100				

Interpro scan analysis of the predicted amino acid sequences of 23 PnRGA concluded that all the PnRGA possessed NB-ARC (Nucleotide-binding and similarity to human Apaf-1, R genes and Ced4) domain (IPR002182) and were classified into P-loop containing nucleoside triphosphatase hydrolase superfamily (IPR027417).

Expression profiling of the PnRGAs

The present study examined the temporal expression analysis of six PnRGAs that are involved in the compatible and incompatible interaction during the *P. capsici* invasion at different time periods. Differences in the transcription levels of the selected PnRGAs over time were observed after inoculating resistant wild species, moderately tolerant and susceptible genotypes of black pepper with highly aggressive isolate of *P. capsici*, 05-06 (Fig. 3). Basal level expressions of CC-NBS-PnRGAs were present in leaves of *P. colubrinum*, IISR Sakthi, O4-P24-1, and Subhakara.

The PnRGAs which are involved in pathogen recognition showed significant and noticeable higher expression in resistant *P. colubrinum*, when compared with the expression in moderately resistant and susceptible black pepper genotypes. PnRGAs (PnRGA1, PnRGA 3, PnRGA 8 and PnRGA 24) displayed a high level of expression in *P. colubrinum* at the very early time point. The highest relative expression of PnRGA in *P. colubrinum* was 22 fold for PnRGA24.

The PnRGA expression kinetics in moderately resistant IISR Sakthi, revealed upregulation of PnRGA1 and PnRGA24 at early time points. Interestingly, in the case of PnRGA24, the expression level remained high as long as 16 hpi. The highest mRNA transcript level of PnRGA in IISR Sakthi was six-fold at 0.5 hpi for PnRGA11.

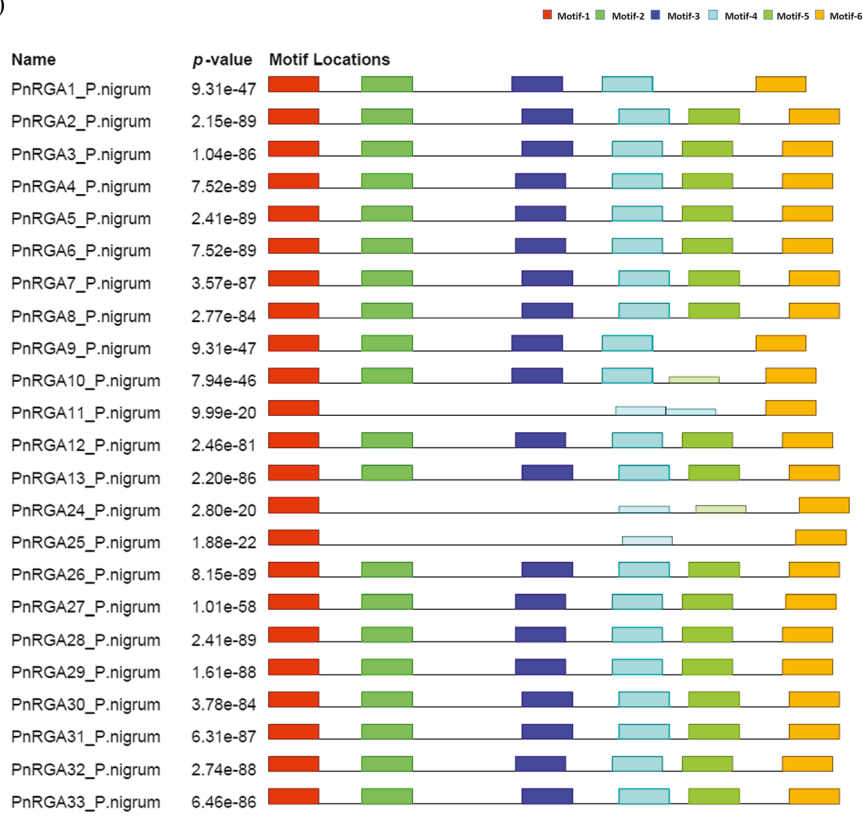
A transient expression of most of the PnRGAs was observed in the case of susceptible genotype, Subhakara at the early time point (0.5 hpi). The mRNA transcript level of PnRGA8 persisted upto 2 hpi and afterwards downregulated compared to mock-inoculated control.

The expression of PnRGA3 was higher in O4-P24-1 up to 12 hpi and afterward transcript level was lower compared to the uninoculated control. Although in O4-P24-1, a higher level of transcription of PnRGA24 was noticed only at 0.5 hpi (5 fold higher), the transcript level remained lower till 72 hpi. The relationships among the differential mean fold-changes of expressions of the six PnRGAs during the time-course were observed in the heatmap (Fig. 4).

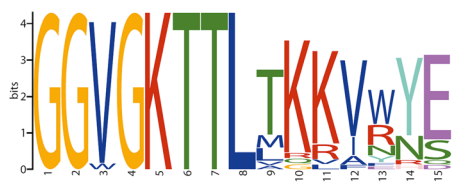
Discussion

Integrated disease management strategy comprising of host resistance, biological control, and phytosanitary measures

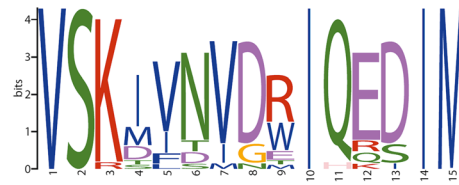
(I)



(II)



Motif-1: Kinase 1a



Motif-2: RNBS A non-TIR



Motif-3: Kinase 2



Motif-4: RNBS-B



Motif-5: RNBS C



Motif-6: GLPL

Fig. 1 I. Distribution of conserved domains in 23 PnRGAs generated using MEME suite, **II.** Sequence logo of six prominent conserved motifs (Motif-1: Kinase 1a, Motif-2: RNBS A non-TIR, Motif-3: Kinase 2, Motif-4: RNBS-B, Motif- 5: RNBS C and Motif- 6:GLPL motifs) in *P. nigrum* non-TIR NBS-LRR type RGAs

are necessary for safeguarding black pepper from its pathogens. The most effective and economical strategy for the management of Phytophthora quick wilt in black pepper relies on identifying or developing host resistant resources. The significant bottleneck towards the development of disease-resistant varieties in black pepper is the perennial nature of the crop, the complexity of the genome, and the existence of *P. capsici* in multiple hosts in the intercropping system. Considering the economic and medicinal properties of black pepper, the characterization of promising tolerant lines in germplasm is of top priority. Identification of RGAs in tolerant black pepper lines is an ideal approach towards the genetic improvement of non-model crops. In this study, 23 RGAs were identified using homology-based PCR cloning of NBS class disease resistance gene analogs in black pepper.

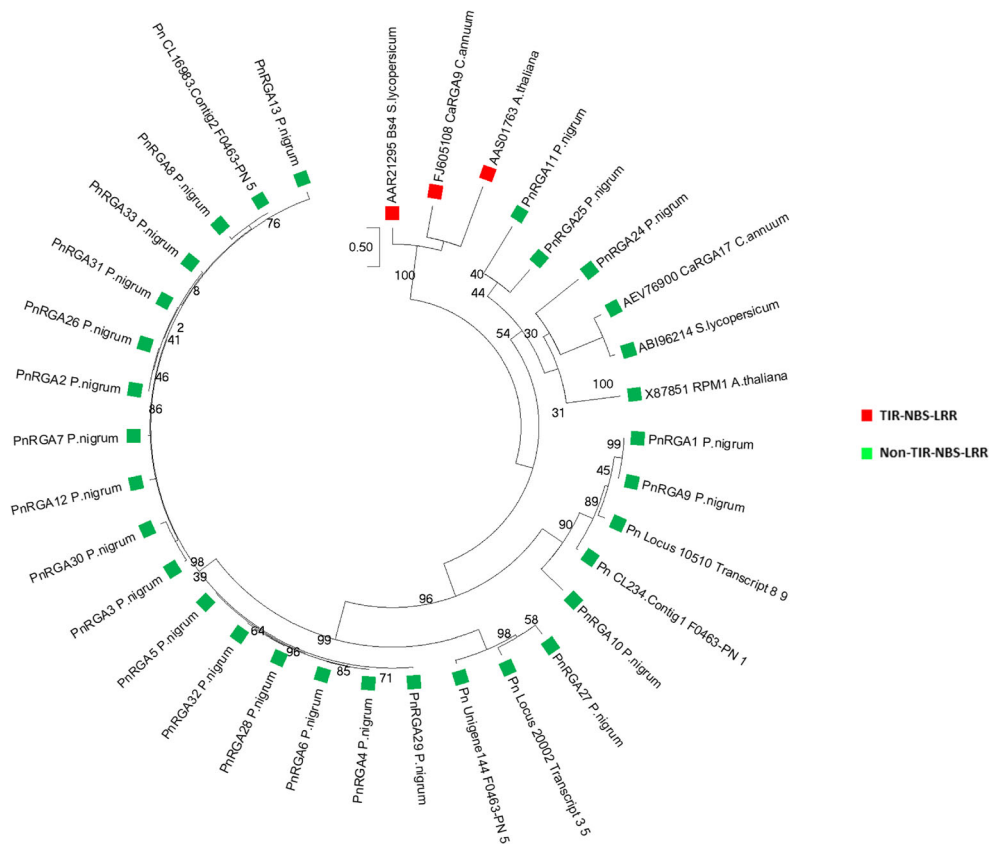
The identified R gene analogs of black pepper were found to be of non-TIR R gene type based on the criteria; the presence of tryptophan in the kinase 2a domain of the selected NBS region between P-loop and GLPL (Meyers et al. 1999). In dicots, the occurrence of both TIR and non-TIR RGA were reported by Meyers et al. (1999), however, later studies by Tarr

and Alexander (2009) revealed both TIR and non-TIR R genes are restricted to basal angiosperms whereas Magnoliidae family, to which black pepper belongs was reported to be devoid of TIR R genes. This may be because magnolids resemble monocots in which TIR R gene types are rare or absent. *Persea americana* and *P. colubrinum*, which belong to Magnoliidae family were found to be devoid of the TIR NBS-LRR class of R genes (Tarr and Alexander 2009; Malik and George 2018). The presence of non-TIR RGA alone in black pepper is also in agreement with earlier reports in Magnoliidae.

The alignment of deduced amino acid sequences of PnRGAs exhibited the typical features of the NBS domain [P loop (Kinase-1), RNBS-A-non-TIR, Kinase 2, RNBS-B, RNBS-C, and GLPL domains], indicating their possible function in disease resistance (McHale et al. 2006). In silico analysis predicted that amino acid sequences of all 23 PnRGAs belong to P-loop containing nucleoside triphosphate hydrolase (P-loop NTPase) superfamily, which functions in ADP binding by hydrolyzing of the beta-gamma phosphate bond of bound nucleoside triphosphate (NTP) (Zhou et al. 2019). So it is possible that identified PnRGAs may participate in ADP phosphorylation, which is a crucial process in pathogen recognition.

The distinction between resistant genes identified at the genetic level and those expressed during resistance response in plants that vary in degree of resistance is a pre-requisite for the functional validation of plant RGAs. Most plant R genes

Fig. 2 Phylogenetic analysis based on the deduced amino acid sequence alignment of 23 Pn RGAs and NBS domains of known TIR and non-TIR R genes by using maximum likelihood algorithm along with jones-taylorthornton (JTT) model. Bootstrap replications are given as numbers along the branches



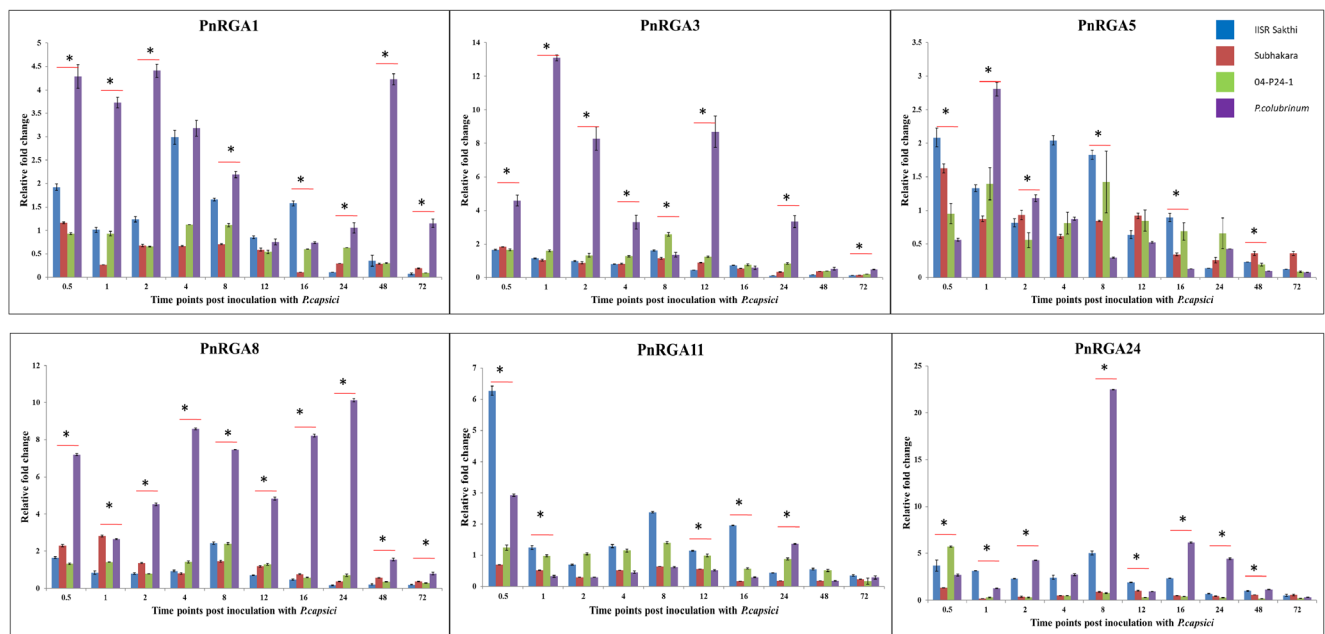


Fig. 3 Relative transcript level of *Piper nigrum* RGAs namely PnRGA1, PnRGA3, PnRGA5, PnRGA8 PnRGA11 and PnRGA24 in IISR Sakthi, Subhakara, 04-P24-1 and *P. colubrinum* at different time points post

inoculation with *P. capsici*. All real time experiments were performed in triplicates ($n = 3$). Data are represented as means \pm SD. Compared with *P. colubrinum* * means $P < 0.05$

are transcriptionally induced in response to pathogen attack, and qPCR could be employed to detect even small fluctuations in gene activation compared to Northern blot analysis (Bustin et al. 2009).

Resistance responses of a plant during attempted infection can be studied by comparing the responses in tolerant/resistant genotype and susceptible genotype with and without pathogen stress. *P. nigrum* genotypes are susceptible to *P. capsici* and lesion appears in leaf within 12 hpi, with progressing margins and after 72 hpi the leaf falls. *P. colubrinum* exhibited hypersensitive response after 12 hpi and lesion does not progress even after 72 hpi when inoculated with highly aggressive isolate of *P. capsici*. This suggests that incompatible interactions occur between *P. colubrinum* and *P. capsici*. Hence, a time frame of 0-72 hpi was chosen for RGA expression studies in *Piper* spp. after pathogen challenge.

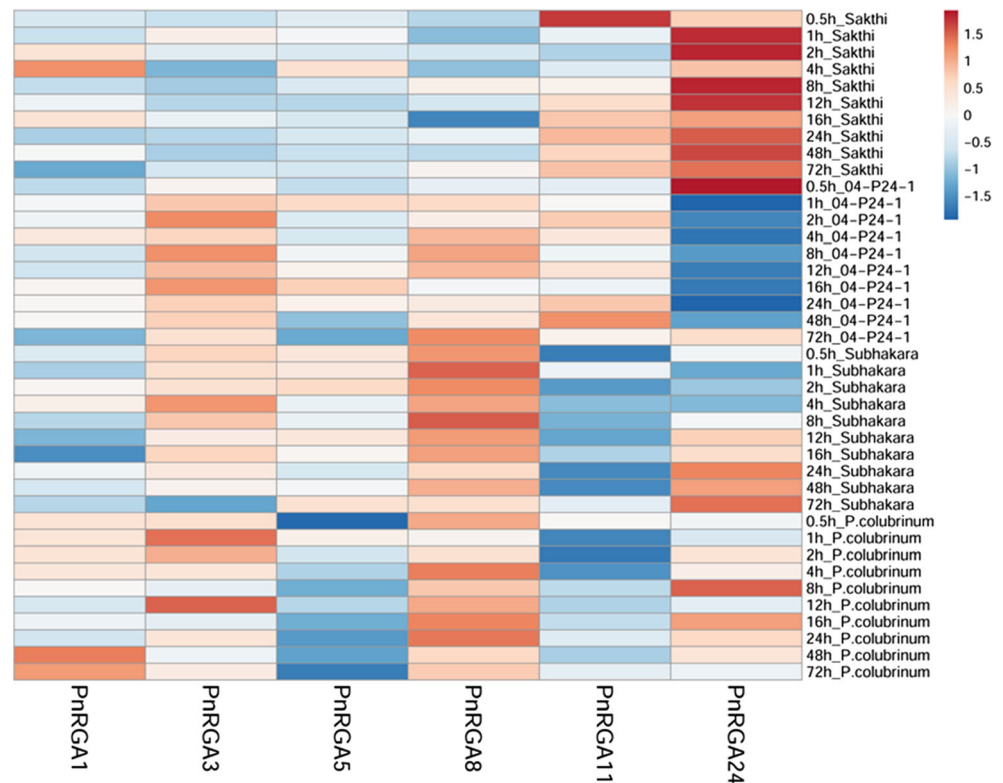
Differential expressions of PnRGAs were observed in resistant (*P. colubrinum*), moderately resistant (IISR Shakthi and 04-P24-1) and susceptible (Subhakara) black pepper genotypes and their relative transcript level varied at different time points. This infers that RGAs are present in all genotypes irrespective of its resistance or susceptible nature; however, its expression will have a significant role in disease resistance. PnRGA transcripts were also expressed in uninoculated leaf samples suggesting that constitutive expression of RGA transcripts occurs for continuously monitoring of pathogen invasion. Low-level transcript accumulation of RGAs under unchallenged conditions was also reported in banana, ginger, and turmeric (Peraza-Echeverria et al. 2008; Nair and Thomas 2007; Kar et al. 2013).

RGA expression in plants should be rapid enough for pathogen recognition and subsequent initiation of defense signaling pathways (Zhou et al. 2019). The expression of PnRGAs was found to be upregulated during the early hours of infection and down-regulated towards the later phases of infection. In *C. annuum*-*P. capsici* pathosystem, CaRGA2 encoding a blight resistance protein was differentially expressed in the resistant and susceptible genotypes following *P. capsici* infection and peak expression was recorded within 12 hpi followed by a decrease in expression (Zhang et al. 2013).

Since *P. colubrinum* is a potential donor for Phytophthora resistance and widely used as rootstock for black pepper propagation, it was essential to determine which of the responsive PnRGA expresses in this genetic background. The mRNA transcript level of PnRGAs was higher compared to *P. nigrum* genotypes after *P. capsici* challenge and this might be due to the hypersensitive response of *P. colubrinum* after 12 hpi.

In *P. capsici*-*S. lycopersicum* pathosystem, after 24hpi, *P. capsici* shifts from biotrophic to necrotrophic lifestyle and distinct transcriptome changes occurred in the host during initial infection and the transition from biotrophy to necrotrophy (24 to 48 hpi). The genes involved in pathogen invasion viz., those involved in germ tube formation and appressoria development were expressed in *P. capsici* during the time frame of 0-24 hpi (Jupe et al. 2013). In our study, we found that most of the PnRGAs were upregulated until 12 h post-inoculation with *P. capsici*. This suggests that RGAs were expressed more during the biotrophic stage of the pathogen. Mahadevan et al. (2016) also observed widespread

Fig. 4 A heatmap representing the fold-change differences in expression among selected six PnRGA at 0.5, 1, 2, 4, 8, 12, 16, 24, 48 and 72 hpi in IISR Sakthi, Subhakara, 04-P24-1 and *P. colubrinum*. Red color represents up-regulation of PnRGAs and blue color represents down-regulation and color intensity indicates fold-change. The mean fold change expression values for each treatment and genotypes were normalized with mentioned reference genes and uninoculated control



colonization of *P. capsici* within 24 hpi and proliferation of mycelia towards other healthy tissues in black pepper variety Panniyur I. We presume that being a hemibiotroph, *P. capsici* changes to the necrotrophic phase of its life cycle with the active involvement of pathogen effectors and it might be responsible for the lowered expression of PnRGA from 24 hpi.

Phytophthora infection in black pepper occurs mainly through soil and aerial infections. Aerial infections occurs on the foliage, shoots, spikes and branches causing blight, spike shedding, defoliation which leads to plant death (Anandaraj 2000). The aerial infection of the plant happens from runner shoots or through the roots close to soil level, leading to black pepper wilt. The root infections culminating in collar rot takes long incubation time and takes nearly 2-3 rainy seasons, whereas the disease spread due to aerial infection in the fields are rapid due to the inoculums transferred by rain splashes and wind blown water droplets (Nambiar and Sarma 1982; Ristaino and Gumpertz 2000).

Our results envisaged that PnRGAs are constitutively expressed irrespective of the pathogen encounter and the transcriptional patterns of PnRGAs after *P. capsici* infection showed that the response was quicker during early infection stages. The future directions of this study should be focused on unraveling the protein functions of the selected RGAs that were differentially expressed in moderately resistant black pepper genotypes after *P. capsici* infection. This study will provide new insights and open new avenues in *Piper* breeding program against Phytophthora resistance. However, more

studies are required to envisage the mechanism of various other gene families which are involved in the resistance response of black pepper during Phytophthora infection.

Acknowledgments The authors extend their gratitude to the Indian Council of Agriculture Research (ICAR) for financial support through ORP on Phytophthora, Fusarium and Ralstonia diseases of horticultural and field crops (PhytoFuRa). The authors acknowledge Dr. Santhosh J Eapen and Mrs. Rosana OB, DISC, ICAR- IISR, Kozhikode for bioinformatics support. The authors are thankful to Dr. Johnson K George, ICAR- IISR, Kozhikode for sharing data of leaf transcriptome of *P. nigrum* L. The authors are also grateful to Huasong Wu, Key Laboratory of Genetic Resources Utilization of Spice and Beverage Crops, Ministry of Agriculture, Wanning, Hainan 571533, China, for sharing data of fruit transcriptome of *P. nigrum* L.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Ameline-Torregrosa C, Wang BB, O'Bleness MS, Deshpande S, Zhu H, Roe B, Young ND, Cannon SB (2008) Identification and characterization of nucleotide-binding site-leucine-rich repeat genes in the model plant *Medicago truncatula*. *Plant Physiol* 146:5–21
- Anandaraj M (2000) Diseases of black pepper (*Piper nigrum* L.). In: Ravindran PN (ed) Black pepper- *Piper nigrum* L., medicinal and aromatic plants-industrial profiles. CRC Press Florida, Boca Raton, p 239

- Anandaraj M, Sarma YR (1995) Diseases of black pepper (*Piper nigrum* L.) and their management. *J Spices Aromat Crops* 4:17–23
- Bailey TL, Bodén M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS (2009) MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res* 37:W202–W208
- Bakker EG, Toomajian C, Kreitman M, Bergelson J (2006) A genome-wide survey of R gene polymorphisms in Arabidopsis. *Plant Cell* 18:1803–1818
- Bayer PE, Golicz AA, Timaz S, Chan CK, Edwards D, Batley J (2019) Variation in abundance of predicted resistance genes in the *Brassica oleracea* pangenome. *Plant Biotechnol J* 17:789–800
- Bhai RS, Eapen SJ, Anandaraj M, Saji KV (2010) Identification of Phytophthora nematode resistant source-from opens pollinated progenies of black pepper (*Piper nigrum*) using a modified protocol. *Indian J Agric Sci* 80(10):893–897
- Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55(4):611–622
- Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19:11–15
- Food and Agriculture Organization of the United Nations (2017) FAOSTAT Database. FAO, Rome Retrieved 1 Dec 2018 from <http://faostat3.fao.org/home/E>
- Gururani MA, Venkatesh J, Upadhyaya CP, Nookaraju A, Pandey SK, Park SW (2012) Plant disease resistance genes: current status and future directions. *Physiol Mol Plant P* 78:51–65
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
- Hu L, Hao C, Fan R, Wu B, Tan L, Wu H (2015) De novo assembly and characterization of fruit transcriptome in black pepper (*Piper nigrum*). *PLoS One* 10(6):e0129822
- Jebakumar SR, Anandaraj M, Sarma YR (2001) Induction of PR proteins and defense related enzymes in black pepper due to inoculation with *Phytophthora capsici*. *Indian Phytopathol* 54:23e28
- Johnson KG, VijeshKumar IP, Anandaraj M, (2012) Transcriptomics approaches for gene discovery in plants - a case study in *Piper*. International Conference on Agricultural and Horticultural Sciences. Hyderabad International Convention Centre, India, Agrotechnology 1(2)
- Joshi RK, Kar B, Mohanty S, Subudhi E, Nayak S (2012) Molecular cloning, characterisation and expression analysis of resistance gene candidates in *Kampferia galanga* L. *Mol Biotechnol* 50(3):200–210
- Jupe J, Stam R, Howden AJM, Morris JA, Zhang R, Hedley PE, Huitema E (2013) Phytophthora capsici-tomato interaction features dramatic shifts in gene expression associated with a hemi-biotrophic lifestyle. *Genome Biol* 14:R63
- Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. *Proc Natl Acad Sci U S A* 93:11746–11750
- Kar B, Nanda S, Kumar PN, Nayak S, Joshi RK (2013) Molecular characterization and functional analysis of CzR1, a coiled-coil-nucleotide-binding site-leucine-rich repeat R-gene from *Curcuma zedoaria* Loeb. That confers resistance to *Pythium aphanidermatum*. *Physiol Mol Plant P* 83:59–68
- Leister D, Ballvora A, Salamini S, Gebhardt C (1996) A PCR based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. *Nat Genet* 14:421–429
- Li P, Quan X, Jia G, Xiao J, Cloutier S, You FM (2016) RGAugury: a pipeline for genome-wide prediction of resistance gene analogs (RGAs) in plants. *BMC Genomics* 17:852
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the 2^{-ΔΔCt} method. *Methods* 25(4):402–408
- Maekawa T, Kufer TA, Schulze-Lefert P (2011) NLR functions in plant and animal immune systems: so far and yet so close. *Nat Immunol* 12:817–826
- Mahadevan C, Krishnan A, Saraswathy GG, Surendran A, Jaleel A, Sakuntala M (2016) Transcriptome- assisted label-free quantitative proteomics analysis reveals novel insights into *Piper nigrum*—*Phytophthora capsici* Phytopathosystem. *Front Plant Sci* 7:785
- Malik N, George JK (2018) Resistance genes in *Piper colubrinum*: In silico survey from leaf transcriptome and expression studies upon challenge inoculation with *Phytophthora capsici*. *Appl Biochem Biotechnol* 184:987–1008
- Marone D, Russo MA, Laido G, Leonardis AMD, Mastrangelo AM (2013) Plant nucleotide binding site- leucine rich repeat (NBS-LRR) genes: active guardians in host defense responses. *Int J Mol Sci* 14:7302–7326
- McHale L, Tan X, Koehl P, Michelmore RW (2006) Plant NBS-LRR proteins: adaptable guards. *Genome Biol* 7:212
- Metsalu T, Vilo J (2015) Clustvis: a web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. *Nucleic Acids Res* 43(W1):W566–W570
- Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding super family. *Plant J* 20:317–332
- Nair RA, Thomas G (2007) Isolation, characterization and expression studies of resistance gene candidates (RGCs) from *Zingiber* spp. *Theor Appl Genet* 116:123–134
- Nambiar KKN, Sarma YR (1982) Some aspects of epidemiology of foot rot of black pepper. In: Nambiar KKN (ed) *Phytophthora* diseases of tropical cultivated plants. Central Plantation Crops Research Institute, Kasaragod, pp 225–232
- Ohmori T, Murata M, Motoyoshi F (1998) Characterization of disease resistance gene-like sequences in near-isogenic lines of tomato. *Theor Appl Genet* 96:331–338
- Peraza-Echeverria S, Dale JL, Harding RM, Smith MK, Collet C (2008) Characterization of disease resistance gene candidates of the nucleotide binding site (NBS) type from banana and correlation of a transcriptional polymorphism with resistance to *Fusarium oxysporum* f.sp. *cubense* race 4. *Mol Breed* 22:565e79
- Purseglove JW, Brown EG, Green CL, Robbins SRJ (1981) *Spices*, vol I. Longman, London
- Ravindran PN, Babu KN, Sasikumar B, Krishnamurthy KS (2000) Botany and crop improvement of black pepper. In: Ravindran PN (ed) *Black pepper- Piper nigrum* L., medicinal and aromatic plants-industrial profiles. CRC Press, Boca Raton, p 23
- Ristaino JB, Gumpertz ML (2000) New frontiers in the study of dispersal and spatial analysis of epidemics caused by species in the genus *Phytophthora*. *Annu Rev Phytopathol* 38(1):541–576
- Sarma YR, Anandaraj M, Venugopal, M (1994) Diseases of spice crops. In: Chadha KL, Rethinam P (eds.), *Advances in horticulture*, Vol 10 (2). Malhotra Publishing House New Delhi, pp. 1015-1058
- Shamina A (1997) Biochemical studies on host-pathogen interactions in black pepper (*Piper nigrum* L.) infected with *Phytophthora capsici*. Ph.D. Dissertation, University of Calicut
- Sievers F, Wilm A, Dineen DG, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal omega. *Mol Syst Biol* 7:539
- Tamura K, Peterson D, Peterson N, Steecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Tarr DEK, Alexander HM (2009) TIR-NBS-LRR genes are rare in monocots: evidence from diverse monocot orders. *BMC Res Notes* 2(1): 197

- Umadevi P, Suraby EJ, Ananaraj M, Nepolean T (2019) Identification of stable reference gene for transcript normalization in black pepper-*Phytophthora capsici* pathosystem. *Physiol Mol Biol Pla* 25(4): 945–952
- Vandana VV, Bhai RS (2018) Differential expression of PR genes in response to *Phytophthora capsici* inoculation in resistant and susceptible black pepper (*Piper nigrum* L.) lines. *Eur J Plant Pathol* 150(3):713–724
- Vandana VV, Bhai RS, Shamina A (2014) Biochemical defense responses of black pepper (*Piper nigrum* L.) lines to *Phytophthora capsici*. *Physiol Mol Plant Path* 88:18e27
- Yang S, Zhang X, Yue JX, Tian D, Chen JQ (2008) Recent duplications dominate NBS-encoding gene expansion in two woody species. *Mol Gen Genomics* 280:187–198
- Zhang Y, Wang J, Zhang X, Chen JQ, Tian D, Yang S (2009) Genetic signature of rice domestication shown by a variety of genes. *J Mol Evol* 68:393–406
- Zhang YL, Li DW, Gong ZH, Wang JE, Yin YX, Ji JJ (2013) Genetic determinants of the defense response of resistant and susceptible pepper (*Capsicum annum*) cultivars infected with *Phytophthora capsici* (Oomycetes; *Pythiaceae*). *Genet Mol Res* 12:3605–3621
- Zhou Z, Bar I, Sambasivam PT, Ford R (2019) Determination of the key resistance gene analogs involved in *Ascochyta rabiei* recognition in chickpea. *Front Plant Sci* 10:644

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