# RPF-III

## PERFORMA FOR SUBMISSION OF FINAL REPORT OF RESEARCH PROJECTS

Part- I : General Information

**800 Project Code** : **Path. XII (813)**

8001 Institute Project Code No. : Path. XII (813)

8002 ICAR Project Code No.

**801 Name of the Institute and Division**

8011 Name and address of Institute : Indian Institute of Spices Research, Calicut –673 012, Kerala

8012 Name of Division / Section : Division of Crop Protection

8013 Location of the Project : IISR, Calicut, Kerala, India

**802 Project Title** : Investigations on stunted and phyllody diseases of black pepper

**803 Priority Area** : Crop Protection

8031 Research Approach :

| Applied Res. | Basic Res. | Process or Tech. Dev. | Transfer of Tech. |
|---|---|---|---|
| ✓ | ✓ | ✓ | ✓ |

804 Specific Area : Identification & development of diagnostics against pathogen

**805 Duration of Project** : 13 years

8051 Date of start : April 1992

8052 Date of Completion : March 31, 2005

**806 Total cost /Expenditure Incurred** : Rs 31,60,000
(Give reasons for variation, if any from original estimated cost)

**807 Executive Summary**

Stunted disease is the third important disease affecting black pepper in the country. Survey indicated the prevalence of the disease in all black pepper growing areas with high incidence and severity in black pepper plantations located at higher altitudes such as Idukki and Wyanad districts of Kerala, and Kodagu district of

Karnataka. The symptoms of the disease included mosaic mottling on the leaf, reduction in leaf size and internode length leading to stunting of whole vines. Analysis of the isolates revealed the presence of two viruses namely *Cucumber mosaic virus* and a Badnavirus. The badnavirus was shown to be transmitted through mealybug, *Ferrisia virgata* and *Planococcus citri* in a semipersistant manner. The vertical transmission through use of infecting cuttings is the major cause for spread of the viruses. The phyllody disease was characterized by the malformation of spikes and spikelets into small leaf like structures. The affected vine showed yellowing symptoms and witches' broom in appearance. Based on cloning and sequencing of 1.2 kb region of 16S rDNA showed that the disease is caused by a phytoplasma belonging to aster yellows group. A reliable nested PCR method was developed for the detection of phytoplasma in affected vines.

**808 Key words** : stunted disease, survey, incidence, transmission, symptoms, virus, phyllody, phytoplasma, nested PCR.

**Part-II : Investigator Profile**
(Please identify clearly changes, if any in Project personnel)

**810 Principal Investigator** :

| | | |
|---|---|---|
| 8101 | Name | : Dr. A.Ishwara Bhat |
| 8102 | Designation | : Senior Scientist |
| 8103 | Division/ Section | : Crop Protection |
| 8104 | Location | : Indian Institute of Spices Research |
| 8105 | Institute Address | : Indian Institute of Spices Research<br>Calicut-673 012, Kerala, India |

**811 Co- Investigator:**

| | | |
|---|---|---|
| 8111 | Name | : Dr. S. Devasahayam |
| 8112 | Designation | : Principal Scientist |
| 8113 | Division/ Section | : Crop Protection |
| 8114 | Location | : Indian Institute of Spices Research |
| 8115 | Institute Address | : Calicut-673 012, Kerala, India |

**812 Co- Investigator:**

| | | |
|---|---|---|
| 8121 | Name | : Dr M.N. Venugopal |
| 8122 | Designation | : Principal Scientist |
| 8123 | Division/ Section | : Crop Protection |
| 8124 | Location | : Indian Institute of Spices Research |
| 8125 | Institute Address | : Indian Institute of Spices Research<br>Cardamom Research Centre, Appangala,<br>Madikeri, Karnataka. |

**Par-III: Technical Details**

**820 Introduction and objectives**

8201 Project Objectives : To study etiology, distribution of the disease in major growing areas and evolving control measures.

8202 Background information and importance of the projects

Among the disease of unknown etiology, stunted disease and phyllody are on the increase in some of the black pepper growing areas of Kerala. The disease was first started in some isolated pockets in Idukki district of Kerala during 1970s. Now it is noticed in Wyanad and Idukki districts the two major black pepper growing districts of Kerala. The symptoms are mottling of leaves, reduction in size of leaves and internodes which give an appearance of stunted growth. It is proposed to study the etiology, distribution of the disease in major black pepper growing areas and evolving control measures.

**821 Project Technical Profile**

8211 Technical programme

(Indicate briefly plan of procedure, techniques, instruments and special materials, organisms, special environments etc.)

Survey for incidence of the disease in black pepper growing areas.

Studies on the etiology of the disease, its transmission and evolving suitable control measures

8212 Total man months involvement of component project workers

| | | |
|---|---|---|
| a) | Scientific | 70 |
| b) | Technical | 39 |
| c) | Supporting | 33 |

**822 Final Report on the Project**

Detailed report containing all relevant data with a summary of results (not exceeding 2-5 pages)

8221 Achievements in terms of targets fixed for each activity

**Survey for the incidence of the disease**

Three districts of Karnataka (Dakshina Kannada, Hassan and Uttara Kannada) and four districts of Kerala (Idukki, Kannur, Kasragod and Kozhikode) covering 216 black pepper gardens were surveyed for the presence of stunted disease and associated insect fauna. The incidence of the disease was highest at Idukki District of Kerala. In Kerala except for four locations (Kodathai, Mulleria, Santapara and Thodupuzha), the incidence of viral diseases was noticed in all the locations surveyed (Table 1). No incidence of viral disease was noticed in any of the plantations surveyed in Dakshina Kannada District of Karnataka while only one plantation in Uttara Kannada District of Karnataka showed the presence of the disease. In Hassan District, Belur Taluk had a higher disease incidence compared to other taluks surveyed. In general disease incidence and severity was higher in black pepper plantations situated at higher altitudes of Kerala (Table 1). Mosaic, reduction in leaf size and internode length leading to the stunting of vine, and bright yellow mottling along veins of the leaves were the two kinds of symptoms observed on diseased vines. All cultivars and improved varieties including hybrids were found susceptible to the diseases. Vines of all ages raised on all kinds of standards were also found affected by the diseases. Among the several weeds found in and around black pepper plantations, a few of them showed typical viral like symptoms, which might act as potential alternate hosts for the virus. Though 12 species of insects were collected from diseased vines no species was specifically associated with diseased vines.

**Table 1. Incidence of viral disease of black pepper in various districts of Karnataka and Kerala**

| State /District | Disease incidence | |
|---|---|---|
| | Range (%) | Mean (%) |
| **Karnataka** | | |
| Dakshina Kannada | 0 | 0 |
| Hassan | 0-20 | 5.2 |
| Madikeri | 0-78 | 14.9 |
| Uttara Kannada | 0-2 | 0.4 |
| **Kerala** | | |
| Idukki | 0-78 | 29.4 |
| Kannur | 2-42 | 19.5 |
| Kasaragod | 0-53 | 18.9 |
| Kozhikode | 0-33 | 10.7 |
| Wyanad | 13-83 | 45.4 |

**Serological analysis of isolates**

When the collected isolates were subjected to direct antigen coated (DAC) ELISA using antisera to different viruses, majority of the isolates reacted with antiserum to either CMV or Banana streak badnavirus (BSV) while a few of the isolates reacted with both the antisera indicating the involvement of two viruses belonging to the genera *Cucumovirus* and *Badnavirus* in the disease. Majority of the isolates from Idukki and Wyanad Districts of Kerala reacted with CMV antiserum while majority of the samples form remaining locations reacted with BSV antiserum indicating the involvement of badnavirus with the diseased vines collected from these locations.

**Crop loss due to disease**

The yield loss due to stunted disease was estimated in a fixed plot at Godukutti, Madikeri. The diseased plants were graded based on the severity of infection into four categories. Average yield per vine in all the four categories were taken and compared with yield obtained with healthy. A yield loss varying from 50 to 85% were seen among infected plants. Similarly number of spikes required per kg also varied in infected vines. In the case of healthy about 165 spikes make on kg while in the case of infected 270 to 410 spikes were required to make one kg thus

indicating poor filling in diseased vines. When berries collected from diseased vines were subjected quality analysis, increase in the essential oil, oleoresin and piperine were observed with berries collected from stunted disease affected plants. Variation in the constituents were seen among plants showing different levels of disease severity, the least being in severely diseased vine.

**Transmission of badnavirus by mealybugs**

Citrus mealybug (*Planococcus citri* (Risso), and striped mealybug (*Ferrisia virgata*) commonly found associated with black pepper (*Piper nigrum* L.) was reared laboratory conditions on matured pumpkins. After three generations on pumpkins, the non-viruliferous young adult female mealybugs were given a 24 h acquisition access on diseased black pepper leaves. Fifteen mealybugs each were then transferred to 30 day old healthy black pepper seedlings and given a 24 h inoculation access period. Plants were sprayed to kill the mealybugs and kept for observation in insect proof glass house. Initial symptoms of the disease such as vein clearing and chlorotic mottle could be seen four weeks after inoculation in about 60% of plants used. Total nucleic acid extracted from these plants when subjected to PCR using *Badnavirus* specific primers, gave an expected product of about 700 bp thus confirming the presence of virus in the plants (Fig. 1).

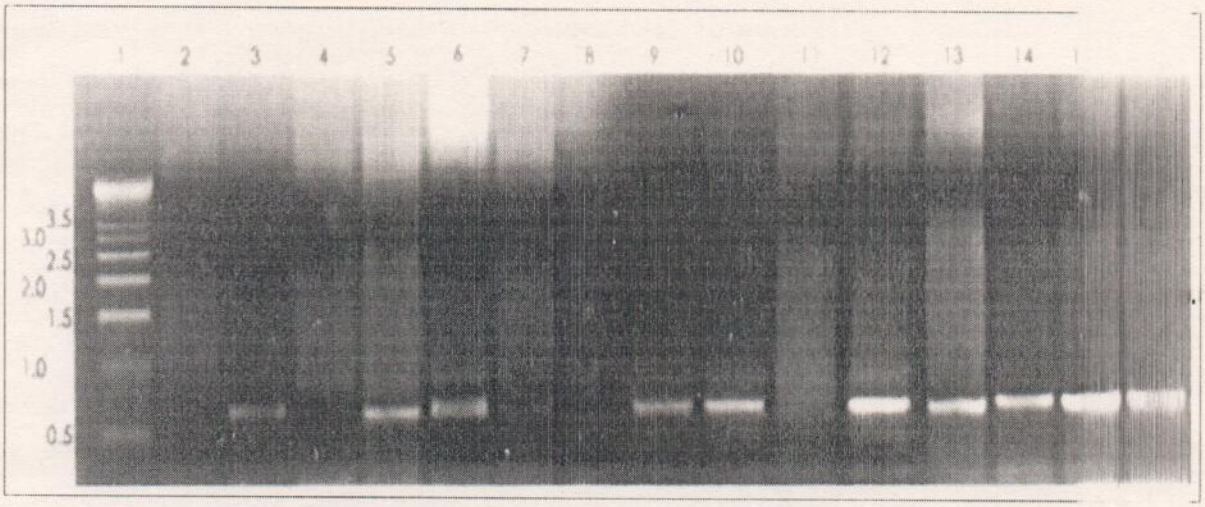


**Figure 1: Detection of virus in black pepper seedlings used for mealybug transmission through PCR. DNA extracted from 15 different seedlings (lanes 2-16) were subjected to PCR test for detecting the presence of *Badnavirus*. Presence of a band at 0.7 kb in the sample indicate the presence of virus. Lane 1: marker DNA ladder; Lane 2: Healthy black pepper (negative) control; Lane 3-15: test plants of black pepper; Lane 16: A known *Badnavirus* infected black pepper (positive) control.**

**Transmission of CMV by aphids**

Transmission studies of *Cucumber mosaic virus* (CMV) from infected black pepper to healthy black pepper using four different aphid species (*Aphis craccivora, A.spiraecola, Pentalonia nigronervosa* and *Toxoptera auruntii*) was not successful.

**Characterization of *Cucumber mosaic virus***

The virus was transmitted through stem cuttings of black pepper and by grafting. It was also sap-transmitted with difficulty from black pepper to black pepper and to a few experimental hosts such as *Chenodpodium amaranticolor*, *C. quinoa*, *Vigna unquiculata*, *V. radiata* and *V. mungo* reacted with local chlorotic /necrotic lesions. Cucumber and five tested solanaceous hosts reacted with systemic mosaic symptoms. The leaf extract of diseased black pepper or virus purified from diseased black pepper leaves in direct antigen coating ELISA and electroblot immunoassay reacted positively with polyclonal antisera of CMV from chilli, banana, brinjal and tomato. The negatively stained purified virus preparation contained non-enveloped isometric virions. The molecular weight of double stranded RNAs isolated from infected black pepper leaves was 2.42, 2.20 and 1.62 X $10^6$ Da. The coat protein of disrupted purified virions in SDS-PAGE was 26.1kDa and of nucleic acid in 1% agarose gel resolved into four species with molecular weight 1.21, 1.10, 0.81 and 0.37 X $10^6$ Da. Particle morphology, antigenic relationships with CMV, coat protein molecular weight and genome characteristics suggested the association of a strain of CMV with stunted disease.

**Characterization of the badnavirus**

The association of a badnavirus with disease affected black pepper leaf samples collected from Kozhikode and Wyanad districts of Kerala was established for the first time on the basis of symptomatology, vector transmission, electron microscopy and serology. The virus induced vein clearing, chlorotic flecks, chlorotic mottling along veins and characteristic curling of leaves leading to reduced vigor and yield. The graft transmitted black pepper plants showed typical symptoms of the disease in 2-3 months. The disease could also be transmitted with difficulty by mechanical inoculation onto black pepper and only 1 of 10 healthy seedlings showed

typical symptoms of the disease. No local or systemic symptoms appeared in any of the other hosts tested. The virus was transmitted from diseased to healthy black pepper plants by grafting and mealybug (*Ferrisia virgata and Planococo*). In DAC-ELISA, none of the samples reacted with CMV, GBNV, PVY and TSV anstisera, suggesting the lack of association of a cucumo-, tospo-, poty- or ilar- viruses with the disease. Further, among the antisera to different badnaviruses tested, none of the samples reacted with RTBV and CoYMV antisera while all the samples reacted with BSV and ScBV antisera suggesting the association of a badnavirus serologically related to BSV or ScBV (Table 2). PYMV antiserum could not be used in the tests

**Table 2:** Detection of badnavirus in field infected black pepper plants in direct antigen coated (DAC) ELISA*

| Place of collection | $A_{405}$ against antisera to | |
|---|---|---|
| | **BSV** | **ScBV** |
| **Kozhikode** | | |
| Sample 1 | 0.17 | 0.10 |
| Sample 2 | 0.19 | 0.11 |
| Sample 3 | 0.33 | 0.09 |
| Sample 4 | 0.56 | 0.12 |
| Sample 5 | 0.11 | 0.11 |
| Sample 6 | 0.22 | 0.19 |
| Sample 7 | 0.19 | 0.11 |
| Sample 8 | 0.20 | 0.13 |
| **Wyanad** | | |
| Sample 1 | 0.10 | 0.13 |
| Sample 2 | 0.48 | 0.19 |
| Sample 3 | 0.44 | 0.28 |
| Sample 4 | 0.12 | 0.16 |
| Sample 5 | 0.10 | 0.09 |
| Sample 6 | 0.22 | 0.19 |
| Sample 7 | 0.26 | 0.21 |
| Sample 8 | 0.31 | 0.19 |
| Healthy black pepper | 0.04 | 0.07 |

* Average of three replications, 1 h after substrate addition.
BSV, banana streak virus; ScBV, sugarcane bacilliform virus

because of its non-availability. Of these, majority of the samples reacted more strongly with BSV antiserum suggesting close antigenic relationship between black

pepper badnavirus and BSV (Table 2). This data was also supported by the electron microscopy of leaf dip preparations of diseased leaves, which showed the presence of bacilliform shaped particles measuring about 30 X 120 nm in size, although particle concentration was very low (one particle per 4-5 squares of the grid).

**Etiology of phyllody disease**

Phyllody affected black pepper showed various kinds of malformation of spikes. The stalk of affected increased in length, the bracts and the flowers were transformed into small leaf like structures and floral buds of the affected spikes were transformed into small branches with nodes and internodes similar to fruiting laterals. Malformed fruiting laterals produced aborted flower buds/small leaf like structures. Using nested polymerase chain reaction (PCR) using primers specific for rDNA of phytoplasmas, the phytoplasma was detected in these samples. A 1.20 kb DNA fragment encoding the portion of phytoplasma 16S rDNA consistently amplified by nested PCR was cloned and sequenced. The sequenced region contained 1230 nucleotides. The sequenced region of 16S rDNA was compared with corresponding region of phytoplasma isolates belonging to different groups from different hosts and regions. Sequence analyses showed that the gene was most closely related to members of aster yellows group (16Sr I) of phytoplasma. The sequence identity with members of aster yellows group (16Sr I) was >98% while that with members of other groups (16Sr II to 16Sr XV and other undesignated groups) ranged from 88 to 96%. Phylogenetic tree constructed using these sequences also revealed that among phytoplasmas, black pepper phytoplasma was most closely related to the members of 16S rDNA group I (aster yellows) forming one cluster that is well separated from other groups. On the basis of sequence identity, it is concluded that phytoplasma infecting black pepper in India belongs to aster yellows group (Fig.2).

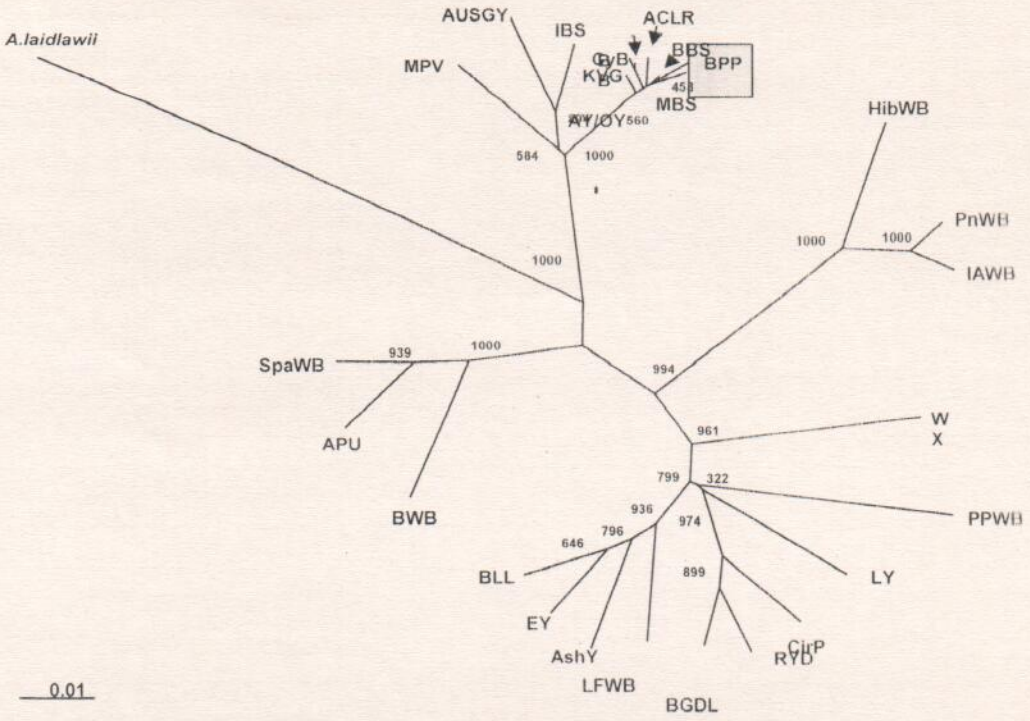


Fig.2. Radial tree drawn by Neighborhood Joining Bootstrap method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of 1.2 kb sequences of 16S rDNA from 29 distinct isolates of phytoplasma representing different groups and black pepper isolate (BPP). *A. laidlawii* was used as the outgroup. Sequences for comparisons were obtained from GenBank. The bootstrap values are shown at the individual nodes.

8222 Questions- Answered

The stunted disease is caused by two viruses namely, *Cucumber mosaic virus* and a badnaviarus serologically related to *Banana streak virus*. Both incidence and severity of the disease was high at black pepper plantations located at higher altitudes. Both the viruses are transmitted vertically through infecting cuttings. Two species of mealybugs were shown to transmit the badnavirus. The phyllody disease was shown to be caused by a phytoplasma belonging to aster yellows group.

8223 Process/ Product/ Technology/ Developed

**Detection of viruses**

ELISA based detection for viruses and a nested PCR based method for phyllody disease developed for use in indexing.

8224 Practical Utility (not more than 150 words)

For management of stunted and phyllody disease use of virus-free planting materials is important. As symptoms can not be reliably used to identify healthy plants, use of ELISA and PCR based methods are needed to identify virus-free plants. Planting materials should be taken only from such virus-free plants in order to check further spread of the viruses.

Constraints, if any: Nil

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

8231 Research papers

Bhat, A.I., Madhubala, R., Hareesh, P.S. and Anandaraj, M. 2005. Detection and characterization of the phytoplasma associated with a phyllody disease of black pepper (*Piper nigrum* L.) in India. *Scientia Horticulturae* (in Press)

Bhat, A.I., Devasahayam, S., Hareesh, P.S. , Preethi, N. and Thomas, T. 2005. *Planococcus citri* (Risso)-an additional mealybug vector of *Badnavirus* infecting black pepper (*Piper nigrum* L.) in India. *Entomon* 30: 85-90.

Bhat, A.I., Devasahayam, S., Venugopal, M.N. and Suseela Bhai, R. 2005. Distribution and incidence of viral diseases of black pepper in Karnataka and Kerala, India. *J. Plantn. Crops* **33:** 59-64.

Bhat, A.I., Sarma, Y.R., P. Sreenivasulu and Pant, R.P. 2004. Occurrence and identification of a *Cucumber mosaic virus* isolate infecting Indian long pepper (*Piper longum*). *J Medicinal & Aromatic Plant Sci.* 26: 279-284.

Bhat, A.I., Devasahayam, S., Sarma, Y.R. and Pant, R.P. 2003. Association of a badnavirus in black pepper (*Piper nigrum* L.) transmitted by mealy bug (*Ferrsia virgata*) in India. *Curr Sci,.* 84: 1547-1550.

Sarma, Y.R. Kiranmai, G., Sreenivasulu, P., Anandraj, M., Hema, M., Venkatramana, M., Murthy, A.K. and Reddy, D.V.R. 2001. Partial characterization and identification of a virus associated with stunt disease of black pepper (*Piper nigrum*) in South India. *Curr. Sci.* 80: 459-462.

8232 Popular articles

Bhat, A.I. 2005. ELISA- a powerful tool for the detection and diagnosis of viruses. *Spice India* 18 (1):36-38.

Bhat , A.I., Devasahayam, S. and Anandaraj, M., 2003. Viral disease- a new threat to black pepper cultivation in India. *Indian J. Arecanut, spices and Medicinal plants*. 5(2): 46-48.

Bhat, A.I. 2003. Diagnostics for virus detection-its importance in spices. *Spice India*. 16 (9): 30-33.

Sarma, Y.R., Bhat, A.I., Devasahayam, S and Anandaraj, M. 2002. Viral diseases can be a future threat to Black pepper. ISS Newsletter, 4: 5-6.

8233 Reports
Nil

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

Parthasarathy,U., Bhat, A.I., Devasahayam, S., Jayarajan, K., Shareef, B.H.A. and Parthasarathy, V.A. 2004. GIS studies on the influence of environmental factors on stunted diseased of black pepper. Paper presented at the National Seminar on "Commercialization of spices, medicinal and aromatic crops" held at IISR, Calicut November 01-02, 2004.

Sarma, Y.R., M. Anandaraj, M. and Devasahayam, S. 1992. Diseases of unknown etiology of black pepper (*Piper nigrum* L.). In: *Proceedings International Workshop on Black Pepper Diseases*. (Eds.) P.Wahid, D.Sitepu, S.Deciyanto and U.Superman, Institute for Spice and Medicinal Crops, Boger, Indonesia, pp 133-143.

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

An animal house was constructed for maintaining rabbits to be used for antiserum production.

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

Molecular characterization of both the viruses involved in stunted disease and development of sensitive and reliable diagnostics for use in certification of planting material need to be taken up. Similarly identification of the vectors involved in the transmission of phyllody disease need to be worked out. Identification of resistant sources against the viruses in the *Piper* germplasm and their use in breeding to get virus resistant lines should be carried out . As an alternative to this, coat protein mediated transgenic approach need to be worked out to get virus resistant black pepper cultivars.

## Part-IV : Project Expenditure
(Summary)

1992 – 2005

**830 Total Recurring Expenditure**

8301 Salaries: (Designation with pay scale)

| | Estimated | Actual |
|---|---|---|
| i) Scientific | - | 19,10,000 |
| ii) Technical | - | 3,20,000 |
| iii) Supporting | - | 1,30,000 |
| Sub-Total | - | 23,60,000 |
| Consumables | | |
| i) Chemicals | - | 2,35,000 |
| ii) Glasswares | - | 75,000 |
| iii) Others | - | 70,000 |
| Sub-Total | - | 2,80,000 |
| 8303 Travel | - | 70,000 |
| 8304 Miscellaneous (other costs) | - | 60,000 |
| 8305 Sub-Total (Recurring) | - | 4,10,000 |

**831 Total Non – Recurring Expenditure**
(Equipments and works)

| | | |
|---|---|---|
| i) Construction of animal house | - | 1,10,000 |

**823 Total** (830 and 831) - 31,60,000

## Part-V : Declaration

This is to certify that the final report of the Project has been submitted in full consultation with the Project workers as per the approved objectives and technical programme and the relevant records, note-books, materials are available for the same.

Signature of the Project Investigator: A. Ishwara Bhat

Co-Investigators :

1. Dr. S.Devasahayam

2. Dr. M.N.Venugopal

Signature & Comments of the Head of the Division/ Section

The work has been done as per the decisions of SRC

Signature & Comments of the Director