RPF – III

FINAL REPORT OF RESEARCH PROJECT

Cloning of *Phytophthora* resistance and defense genes from *Piper colubrinum*

Indian Institute of Spices Research,

Marikuunu P.O, Calicut – 673 012.

RPF - III
(FINAL REPORT OF RESEARCH PROJECTS)

Part- I : General Information

800 Project Code :

8001 Institute Project Code No. : **ICAR-CIB 2**

8002 ICAR Project Code No. : **3030771021**

801 Name of the Institute and Division

8011 Name and address of Institute :**Indian Institute of Spices Research (IISR)**
P.B. No 1701, Marikunnu PO,
Calicut, PIN-673 012, Kerala

8012 Name of Division / Section : **Division of Crop Improvement and Biotechnology**

8013 Location of the Project : **IISR Calicut.**

802 Project Title : **Cloning of *Phytophthora* resistance and defense genes from *Piper colubrinum***

803 Priority Area

8031 Research Approach: : Basic Research.

804 Specific Area : Plant molecular biology

805 Duration of Project :

8051 Date of start : Nov.2004

8052 Date of Completion : April 2008

806 Total cost /Expenditure Incurred: **Rs. 15,24,999**

807 Executive Summary

In an attempt to isolate disease resistance genes, mRNA population from *P. colubrinum* plants challenged with *P. capsici* were compared with samples from uninoculated plants. Degenerate primers based on conserved sequence motifs of disease resistance genes were designed for amplification of a part for the resistance gene. Isolation of the part (internal region) of the resistance gene sequence (252 bp. fragment corresponding to 84 a. acids) was achieved by this targeted gene amplification. Sequence comparison of the deduced amino acid sequence of the fragment showed sequence similarity with already identified disease resistance genes in public databases.

Attempts were also made to amplify chitinase gene differentially amplified in *P. colubrinum* - *P. capsici* interactions. A 207 base pair cDNA corresponding to 69 amino acids from a putative chitinase gene was amplified, cloned and sequenced.

808 Key words **:** disease resistance genes, *P. colubrinum*

Part-II : Investigator Profile

(Please identify clearly changes, if any in Project personnel)

810 Principal Investigator :

8101 Name : Dr. Johnson George K

8102 Designation : Senior Scientist

8103 Division/ Section : Crop Improvement and Biotechnology

8104 Location : Indian Institute of Spices Research,

8105 Institute Address : Calicut, Kerala - 673012.

811 Co- Investigator:

8111 Name : Dr. M. Anandaraj

8112 Designation : Project Coordinator (Spices)

8113 Division/ Section : Crop protection

8114 Location : Indian Institute of Spices Research,

8115 Institute Address : Calicut, Kerala - 673012.

Par-III: Technical Details

820 Introduction and objectives

8201 Project Objectives :

The major objectives were,

(i) To identify the presence of *Phytophthora* resistance gene and defense genes like chitinase in *Piper colubrinum* .

(iii) To isolation and clone full length cDNA of these resistance and the defense genes.

8202 Background information and importance of the projects

Phytopthora capsici, a fungus that causes foot rot disease in black pepper Is the most devastating of all pepper diseases and no major cultivar with adequate resistance is developed in India or elsewhere. It occurs everywhere black pepper is grown. *Phytopthora* has been classified under oomycetes. With recent efforts in genomics and functional genomics and the resulting resources, genetic research on oomycetes has entered an exciting phase. Control of foot rot in black pepper has proven difficult partly due to a lack of sustainable sources of genetic resistance. The appearance of highly aggressive and fungicide insensitive strains in cultivators garden can result in a new wave of severe and destructive disease spread. Spores transported in the wind or infected planting materials carried to new areas can cause infestation. The disease first appears as a few grayish specks on the plant's leaves, and then a cottony film appears. Under certain climatic conditions (high humidity and cool to warm temperatures), the disease can easily lead to the destruction of a whole field of black pepper. It may also be mentioned that the major reason for wiping out pepper cultivars from the plains of Kerala is mainly attributed to the susceptibility to this dreaded pathogen.

Plants defend themselves from attack by microbial pathogens by activating a battery of defense responses after infection. Gene-for-gene resistance is a particularly strong form of plant disease resistance. Plants carry specific resistance (*R*) genes that are able to recognize pathogens carrying corresponding avirulence (*avr*) genes. This reaction triggers a rapid defense response that generally includes the programmed cell death of plant cells that are in contact with the pathogen, a phenomenon called the hypersensitive response (HR).

One of the *Piper* species collections at IISR viz., *P. colubrinum* is found to be highly resistant to all known strains of *P. capsici.* Of the two main types of resistance, hypersensitive response is the more straightforward - a process thought to involve "gene-for-gene recognition," in which a single resistance gene in the host recognizes a protein produced by a particular gene in the pathogen. Activation of the HR is believed to trigger a systemic resistance response known as systemic acquired resistance (SAR). This response includes the accumulation of the signal molecules throughout the plant and the consequent expression of a characteristic set of defense genes known as pathogenesis related (PR) proteins. Plants expressing SAR are more resistant to subsequent attack by a variety of otherwise virulent pathogens. The R genes in *P. colubrinum* cannot be directly utilized in the breeding programme due to sexually incompatibility with black pepper.

Disease resistance programs based on *R*-genes and defense genes will greatly benefit by molecular biological investigations. The *R*-genes and defense genes could be then transferred into elite black pepper germplasm for durable resistance and sustainable production.

821 Project Technical Profile

8211 Technical programme

Item of work
1. Standardization of RNA isolation protocol
2. Targeted amplification for cloning internal region of the resistance gene
3. Studies on differential induction of chitinase activity in response to inoculation with *Phytophthora capsici*
4. Primer designing and chitinase gene amplification

8212 Total man months involvement of component project workers

a) Scientific – 14 man months
b) Technical- (SRF)- 36 man months
c) Supporting- nil

I. RNA isolation: An modified method based on Trizol (Sigma) was tested for isolation of mRNA from *P. colubrinum* with the inclusion of beta-mercaptoethanol (1%) in the isolation buffer. Even though the method enabled isolation of good quality RNA, the RNA recovery was less compared to the method developed by Johnson *et al* (2005). The sporulating *P. capsici* cultures maintained on carrot agar medium was used for challenging *P. colubrinum* and the inoculated leaves were taken after 12 hours for RNA isolation.

Isolation of high quality RNA from *Piper* is difficult due to the presence of polysaccharides and polyphenolics, which coprecipitate with RNA, making it unsuitable for RT-PCR experiments. A rapid and efficient protocol for RNA isolation from *Piper* leaves which is a modified extraction protocol described by Chomczynski and Sacchi (1987). RNA was extracted from *piper* species with strong denaturing buffer containing guanidinum thiocyanate and PVP followed by precipitation with pre-cooled sodium acetate and extraction with phenol:chloroform:isoamylalcohol. RNA was precipitated with isopropanol. The extraction procedure reliably yield high quality RNA suitable for RT-PCR experiments.

The protocol and the major steps involved are as follows:

- Pulverise 2 g of the frozen leaves in the presence of liquid nitrogen in a pre cooled mortar and pestle with 250 mg PVP (polyvinyl pyrollidone).
- Transfer the ground leaf material to a polypropylene tube containing 5 mL of denaturing buffer.
- Add 0.5 mL, 2 M Sodium acetate (pH4.0) and mix by inverting the tube.
- Add 5 mL of water saturated phenol and mix the tubes gently by inverting.
- Add 1 mL of chloroform: isoamyl alcohol (24:1) and mix the tubes.
- Incubate the tubes on ice for 20 min.
- Centrifuge the tubes at 10,000g for 20 min at 4ºC.
- Carefully transfer the supernatant to a fresh polypropylene tube.
- Add equal amount (5 mL) of cold isopropanol, mix well and incubate the tubes at -20°C for 1 hour to precipitate RNA.
- Centrifuge the tubes at 10,000 g for 20 min at 4ºC.
- Dissolve the pellet containing total RNA in 1.5 mL of denaturing buffer and distribute 0.5 mL in three microfuge tubes.

- Add equal volumes of cold isopropanol to each tube, mix well and incubate at -20°C for 1 hour.
- Centrifuge at 10,000 g for 15 min at 4ºC and discard the supernatant.
- Resuspend the RNA pellet in 75% ethanol and incubate at room temperature for 15 min.
- Centrifuge the tubes for 10 min at 10,000 g at 4°C and aspirate the supernatant.
- Vacuum dry the RNA pellet for 15 min and dissolve in 50 µL of nuclease free water or 100% formamide (for long time storage).
- Keep overnight at 4ºC for dissolving the pellet.
- Aliquot the RNA in 1.5 mL tubes and store at -80°C until use.

The protocol developed rendered a rapid and simple procedure for isolating good quality RNA from *Piper* leaves. Using this protocol, it will be possible to obtain 100µg total RNA from 1g (fresh weight) of *P.colubrinum.* In addition , the spectrophotometric reading A_{260}/A_{280} ratio ranged between 1.8 and 2.0 indicating little or no protein contamination. The integrity of RNA was judged by denaturing agarose gel electrophoresis which showed clear, discrete ribosomal RNA with no apparent RNA degradation. The quality of RNA was also tested by RT-PCR. Oligo $dT_{(18)}$ for the first strand synthesis followed by arbitrary 13mer primers for the second strand synthesis. High intensity bands were observed in agarose gels (2%) stained with ethidium bromide.

II. Targeted amplification for cloning internal region of the resistance gene:

Targeted amplification of an R gene from mRNA population isolated from *P. colubrinum* challenged with *P. capsici* was done using degenerate primers, designed for the purpose. Out of 14 degenerate primers designed based on conserved sequence motif of disease resistance genes, the primer set IA and IV A gave positive results.

First strand cDNA synthesis was performed using a degenerate primer-IV A, second strand synthesis and subsequent amplification using forward and reverse resistance gene specific degenerate primer set IA and IV A. Cloning of the fragment was done using a vector suitable for PCR product cloning. sequencing of the differentially amplified fragment was done using ABI prism technology.

Sequencing revealed the size of the fragment as 252 bp. corresponding to 84 amino acids. Sequence comparison of the deduced amino acid sequences of the fragment was done with NCBI-Blast 2 and SAWTED PSI-BLAST tools. The list of matching sequences are given in the **table 1**. The sequences could match with already identified disease resistance genes in public databases. Homologue of known structure to the sequence using SAWTED-enhanced PSI-BLAST server (table 2) indicated that the sequence is also close to *Phytophthora* resistance gene viz., blight resistance protein RPI gene from *Solanum bulbocastanum* and R1 gene from *Solanum tuberosum*. Sequence similarity was also found between other resistance genes from cacao, *Saccharum*,etc. The fragment, having shown significant similarity to NBS-LRR containing resistance proteins confirms that the fragment belongs to the resistance gene. Further work for isolation of 5' end of the resistance gene did not give expected results and a fragment (~500 bp) isolated and cloned from the RACE reaction was found to be the result of mispairing (confirmed by sequencing).

A similar approach for isolation of resistance gene was also followed for isolation of chitinase gene. A degenerate primer pair (CH 1R & 3F) based on semi-conserved region of chitinase gene yielded in the amplification of a frament of 207 bp corresponing to 69 amino acids was identified in a resistance reaction. The fragment was eluted from the gel, cloned using PCR product cloning protocol and sequenced. The sequence was also found to be related to chitinase gene (most related ; a chitinase gene from a coniferous plant)

Table 1. List of matching sequences and their identity (%) with the R gene fragment based on **NCBI-Blast2.**

Source	Length	Identity%
NBS-LRR resistance gene-like protein ARGH34 (Fragment).	88	43
Resistance protein candidate (Fragment).	88	44
Resistance protein candidate (Fragment).	88	44
NBS-LRR disease resistance protein homologue (Fragment).	179	44
NBS-LRR disease resistance protein homologue (Fragment).	940	44
NBS-LRR protein (Fragment).	181	45
Putative NBS-LRR type disease resistance protein.	917	47

Disease resistance-like protein (Fragment).	177	46
Disease resistance-like protein (Fragment).	177	44
NBS-LRR disease resistance protein-like.	306	46
NBS-LRR protein (Fragment).	173	43
NBS-LRR protein (Fragment).	180	44
NBS/LRR resistance protein-like protein (Fragment).	244	40
NBS/LRR disease resistance-like (Fragment).	97	40
Putative disease resistance RPP13-like protein 4.	852	41
Disease resistance gene analog PIC11 (Fragment).	172	45
NBS-LRR disease resistance protein RPM1-like protein (Fragment).	177	37
Disease resistance gene homolog 9N.	926	38
NBS/LRR disease resistance protein RPM1.	921	36
NBS/LRR resistance protein-like protein (Fragment).	255	40
NBS/LRR resistance protein-like protein (Fragment).	255	40
NBS/LRR resistance protein-like protein (Fragment).	250	40
NBS/LRR resistance protein-like protein (Fragment).	230	40
Hypothetical protein (Fragment).	183	44
OSJNBa0065H10.8 protein.	974	42
NBS-LRR protein (Fragment).	132	40
Putative RPR1.	927	39
Putative disease resistance protein RPR1.	906	42
Disease resistance gene homolog 1A.	927	37
Putative disease related protein 2.	935	40
Disease resistance protein RPM1 (Resistance to Pseudomonas syringae protein 3).	926	36
NBS-LRR resistance gene-like protein ARGH35 (Fragment).	88	42
Putative NBS-LRR disease resistance protein.	1026	40
Table 1 Continued---------- NBS-LRR protein (Fragment).	173	40
Resistance gene alike (Fragment).	182	41
NBS-LRR disease resistance protein homologue (Fragment).	185	40
Putative citrus disease resistance protein Pt3 (Fragment).	172	39
Putative citrus disease resistance protein Pt4 (Fragment).	172	39
Hero resistance protein 1 homologue.	1282	43
Putative NBS-LRR disease resistance protein.	974	43
NBS-LRR protein (Fragment).	173	42
Disease resistance protein.	1257	40
Disease resistance gene homolog Mi-copy2.	1206	40
Tospovirus resistance protein D.	1263	42
Tospovirus resistance protein E.	1241	42
Disease resistance-like protein (Fragment).	176	38
Root-knot nematode resistance protein.	1257	40
Hero resistance protein 2 homologue (Fragment).	1147	46

Tospovirus resistance protein C.	1271	41
NBS-LRR protein (Fragment).	180	36

Table 2. The SAWTED alignment score and database hits selected at random with E-values better than 0.1.

	query		bits	E-value	N	100.0%
7	gi|32470638|gb|AAP45165.1|	putative disease resistant ...	118	5e-26	1	34.1%
26	gi|39636705|gb|AAR29069.1|	blight resistance protein R...	115	3e-25	1	32.9%
42	gi|2792208|gb|AAB96979.1|	NBS-LRR type resistance pro...	113	1e-24	1	35.4%
53	gi|7248786|gb|AAF43667.1|	NBS-LRR-like protein [Oryza...	112	2e-24	1	30.5%
61	gi|15788516|gb|AAL07816.1|	NBS-LRR-like protein [Horde...	110	8e-24	1	31.7%
79	gi|39636816|gb|AAR29076.1|	blight resistance protein T...	108	2e-23	1	30.5%
81	gi|34910060|ref|NP_916377.1|	putative rust resistance pr...	108	3e-23	1	31.7%
95	gi|54291758|gb|AAV32127.1|	putative NBS-LRR type resis...	107	6e-23	1	30.5%
99	gi|32470636|gb|AAP45163.1|	putative disease resistant ...	107	6e-23	1	29.3%
109	gi|21616918|gb|AAM66421.1|	NBS-LRR protein [Oryza sati...	106	1e-22	1	31.7%
114	gi|13487351|gb|AAK27507.1|	rust resistance protein Rp1...	106	1e-22	1	30.5%
122	gi|32423726|gb|AAP81259.1|	rust resistance protein Rp1...	106	1e-22	1	30.5%
138	gi|5305461|gb|AAD41662.1|	resistance protein [Oryza s...	105	3e-22	1	31.7%
141	gi|12744957|gb|AAK06859.1|	rust resistance protein Rp1...	105	3e-22	1	29.3%
152	gi|24459853|emb|CAC82602.1|	disease resistance-like pro...	104	4e-22	1	47.6%
154	gi|50948143|ref|XP_483599.1|	putative resistance gene an...	104	6e-22	1	31.3%
160	gi|38045772|gb|AAR08858.1|	resistance protein candidat...	103	1e-21	1	29.3%
167	gi|10177352|dbj|BAB10695.1|	disease resistance protein ...	103	2e-21	1	35.4%
183	gi|37535878|ref|NP_922241.1|	putative disease resistant ...	102	3e-21	1	28.0%
187	gi|18181934|dbj|BAB83872.1|	disease resistance protein ...	101	3e-21	1	31.7%
195	gi|54291872|gb|AAV32240.1|	putative NBS-LRR type disea...	101	4e-21	1	26.8%
205	gi|6573285|dbj|BAA88265.1|	unnamed protein product [Ar...	101	5e-21	1	31.7%
211	gi|8843900|dbj|BAA97426.1|	disease resistance protein ...	101	5e-21	1	36.6%
212	gi|53749439|gb|AAU90295.1|	putative disease resistance...	101	5e-21	1	32.9%
215	gi|51090834|dbj|BAD35362.1|	putative disease resistance...	100	7e-21	1	29.5%
226	gi|26190258|emb|CAD29728.1|	hero resistance protein [Ly...	100	1e-20	1	41.5%
230	gi|3928862|gb|AAC83165.1|	disease resistance protein ...	100	1e-20	1	36.6%
245	gi|6520229|dbj|BAA87956.1|	PRM1 homolog [Arabidopsis t...	99	2e-20	1	34.1%

III. RACE (Rapid Amplification of cDNA Ends) and gene walking for identification of 5' end of the *Phytophthora* resistance gene:

RACE (Rapid Amplification of cDNA Ends) allows the isolation and characterisation of the extreme 5' end of the transcript. The method is used to extend partial cDNA clones by amplifying the 5' sequences of the corresponding mRNAs. The technique requires knowledge of only a small region of sequence within the partial cDNA clone. The primer designed based on the nucleotide composition of the partially sequenced *Phytophthora* resistance gene was used in the reaction. The product from the RACE reaction was cloned and revealed that it is derived from the result of priming of the desgned primer alone. The experiments are being repeated. As an alternate approach, primers were designed for gene walking using DNA. Fragments of different sizes were found in the amplification reactions and are being cloned and sequenced.

IV. Studies on differential induction of chitinase activity in response to inoculation with *Phytophthora capsici:*

Plant chitinases have been of particular interest since they are known to be induced upon pathogen invasion. Inoculation of *P. colubrinum* leaves with the foot rot fungus, *P. capsici* leads to increase in chitinase activity. A marked increase in chitinase activity in the inoculated leaves was observed, with the maximum activity after 60 hours of inoculation and gradually decreased thereafter. Older leaves showed more chitinase activity than young leaves. The level of chitinase in black pepper (*Piper nigrum* L.) upon inoculation was found to be substantially high when compared to *P. colubrinum.* However, hyphal extension assays revealed no obvious differences in the ability of the protein extracts to inhibit growth of *P. capsici in vitro*.

Assay of chitinase activity

Chitinase activity in the leaf extracts of chitinase was performed using colloidal chitin as substrate. The increased reducing ends, which were released by *P. colubrinum* chitinase were determined according to the method of Nelson-Somogyi (Somogyi, 1952). The absorbance was taken at 620 nm. One unit of chitinase activity was defined as an amount capable of releasing reducing ends corresponding to 1 µg GlcNAc from colloidal chitin at pH 5.0 in one hour.

The differential activity of chitinase in *P. colubrinum* was analyzed on young and old leaves. The chitinase enzyme activity calculated is given in the table 3. While enzyme activity in the young leaves (top of the plant) was found to be less, increasing amounts of chitinase were present in the old leaves (Fig. 2.)

Table 3. Chitinase activitiy in young and old leaves of *P. colubrinum*

Sl. No.	Sample	Chitinase activity (Units/ml)
1	Young leaves	0.813 ± 0.23
2	Old leaves	1.695 ± 0.14

Fig. 2. The chitinase activities in protein extracts *P. colubrinum* leaves. 1- young leaves, 2- old leaves. Each mean and error bar were obtained from samples of four repeated blocks.

Optimum pH and temperature of chitinase activity

To characterize the chitinase isolated from *P. colubrinum*, the chitinase activity over a wide range of pH and temperature were examined. Leaf extracts were used as the source of the enzyme. For determining the pH effect, chitinase activity assays were carried out in buffers with pH values ranging from 4 to 6.5. The highest activity was observed at pH 4.5 (Fig. 3). At pH 5 and 5.5, the activity was approximately 90% of that of pH 4.5. The chitinase activity was found to decrease strongly after pH 5.5.

The optimum temperature of *P. colubrinum* chitinase was determined by incubating protein extracts at different temperatures ranging from 25 to 40°C. The optimum temperature was found to be 30°C, at which the chitinase activity was the maximum (Fig.4).

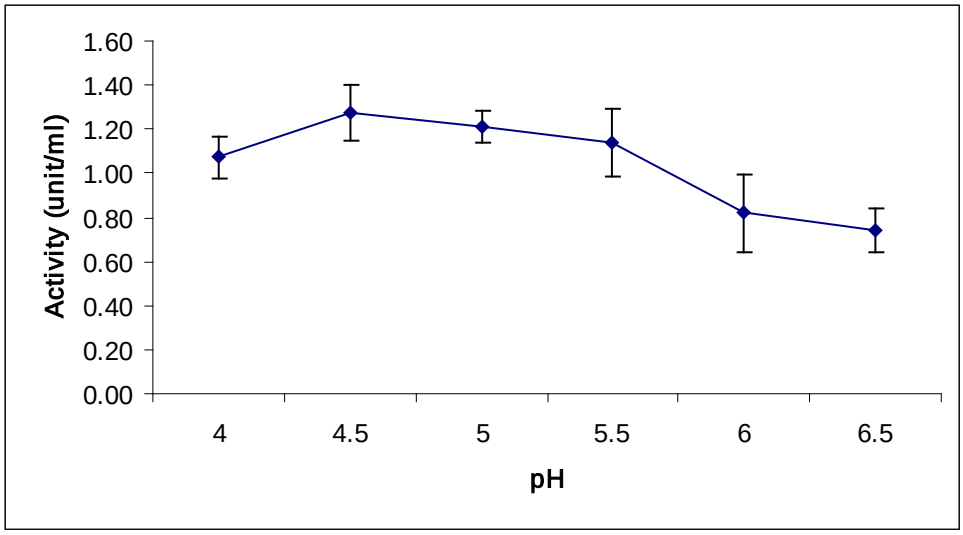

Fig. 3. The effect of pH on chitinase activity. Leaf extract of *P.colubrinum* and colloidal chitin in buffers at different pHs was incubated at 37°C for 30 min, and the activity was measured at 620 nm. Each mean and error bars were obtained from samples of four repeated blocks.

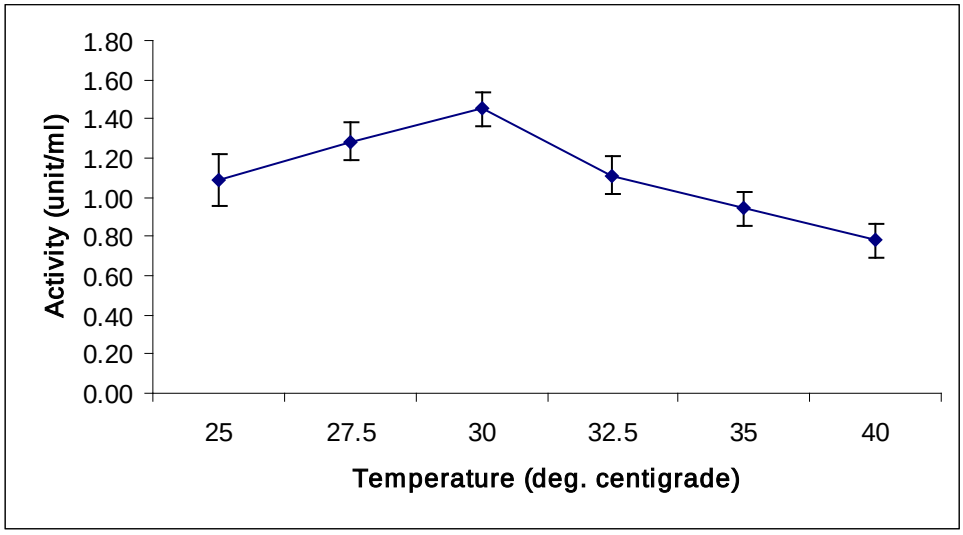

Fig.4. The effect of temperature on chitinase activity. Leaf extract of *P.colubrinum* and colloidal chitin was incubated at different temperatures for 30 min, and the activity was measured at 620 nm. Each mean and error bars were obtained from samples of four repeated blocks.

Activity staining of chitinase

The protein extract of *P. colubrinum* was fractionated on glycol-chitosan containing SDS-polyacrylamide gels. After renaturing and staining the gel with calcoflour white M2R, four major activity bands were observed in the gel when observed under exposure to UV light.

The molecular weight of the bands were found to range between 30-40 kDa. The possible explanation of this discrepancy could be that the chitinase detected is a protein complex consisting of multiple isoforms.

Differential accumulation of chitinase

Inoculation with Phytophthora capsici: *Visual estimation of hypersensitive reaction*

Necrotic cells associated with the hypersensitive reaction was observed in the cells after inoculation with *P. capsici.* The intensity of the necrotic spots were higher in the leaves inoculated for 48 hours and above with the pathogen, whereas only a small number of single necrotic cells was observed in the leaves inoculated for a period of 24 hours with the pathogen. Necrotic spots could not be detected in the leaves inoculated with *P. capsici* for 12 hour duration.

Chitinase activity of leaf extracts

The chitinase activity of the extracts of *P. colubrinum* leaves harvested at different time intervals after inoculation with *P. capsici* are given in the Fig. 5. Maximum levels of chitinase activity in *P. colubrinum* was observed 60 hours post inoculation after which the levels of chitinase gradually decreased. The chitinase activity was found to be low in uninoculated plants.

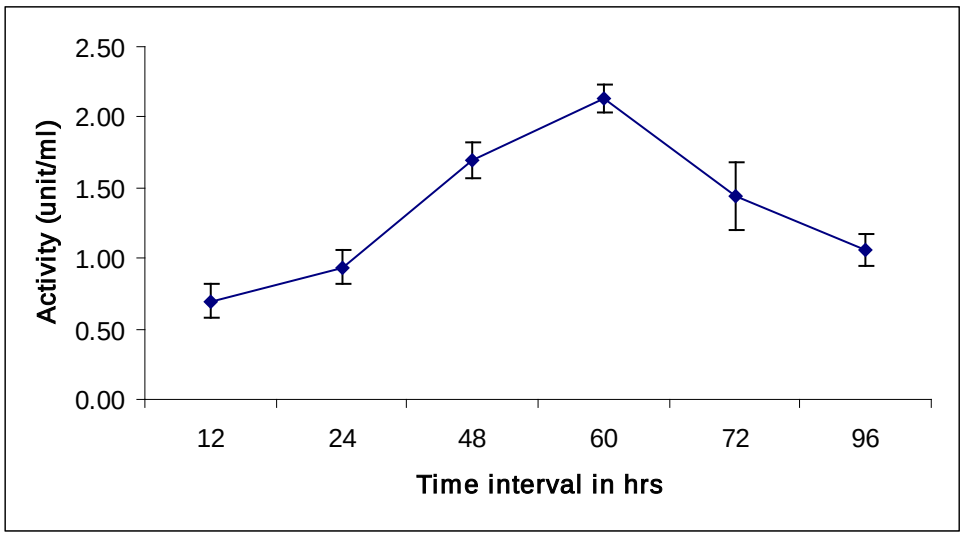

Fig. 5. Chitinase activities of leaf extracts from *P. colubrinum* after 12, 24, 48, 60, 72 and 96 hours of inoculation with *P. capsici*. Values presented are the means of 4 replicates. Error bars represent the standard errors of the mean.

The chitinase activity of *P. colubrinum* and *P. nigrum* after 48 hours of inoculation with *P. capsici* was determined. The chitinase activity of *P. nigrum* was found to be higher than *P. colubrinum* (Fig. 6). The chitinase

activity obtained from *P. colubrinum* and *P. nigrum* is given in the table 3. The result showed that chitinase activity of *P. nigrum* was found to be approximately 30% higher than that of *P. colubrinum*.

Table 3. Chitinase activitiy of *P. colubrinum* and *P. nigrum* after 48 hours of inoculation with *P. capsici.*

Sl. No.	Sample	Chitinase activity (Units/ml)
1	*P. colubrinum*	1.378 ± 0.23
2	*P. nigrum*	1.813 ± 0.25

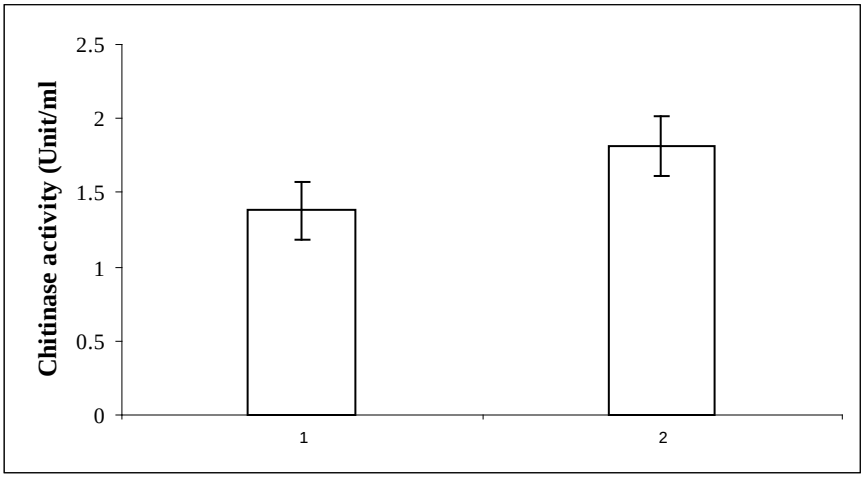

Fig. 6. The chitinase activities in protein extracts of *P. colubrinum* and *P. nigrum*. 1- *P. colubrinum*, 2- *P. nigrum.* Each mean and error bar were obtained from samples of four repeated blocks.

Antifungal assay

To check the ability of the chitinase extract to inhibit the growth of *P. capsici in vitro*, hyphal extension assays was carried out. 100, 150 and 200 µg of protein extracts of *P. colubrinum* were applied on filter paper discs. Extraction buffer was used as negative control. After 24 hours of incubation at 21°C in the dark, the plate was observed for any zone of inhibition. However, the results showed that no inhibition was observed in the fungal growth when compared to the control (Fig. 21). From the results, it could be concluded that the protein extract of *P. colubrinum* was unable to inhibit the growth of *P. capsici in vitro.*

RT-PCR for confirmation of presence of specific RNA

In order to confirm whether the induction of chitinase was the result of gene activation, RNA was isolated from the inoculated (18 hour inoculation) and non inoculated leaves of *P. colubrinum*. Good quality RNA was observed on 1% denaturing agarose gel stained with ethidium bromide. RT-PCR was carried out using oligo dT in the first strand synthesis followed by oligo dT and a chitinase specific primer (primer CHIT F1) in the second strand synthesis. The RT-PCR products were analysed on 1.5% agarose gel. The result showed that abundance of chitinase mRNA was low in control leaves whereas, 18 hours post inoculation, a strong increase in chitinase cDNA was observed in the inoculated leaves. A 540 bp chitinase specific cDNA fragment was amplified in the inoculated leaves and was absent in the uninoculated control.

V. Primer designing and chitinase gene amplification:

Chitinase sequences (both protein and nucleic acid) were obtained from SWISS-PROT and TrEMBL (Protein knowledge base) in ExPASy (Expert Protein Analysis System) Molecular Biology Server. Barley chitinase structure was taken as a model to design primers. Sequence homologues was obtained by FASTA search in PDB. The chitinase sequences were subjected to Basic Local Alignment Search Tool-(BLAST) heuristic search algorithm to search the sequence databases at NCBI to get the sequence alignments with E-values to identify those sequences that shared the highest percent identity with the query sequence. Then the chitinase sequences were submitted for multiple alignment program provided by different online softwares CLUSTAL-W by Genebee software. The conserved amino acid sequences was used to design degenerate primers using primer Premier 5 software. A total of 12 primers were designed based on conserved sequence motiffs of chitinase gene. RT-PCR was done employing the designed primers to amplify the chitinase specific cDNA fragment. A cDNA product of 313 bp was obtained (fig. 7). These cDNA fragments were eluted from the gel and reamplified using the respective primers. These cDNA fragments were cloned into pTZ5R/T vector (Fermentas) and transformed into TOP10F' cells. The recombinant plamids were purified and the presence of clones were confirmed by PCR using the respective primers. Sequencing of the clone was done using ABI prism technology(table1). Sequence comparison of the deduced amino acid sequences of the fragment was done with NCBI-Blast 2 and revealed to be closely related to different classes of chitinase genes isolated from other plants (table 3).

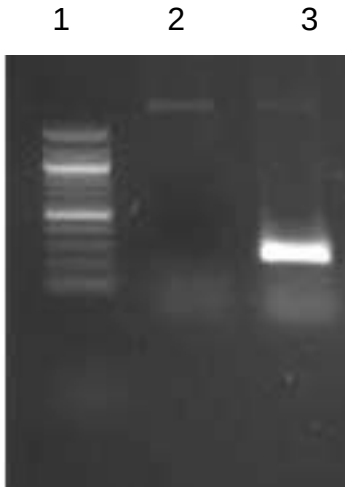

Fig 7. Amplification product of the chitinase gene fragment. Lane1- marker, lane 3- chitinase gene fragment (313 bp).

Table 4. List of matching sequences and their identity (%) with the chitinase gene fragment based on NCBI-Blast2.

Alignment	DB:ID	Source	Length	Score	Identity%	Positives
1 ☑	UNIPROT:O82552_CAPAN	Chitinase class II (Chi2).	253	115	66	79
2 ☑	UNIPROT:Q6SPQ7_BAMOL	Chitinase (EC 3.2.1.14).	334	115	42	54
3 ☑	UNIPROT:Q9SDW1_PRUPE	Basic chitinase type I (Fragment).	156	114	43	53
4 ☑	UNIPROT:Q84LQ7_SOLDU	29 kDa chitinase-like thermal hysteresis protein (Fragment).	267	112	66	76
5 ☑	UNIPROT:Q688M5_ORYSA	Putative chitinase.	334	111	63	76
6 ☑	UNIPROT:Q8VWZ5_ORYSA	Chitinase (EC 3.2.1.14).	333	111	63	76
7 ☑	UNIPROT:Q9ZWS3_TOBAC	Chitinase 134.	265	111	40	54
8 ☑	UNIPROT:Q42992_ORYSA	Chitinase precursor (EC 3.2.1.14).	333	111	63	76
9 ☑	UNIPROT:Q6SZS3_ORYSA	Chitinase (EC 3.2.1.14).	333	111	63	76
10 ☑	UNIPROT:Q42428_CASSA	Chitinase Ib (Endochitinase).	316	111	42	54
11 ☑	UNIPROT:Q5RLX9_MEDSA	Chitinase.	328	110	32	43
12 ☑	UNIPROT:P93680_PERAE	Endochitinase precursor (EC 3.2.1.14).	326	110	40	54
13 ☑	UNIPROT:Q4PJV8_9ROSA	Chitinase.	317	110	66	76
14 ☑	UNIPROT:Q7X9F6_9FABA	Class Ib chitinase.	326	110	38	55
15 ☑	UNIPROT:P93327_MEDTR	Chitinase.	325	109	66	76
16 ☑	UNIPROT:P94084_MEDSA	Class I chitinase.	327	109	66	76
17 ☑	UNIPROT:Q1T5W7_MEDTR	Glycoside hydrolase, family 19; Chitin-binding, type 1.	325	109	66	76
18 ☑	UNIPROT:Q9FEW1_NICSY	Endochitinase precursor.	324	109	66	76
19 ☑	UNIPROT:Q7X9F5_9FABA	Class Ia chitinase.	326	109	66	76

8221 Achievements in terms of targets fixed for each activity

1. Total RNA , mRNA isolation and RT-PCR protocols were standardized in *Piper*.
2. Primers designed based on partially conserved regions of Phytophthora resistance and other fungal resistance genes were used in successful amplification of 3' side of the resistance gene from *Piper colubrinum.*
4. Primers were designed based on conserved sequence motifs of chitinase genes and partial cDNA corresponding to the genes were successfully amplified from *Piper colubrinum* using these primers.
5. Chitinase activity was determined in *P. colubrinum* using colloidal chitin as substrate and the chitinase activity of *P. colubrinum* was found to increase substantially, upon challenging the plants with *Phytophthora capsici*. The maximum activity was observed 60 hours post inoculation.

8222 Questions- Answered

The results presented here clearly demonstrate that RNA profiling is a powerful technique that is capable of cloning R genes and other genes. The technique can be used across species with little or no modifications of the protocol.. This motif-directed RNA profiling may be carried out on any set of targets for amplification in the transcriptome with sufficient conservation to allow selective binding of a (degenerate) primer. Targets may include interesting gene families in plants as well as other organisms.

8223 Process/ Product/ Technology/ Developed

Targeted amplification of an R gene from mRNA population isolated from *P. colubrinum* challenged with *P. capsici* was done using degenerate primers, designed for the purpose. Sequence comparison of the deduced amino acid sequences of the fragment found match with already identified disease resistance genes in public databases.

Degenerate primers designed for amplification based on conserved sequence motifs of chitinase genes resulted in the cloning of a part for the gene of 313 base pairs. Sequence comparison of the deduced amino acid sequence of the fragment showed sequence similarity with already identified chitinase genes in public databases

8224 Practical Utility
(not more than 150 words)

Piper colubrinum , the exotic wild *Piper* shows high degree of resistance to *Phytophthora capsici* and other disease causing organisms and is a potential source of resistance genes. The transcript-based cloning method experimented under this project is a valid and viable approach for gene cloning in *Piper* and in other crop plants. This method allows faster and more efficient gene cloning than is typically achievable through positional cloning (a laborious process) methods. This method of gene identification is independent of gene position, this method does not require the construction of a genetic map.

8225 Constraints, if any --

823 Publications and Material Development
(One copy each to be supplied with this proforma.)

8231 Research papers

Johnson George, K., Sandeep Varma, R., Ganga, G., M. Anandaraj and Parthasarathy V.A. 2005. A method for isolation and reverse transcription of high quality RNA from *Piper* species. Jl.Spices and Arom. Crops 14 (1): 10-14.

Sandeep Varma, R., Johnson George, K., Balagi, S. and Parthasarathy V.A. (2009) Differential induction of chitinase in *Piper colubrinum* in response to inoculation with *Phytophthora capsici*, the cause of foot rot in black pepper. Saudi Journal of Biological Sciences (2009) 16, 11–16.

8232 Popular articles - Nil

8233 Reports -Nil

8234 Seminars, conferences and workshops (relevant to the project) in which the scientists have participated. (List abstracts forwarded)

Johnson George K., M. Anandaraj and V.A.Parthasarathy. Cloning *Phytophthora* resistance and chitinase genes from *Piper colubrinum* based on RNA profiling. National Seminar on Horticultural Biotechnology, IIHR, Bangalore, 8 Dec. 2007.

824 Infrastructural facilities developed
(Details of field, laboratory, note books and final material and their location)

PCR and electrophoresis equipments procured under the programme are under use in the centralized facility of IISR.

825 Comments / Suggestions of Project Leader regarding possible future line of work that may be taken up arising out of this Project.

Pogrammes continued under the new project (Phytofura) under network mode

Part-IV : Project Expenditure
(Summary)

830	**Total Recurring Expenditure**	
8301	Salaries: (Designation with pay scale)	
		Actual (Rs)
	i) Scientific Year	(2004-05 to 2007-08)
	ii) Technical	6,21,501
	iii) Supporting	
	iv) Wages	
Sub-Total		6,21,501
8302	Consumables	5,44,143
Sub-Total		5,44,143
8303	Travel	
8304	Miscellaneous Instt. Charges (other costs)	1,01,580
830Sub-Total		12,67,224
	(Recurring)	
831	**Total Non – Recurring** Expenditure (Equipments and works)	
	i) Equipments	2,57,775

823	2,57,775
Total	
Total (830 and 831)	**15,24,999**