MOLECULAR CHARACTERIZATION AND PREPARATION OF MOLECULAR MAPS IN BLACK PEPPER

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Final Report

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Submitted to

National Agricultural Technology Project

By

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Molecular characterization and preparation of molecular maps in black pepper

1. **File No. / Sanction letter # : F. No.28(1)99 - NATP / CGP-I/32; dated31.5.2000**

2. **Title of the Sub Project :** **Molecular characterization and preparation of molecular maps in black pepper**

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4. **Mode of research :** **CGP**
5. **Period of report** : Final (2000-2003)
6. **Date of start of the project** (as per sanction letter from NATP) : April 1, 2000
   but actually started in Aug 2000
7. **Date of termination** (as per sanction letter) : March 31, 2003

8. Objectives of the project :

a. Molecular profiling of black pepper germplasm collections using RAPD and AFLP markers and estimation of genetic diversity and to use these molecular profiles for registering important varieties and cultivars
b. To develop two mapping populations *viz* karimunda (subhakara) x Panniyur 1 and karimunda (subhakara) x P-24 (tolerant to *Phytophthora*), develop RAPD and AFLP profiles and to delop a preliminary molecular map using polymorphisms expressed by RAPD and AFLP markers using JOINMAP/ MAPMAKER softwares.

9. Executive summary of the work done :

Black pepper is the most important spice for India with a production of 19,250 MT and an export of Rs. 32, 632.75 lakhs during 2000-2001. India is the center of diversity for cultivated black pepper. Many wild forms of black pepper and related species still occur in the Western Ghat region of South India, which is considered the centre of origin of cultivated black pepper.

The objective of the project is - to develop DNA profiles in black pepper species and important cultivars to study interrelationships and genetic diversity, and for using these molecular profiles for registering important varieties and cultivars and

- to prepare a preliminary molecular map in black pepper with a long term objective of tagging resistant genes to Phytophthora capscii along with important agronomic characters.

For developing DNA profiles using RAPD and AFLP markers in black pepper germplasm twenty four different species(both male and female lines) that co-exist with Piper nigrum and 50 important cultivars and varieties representing all the major cultivars of germplasm and released varieties were selected. DNA was isolated from one hundreds genotypes of black pepper

Optimization of PCR programming, PCR-components and annealing temperature for good and consistent amplification in black pepper DNA was completed. One hundred Operon random primers were screened and sixty-two primers were

polymorphic. The identified polymorphic primers, 25 primers, were used to develop RAPD profiles in Piper species, important cultivars and varieties of black pepper. Data was scored and studies on species interrelationships and cluster analysis were done using NT Sys software. RAPD Profiles for many important cultivars and species were developed as additional information for registering these varieties and important cultivars.

These lines are also being evaluated morphologically, which is a long-term programme. Protocol for development of AFLP profiles was standerdised but AFLP data could not be completed to add to RAPD data. This work is being continued as institute project.

RAPD profiles of 11 Phytophthora tolerent lines and with 11 suceptible lines were compared,along with their bulks, in an effort to identify RAPD markers linked to Phytophthora resistence. One marker at 700 bp region was found to be present in tolerant lines and absent in suscepts. Two of the lines did not follow the pattern.

For developing preliminary molecular maps, two mapping populations were developed. The first cross involves Panniyur-1 as female parent and subhakara as male parent as these varieties differ from each other in many morphological characters like wine growth, nature, canopy width, leaf size, leaf L/B, size of laterals, leaf laminar shape, leaf base, shoot tip color, spike length, protogynous nature and berry size in which they clearly differs.

A second cross was also made involving a resistance genotype P-24 as female and a suscept (karimunda) as a male as one of the main objectives is to tag resistance to Phytophthora capsici along with important agronomic characters. This population may help in tagging Phytophthora resistant gene. Since black pepper is a perennial and most of the cultivars are known to give segregating populations when selfed. Indicating residual heterozygosity, selfed progenies of one hundred individuals of each parents, were also developed, to locate the 'traits' which are heterozygous (segregate in progenies) in black pepper,.

Among the one thousand hybrids developed, two hundred and eighteen hybrids were transferred to the pots and morphological characters like seedling length, root length, number of roots, shoot tip color, cotyledon, second and third leaf shape, leaf

shape, base, tip, growth and branching pattern were recorded. One hundred randomly selected progenies will be planted in the field for recording the segregation data on various yield, quality and resistence to pests and pathogens. (Since pepper flowers only after third year, this takes a few years and hence will be continued as an Institute project).

Simultqneously DNA was isolated from these randomly selected one hundred progenies. RAPD profiles were developed for all these progenies and their parents, using 30 polymorphic primers , and the segregating data was recorded on polymorphic loci. This data will be used to develop preliminary molecular maps.

Preparation of molecular maps is a long term project. Hence this work is being continued as institute project.

10. **Objective wise achievements of the project :**

Research Achievements

1. ***Molecular profiling of black pepper germplasm collections using RAPD and AFLP markers and estimation of genetic diversity***

Collection of germplasm

Black pepper germplasm was collected from our Spices germplasm repository and also from different areas. One hundred genotypes of black pepper including twenty-eight wild and related species, ten released varieties, sixty-nine important cultivars and three promising hybrids were involved in the study. The details of germplasm used in the study are given in Table-1. Some of the collections of cultivars like narayakkodi, neelamundi, thevanmundi, P-24, HP-780; kalluvally, uthirancotta, Karimunda; pournami; and kottanadan, are resistant to *Phytophthora*, pollu beetle, root knot nematode and drought.

Isolation of DNA

DNA isolation protocol was standerdused and good quality DNA was isolated from pepper germplasm following modified protocol of Ausubel et al*., (1995).* The isolated DNA samples were dissolved in TE buffer and its quality and quantity was tested

on 0.8% agarose gels along with λ DNA. Comparison with standard λ DNA marker indicated high molecular weight DNA (Fig.2). The DNA quantities in different species, released varieties and cultivars of *Piper* are given in the Table-1. The yield of DNA ranged from 25 – 250 ng/µl.

The standardized protocol is given below as a flow chart.

4 g of leaf material + ground to fine powder using liquid N_2

↓

Scoop powder to preheated CTAB buffer containing β-mercaptoethanol & RNase

↓

Incubate at 65 ^{0} C for 45-60 min

↓ Mix gently & thoroughly

Add equal V of Chloroform : isoamyl alcohol (24:1)

↓ Mix gently & thoroughly

Centrifuge at 10,000 rpm for 10 min

↓

Upper layer + add 0.8 V of isopropanol

↓ Mix gently. If DNA is not seen keep at – 20 ^{0} C for 30 min.

Scoop DNA strands and dry it

↓

Dissolve in sterile distilled water/ TE buffer

↓

Centrifuge at 5000 rpm for 2 min

↓

Upper layer – Test for DNA

Table-1 Details of DNA isolations from black pepper germplasm

S. No.	Name of the Species	Acc. No.	Important Character	DNA ng / µl
1.	*Piper longum* ♀	-	›mmon in low land forests. Spikes cylindric, erect about 2-4 cm long, creamy white to yellowish white when young, peduncle about 1-2 cm long, downy, bracts peltate, orbicular, glaborous, pedicillate; flowers laterally fused; carpel single, ovary obovate, style absent, stigma 3-4 lobed, short, papillate. Fruits very small, fused laterally, spicy and pungent. Dried spikes and roots extensively used in indigenous medical systems	125
2.	*Piper longum* ♂	3027	Similar to *P. longum* female but spikes much longer, about 6-10 cm long, yellow on maturity, stamens 3-4.	150
3.	Piper longum ♂	-	-do-	125
4.	*Piper hapnium* ♀	3218	Endangered species. Similar to *P. longum*, but climbs on trees. Spikes cylindric, erect, 3 cm long, shining white, robust and fused laterally; peduncle puberculous; Young spike light yellow, mature spike green, 3-4 cm long, often curved; bracts peltate, pedicelled, orbicular, glabrous above. Carpel single, ovary obovate, stigma astylous, 3-4 lobed short, papillate. Berries partly projedcting out from the rachis.Fruit small 1-2 mm in diameter, obovate, pungent	200
5.	*Piper hapnium* ♀	Original	-do-	50
6.	*Piper hapnium* ♂	3119	Similar to *P. hapnium* female but spikes slender, 1-1.5 inches long, coffee colored and slightly sickle shaped. Stamens two and dithecous.	100
7.	*Piper peepuloides*	-	", male spikes slender, leaf opposed, 0.75 – 2" on peduncles 0.1-0.15 "	50

			with close peltate shortly stoutly pedicelled bracts.025" diameter, stamens 2-4 to each bract. Female spike very short 0.2-0.7 on peduncles, 0.1-0.2 " cylindric in fruit with very close berries.	
8.	*Piper chaba* ♀	-	A glaborous fleshy climber with adhesive roots; originally form Indonesia. Small cordate juvenile leaves, large lanceolate leaves on laterals. Spikes are erect, cylindro-conic, widest at the base, spike pungent used in medicine.	125
9.	*Piper mullesua* ♀	5405	High elevation species. Juvenile leaves very small, cordate, spikes erect, globose or oblong about 1 cm long, bracts orbicular, peltate and pedicelled, carpel single, ovary ellipsoid, style absent, stigma 3 lobed, minute, papillate, fruits very small, almost ellipsoidal, seeds minute spicy and mildly pungent.	250
10.	*Piper mullesua* ♂	5406	Similar to *P. mullesua* female but spikes filiform, 3-5 cm long, stamens 2, filaments short, anther lobes single, reniform, dorsifixed; pollen sacs 2, dehiscing by longitudinal slits.	125
11.	*Piper silentvalleyensis*	5407	Rare and endangered species. It is bisexual species similar to *P. mullesua* in external appearance but differs from it in having elongated erect fruiting spikes and bisexual flowers. Spikes 2.5-5.5 cm long, erect.	150
12.	**Piper attenuatum** ♀	3195	Spike thin, very long, filiform, pendent, 7-15 cm, peduncle about 2 cm, glaborous. Bracts sessile, adnate to the rachis, obovate to elliptic, margins free, glaborous; ovary single, oblong, style absent, stigma 3-4 lobed, recurved, papillate, ovule single. Mature fruits round to oblong, deciduous, tastes bitter.	37.5
13.	**Piper attenuatum** ♀	699	-do-	50.0
14.	**Piper attenuatum** ♀	-	-do-	37.5
15.	**Piper attenuatum** ♂	4696	Similar to *Piper attenuatum* female but spikes 8-18 cm, stamenms 3-4 dithecous, dehiscing longitudinally.	62.5
16.	**Piper attenuatum** ♂	396	-do-	*50.0
17.	*Piper argyrophyllum* ♀	4403	Very common in all the forests except at higher elevations and in sea-level forests. Spike thin, filiform, pendulous , spikes 5-10 cm, glaborous or puberulous. Bracts sessile, adnate and almost confluent with the rachis, obovate to elliptic; carpel single, style absent, ovary oblong, stigma 4 lobed, short , recurved and papillate. Berry ovate, becomes spherical in full maturity. Differs from *P. attenuatum* in having 5 nerved nature of leaf base and shorter, greenish white fruiting spike and silvery scales on the underside of the leaves.	25.0
18.	*Piper argyrophyllum* ♀	-	-do-	150
19.	*Piper argyrophyllum* ♀	623	-do-	62.5
20.	*Piper argyrophyllum* ♂	379	Similar to *P. argyrophyllum* female but spikes 8-16 cm, stamens 3, anthers dithecous.	25.0
21.	*Piper hymenophyllum* ♀	505	Prominent pubescent branchlets and leaves, hairs more pronounced on the young shoots. Spikes thin, filiform, 6-16 cm long, peduncle pubescent, bracts sessile, adnate to the rachis, obovate to elliptic; style absent, ovary oval, stigma 3-4 lobed, recurved and papillate. Mature fruits spherical.	120
22.	*Piper hymenophyllum* ♀	5345	-do-	75
23.	*Piper hymenophyllum* ♂	4327	Similar to *P. hymenophyllum* female but spikes 5-13 cm long, stamens 3, anthers dithecous.	25/62.5
24.	*Piper hymenophyllum*♂	245	-do-	25
25.	*Piper hymenophyllum*	-	-do-	100
26.	*Piper galeatum* ♀	3339	Found in medium elevations of 500-800 m. Spike filiform, pendulous, young ones green to pale purple, mature yellowish white, glaborous, peduncle short, glaborous. Bracts prominent, connate forming a fleshy cup or boat shaped structure shortly stipitate and recurved; glabrous, style absent, carpel single, ovary obovate, stigma 3-4 lobed. Fruits green on ripening turn bright yellow and then to orange red, oblong, bold, taste bitter first and slightly pungent later.	50
27.	*Piper galeatum* ♀	4577	-do-	75
28.	*Piper galeatum* ♀	-	-do-	100/75

29.	*Piper galeatum* ♂	325	Similar to *P. galeatum* female but spikes long, about 10-15 cm, even longer, sometimes reaching upto 25 cm. Stamens two, anther lobes two.	20
30.	*Piper trichostachyon* ♀	3340	Common at elevations up to above 1000 m. Spikes filiform, minutely hairy, 4-9 cm, peduncle glabrous, bracts decurrent, connate forming a fleshy cup or boat shaped structure, hirtellous; carpel one, style absent, stigma 3-4 lobed, lobes short papillate. Fruits bold, spherical or oblong, tastes bitter first, pungent later, color changes from green to yellow and then to orange red on ripening; closely related to *P. galeatum*.	50
31.	*Piper trichostachyon* ♂	661	Similar to *P. trichostachyon* female but spikes 4-10 cm, stamens two, short, dithecous.	50
32.	*Piper sugandhi* ♀	4660	Spikes slender, fleshy , filiform and pendant or recurved, slightly thicker than male spikes, 5-10 cm long. Flowers held at right angles to the rachis, stipitate, bracteate, bracts deeply copular with free margins. Ovary ovoid, monocarpellary, embedded inside the copular bract exceptfor the tip; style absent stigma 3 lobed, fleshy, white when young. Fruits oblong, bold, pungent as in black pepper, turns yellow and then to red on ripening. Allied to *P. nigrum, P. galeatum* and *P. trichostachyon* but differs from it in having stipitate flowers, deeply copular bract and pungent fruits.	150
33.	*Piper sugandhi I* ♀	-	-do-	50
34.	*Piper sugandhi II* ♀	-	-do-	*25
35.	*Piper sugandhi* ♂	4533	Similar to *P. sugandhi* female but spikes 10-14 cm long, stamens two, filaments short and thick, embedded in the copular bract, anthers projecting out at maturity; dithecous, dehiscing by apical longitudinal slits.	37.5
36.	*Piper psuedonigrum* ♀	3284	Similar to *P. sugandhi* but having pedicellate flowers.	75
37.	*Piper nigrum* ♀	269	Found extensively in evergreen forests of Western Ghats. Spike filiform, pendulous, young ones green or whitish green, or light purple; mature ones green, pale purple or pale yellow, spike length varies much. Peduncle glabrous, bracts oblong, decurrent, sessile with free upper margin, develop into a shallow cup in female spikes, rachis and bracts glabrous; carpel single, ovary spherical, style absent, stigma 3-5 lobed, papillate. Fruit a drupe, green when young changes to red on ripening, seeds spherical, pungent.	50.0
38.	*Piper nigrum* ♀ *?*	-	-do-	20.0
39.	*Piper nigrum* ♂	4677	Similar to *P. nigrum* female. Stamens two, anthers dithecous.	*50.0
40.	*Piper nigrum* ♂	4579	-do-	25.0
41.	**Piper nigrum** ♂	-	-do-	25.0
42.	*Piper nigrum (bisexual)*	-	-	62.5
43.	*Piper schmidtii* ♀	5403	Found at elevations above 1500 m. Leaves very thick and coriaceous, venation very prominent. Spikes filiform, pendent, female spikes thick 6-16 cm, bracts peltate with raised margins; ovary oblong or conical, ovule single, style absent, stigma 3-4 lobed, papillous; fruits oblong , bitter in taste, turning to yellow and finally to orange on ripening	250
44.	*Piper schmidtii* ♂	5404	Similar to *P. schmidtii* female but spikes thinner about 10-15 cm in length, stamens two, filament thick, anthers opening by longitudinal slits.	250
45.	*Piper wightii* ♀	5410	Occurs at elevations of above 1500 m only, leaf ribs prominent on the under side, sparse silvery scales. Spikes filiform, pendulous, medium long, 4-8 cm, peduncle about 1-1.15 cm long, flowers arranged spirally, bracts oblong, narrowed towards the base, overlapping with the successive bracts, adnate to the rachis, margins free; ovary conical, stigma 4 lobed, persistent, style absent. Fruits conical, almost spherical when mature with persistent stigma.	250
46.	*Piper wightii* ♂	5409	Similar to *P. wightii* female. Anthers 2-3, stalked, filament thick, dehisce by longitudinal slits	250
47.	*Piper barberi* ♀	613	Endangered and very distinct South Indian *Piper* not related to any other South Indian species. Reticulately veined leaves and spikes borne on slender, long dangling peduncle, juvenile shoots slender with	150

			persistent scale leaves, orthotropic shoots with small leaves, female spikes 4-7 cm, cylindrical, pendulous, borne on long slender peduncle, bracts peltate, orbicular; ovary sessile, one celled, one ovuled, stigma 3 lobed, papillate, style absent, fruits fleshy drupe red on ripening.	
48.	*Piper barberi* ♀	614	-do-	25
49.	*Piper barberi*	614 A	-do-	25
50.	*Piper bababudani ?*	3285	Long spike with thick central core collected from bababudani hills, Karnataka.	25 &125
51.	*Piper betel*	-	A perennial climber. Leaves used as masticatory, highly variable, 5-20 cm long, ovate to broadly ovate or cordate with unequal bases, tip acuminate or acute, entire, glabrous and shining, yellowish green to bright green.	50
52.	*Piper betel* ♀	-	Flowering rare as plants are replanted in every 4-5 years under cultivation. Spikes cylindrical and pendulous. Fruits observed very rarely.	125
53.	*Piper cubeba (?)*	-	Native to Indonesia. Leaves glabrous, ovate-oblong with cordate or rounded base, fruit subglobose and stalked. Very distinct in having chromosome number of 2n = 24.	125
54.	*Piper colubrinum*	-	part of South America. It is resistant to *Phytophthora capsici* and *Radopholus similes.* Fused berries, elliptical, spike length 60 mm, conical and glabrous spikes, peltate bract.	62.5
55.	*Piper colubrinum ?*	-	-	125
56.	*Piper magnificum*	-	Origin in Peru, South America. Small, erect shrub having winged stem, leaves ovate to broadly elliptic or sub orbicular, apex broadly acute, base cordate to auriculate, deep green above, glossy, bright maroon beneath. Petiole broadly winged.	37.5
57.	*Piper arboreum*	-	labrous spike, peltate orbicular bracts, fused and elliptical berries.	*75
58.	*Piper ornatum*	-	Native of Indonesia. A creeping/ climbing shrub, leaves broadly cordate to suborbicular, peltate, apex attenuate, base cordate, mottled green, pink and silver above, light maroon beneath.	62.5
59.	*Piper Spp. ?*	3090	-	150
60.	*Piper Spp.*	3092	-	25
61.	*Panniyur-1*	-	A hybrid between Uthirankotta and Cheriyakaniakadan and grows in all pepper gowing tracts of Kerala. It is a vigorous vine with typical yellowish green shoot tips and cordate leaves. The spikes are long with high piperine content.	25
62.	*Panniyur-1(Parent)*	-	-do-	100
63.	*Panniyur –1 (MP Block)*	-	-do-	250
64.	*Panniyur-1 (S)*	-	-do-	100
65.	*Panniytur-2*	-	Selection (Cul.141) from open pollinated progeny of cv. Balancotta and grows in all pepper growing tracts of Kerala. It is a shade tolerant. A high yielding variety with high piperine.	75
66.	*Panniyur- 2 (P)*	-	-do-	25
67.	*Panniyur-3*	-	Hybrid (Cul.331) between Uthirankotta and Cheriyakaniakadan and grows in all pepper growing tracts of Kerala. A variety with very long spikes bold berries and high piperine content. Late maturing. Prefers open situations.	50/100
68.	*Panniyur-4*	-	Selection from Kuthiravally type-2 and grows in all pepper growing tracts of Kerala. Performs well even under adverse climatic conditions including partial shade, stable yielder.	25
69.	*Panniyur-5*	-	Selection from open pollinated progeny of *Perumkodi*. Tolerant to nursery diseases and shade.	250
70.	*Subhakara*	-	Selection from Karimunda (KS 27) and grows in all pepper growing tracts of Kerala and Southern Karnataka. A selection with high quality and wider adaptability.	100
71.	*Sreekara*	-	Selection from Karimunda (KS 14) and grows in all pepper growing tracts of Kerala and Southern Karnataka. Adaptable to various climatic conditions in all the pepper growing tracts. Gives high quality pepper.	75
72.	*Panchami*	-	Selection from Aimpiriyan (Coll. 856) and grows in all pepper	100

			growing tracts of Kerala and Karnataka . Performs well in plains and hilly regions, not suitable for heavy shaded areas, late maturing. Large, dark green, cordate to ovate leaves, newly emerging shoot tips purple, spikes medium long, fruits arranged in five distinct rows, 90 % are bisexual flowers and remaining pistillate. A high yielding variety with excellent fruit set. Spike twisted in appearance due to high fruit set. Oleoresin content is high.	
73.	*Pournami*	812	Selection from the germplasm (Coll. 812) and grows in all pepper growing tracts of Kerala and Karnataka. Tolerant to root knot nematode. A moderately high yielding vine with high oleoresin content.	100
74.	*Palode -2*	5055	Clonal selection from cultivar Kottanadan (2559). A variety with high quality and suitable to all pepper growing areas. Oleoresin content is high.	100
75.	*Arakkulam munda*	-	Light purple shoot tips, ovate leaves with acuminate tips, spikes 8-12 cm long, bisexual types, early maturing variety, medium yield and quality	87.5
76.	*Balankotta*	-	Tolerant to shade, performs well as mixed crop in arecanut gardens, large elliptic, lanceolate, slightly drooping leaves, bisexual mostly with hermaphrodite flowers, fruits bold, round and light green , medium yield, bold fruit, medium quality.	125
77.	*Cheppukulamundi*	818	Ovate, cordate leaves, medium long spikes, setting moderate, medium yield and quality.	75
78.	*Cholamundi ?*	1369	Small lanceolate leaves, spikes medium, setting often poor, fruits small, medium quality, predominantly female.	12.5
79.	*Jeerakamundi*	4191	Small, lanceolate leaves, spikes small, setting poor, spiking intensity high.	37.5
80.	*Kalluvally*	1513	A hardy cultivar, minutely hairy in nature, leaves medium sized, elliptical with round base and even margins, young shoot tips purple, predominantly bisexual and occasionally pistillate, spikes od different lengths in different types of kalluvally. Medium yield and quality.	50
81.	*Kalluvally*	880	-do-	125
82.	*Karimunda-1 (Parent)*	-	Originally from south Kerala, now very popular through out Kerala; tolerant to shade, performs well as mixed crop, widely adaptable, good yielder, medium quality. A prolific and regular bearer, leaves small and oval, spikes medium to small compactly set, bisexual type with male and female flowers coming to maturity simultaneously, berries small and round.	100
83.	*Karimunda-2 (Parent)*	-	-do-	12.5
84.	*Kottanadan*	1487	Performs well in plains and hilly regions up to 700-800 m MSL; widely adapted and high yielding, high quality. Leaves dark green, ovate with even margins spikes medium long, curved, bisexual type with mostly hermaphrodite and rarely pistillate flowers, a regular bearer.	125
85.	*Kottanadan*	2438	-do-	50
86.	*Kottanadan*	2471	-do-	50
87.	*Kottanadan*	2501	-do-	25
88.	*Kottanadan*	2572	-do-	25
89.	*Kuthiravally*	849	Leaves ovate, large, even margins and round base, a bisexual type with occasional pistillate flowers, fruits small. A stable yielder, long spikes, good setting, high quality.	100
90.	*Malamundi*	1159	Leaves ovate with round base, spikes medium long, peduncle small; flowers bisexual and female almost in equal proportion. Fruits medium, good setting.	100
91.	*Mundi*	993	Leaves ovate, spikes short to medium, fruit set moderate; fruits medium, quality medium.	12.5
92.	*Narayakkodi*	-	Common in all pepper growing tracts; said to be field tolerant to *Phytophthora* foot rot, persistent stigma on the fruits, a bisexual type with presence of pistillate flowers in every spike, a regular bearer, fruits ovate and small, medium yield and quality.	125
93.	*Neelamundi*	809	Reported to be field tolerant to foot rot. Suitable for high elevation	25

			areas, leaves ovate, medium to large, a bisexual type, spikes medium sized, fruits round and medium sized, moderate yielder, medium quality.	
94.	*Perambramunda*	141	Resembles *Neelamundi*, berries bold, medium long spikes, medium yield and quality.	125
95.	*Perumkodi*	803	Leaves ovate to ovate-elliptic, spikes medium, setting poor, fruits bold, quality medium, alternate bearer.	25
96.	*Poonjaran rmunda*	845	Leaves broadly ovate, base cordate, long spikes, moderate yielder, alternate bearer.	100
97.	*Thevanmundi*	1041	Leaves moderately large, ovate, spike medium, setting good, good spiking, berries medium oblong, good yield, quality medium. Tolerant to *Phytophthora*.	25
98.	*Thommankodi*	966	A vigorous cultivar, leaves ovate to widely ovate in the main stem, medium large in lateral; spike long (13-14 cm), setting good, fruits medium, globose, closely resembles *Kuthiravally*; good yielder and quality.	12.5
99.	*Valiakaniakkadan*	-	Spikes medium to long, berries bold, medium yielder, alternate bearer.	125
100.	*Vattamundi*	4184	Vigorous vine, leaves medium, widely ovate, spikes medium, setting moderate, berries bold, round, medium yield and quality	100
101.	*Arikotta*	1244	-	100
102.	*Cheriyakaniakkadan*	853	Small lanceolate leaves, tips acuminate, spikes short, fruits small; early maturity, poor yielder, quality medium.	75
103.	*Cherukodi*	982	-	75
104.	*Kanjirakodan*	1165	-	50
105.	*Kallubalancotta*	1252	High yielder, bold berries, long spike, tendency for alternate bearing.	75
106.	*Karimkotta*	1592	A common hardy cultivar of Malabar, poor yielder, alternate bearer. Leaves large, dark green, ovate, bisexual type, spikes long, fruit set moderate and berries bold.	125
107.	*Karivilanchi*	1330	Medium long ovate leaves; predominantly female, fruit bold, oblong, medium quality, poor yielder.	37.5
108.	*Kumbhachola*	1114	Very long spike, good bearing.	37.5
109.	*Kumbhanadan*	1326	Late maturing, regular yielder.	62.5
110.	*Konnomkara*	977	Good bearing.	100
111.	*Maramodiyan*	1157	Low yielding plant with very high vegetative growth.	125
112.	*Maramonakki*	1106	Heavy bearing, thick leaves, good drying (50 %).	125
113.	*Murithothan*	1315	-	125
114.	*Nedumchola*	1058	Leaves are smaller among the cultivars, ovate to obovate, base round, spikes very short, 4-6 cm, berries very small, slightly obovate; poor yielder and characteristically small statured vein.	50
115.	*Neyyattinkaara mundi*	816	Good bearing, heavy yielding.	150
116.	*Orumaniyan*	973	Low yielding plant with very small spike and rare fruit set.	100
117.	*Padappan Type IV*	1124	High yielder, alternate bearing.	50
118.	*Perumkarimunda*	910	Good bearing, highly susceptible to *Phytophthora.*	50
119.	*Pirimunda*	1255	Similar to *Orumaniyan* but with smaller spike.	31.25
120.	*Vadakkan*	4132	A natural triploid; vigorous vine; leaves ovate to ovate elliptic, long petioled, spikes medium, setting poor, fruit very bold, medium quality, spikes light purplish.	50
121.	*Vally*	885	-	125
122.	*Vellamunda*	1037	Spike with female and bisexual flowers.	125
123.	*Velutha kaniakkadan*	841	Similar to *Kaniakkadan* but not	75
124.	*Vokkalu*	1213	Short spike, bold berries used for white pepper preparation. Smallest spike among all black pepper cultivars. Each spike (3-4cm) bears only 1-6 berries.	50
125.	*Uthirankotta*	929	Predominently female, poor yield.	31.25
126.	*Chenganoorkodi*	982	-	125
127.	*Chumalakodi*	990	Very old local cultivar.	100
128.	*Chumalanampan*	-	High yielding. More similar to *Chumalakodi*.	100
129.	*Kaniakkadan*	887	Landrace.	100
130.	*Karuthapirimunda*	1253	Small spike, good filling.	75
131.	*TMB VI*	1262	Introduced from Panniyur Research Station.	31.25

132.	*Thulakkodi*	963	-	125
133.	*Valiakarimunda*	1353	Very good setting, dark green berries.	100
134.	*Bilimalligesara*	1230	Most popular cultivars in Karnataka. Moderate yielder, medium quality.	75
135.	*Doddigae*	867	A cultivar grown in Karnataka state, leaves ovate, poor yielder.	125
136.	*Karimalligesara*	4060	Most popular cultivars in Karnataka. Moderate yielder, medium quality.	100
137.	*Kudiragunda*	1223	Medium large leaves, small spikes and medium berries.	50
138.	*Uddaghere*	1268	A popular and high yielding cultivar from Uttara Kannada and Shimoga districts of Karnataka; good yield, moderate quality.	125
139.	*Kuching*	891	Native of Malaysia. Most popular, a bisexual type with occasional pistillate flowers, spikes small, berries medium sized and slightly oblong, high yielder.	25
140.	*Kurumalai*	1205	Performs well as inter crop in coconut and arecanut gardens. Leaves ovate-lanceolate with round base and acuminate tip, spike medium length, fruit round. Not suitable for plains, good yielder, medium quality.	87.5
141.	*Belantung*	1548	Native of Indonesia. Medium yielder.	50
142.	*Exotic collection*	1639	Native of Indonesia.	100
143.	*LDK*	1577	Native of Indonesia. Medium yielder.	50
144.	*Palulauta*	1260	Native of Indonesia. Poor yielder.	125
145.	*OPKm*	-	Superior lines suitable for the planes with vigorous growth, broad leaves and very long spikes. High yielder.	25
146.	*P-24 (Parent)*	-	A medium yielding, *Phytophthora* resistant.	25
147.	*P-24 (Dr AR)*	-	-do-	100
148.	*HP*	127	Hybrid between panniyur-1 x karimunda.	50
149.	*HP*	780	Hybrid between perambramunda x karimunda. *Phytophthora* tolerant line.	125
150.	*HP*	1411	Superior lines suitable for the planes. Hybrid characterized by long compact spike and bold berries. High yielder.	125

Purification of DNA

To remove RNA contamination from the extracted samples RNAse treatment was given followed by phenol : chloroform : isoamyl alcohol (25:24:1) extraction and precipitation of DNA with isopropanol. Finally it was resuspended in TE buffer and tested for quality and quantity of DNA. It was found that after purification, the RNA contamination was reduced substantially.

Optimization of RAPD

Optimization of PCR components

The following parameters were kept constant

1. Assay buffer - 1 X
2. Primer - 10 picomoles

Three different variables such as dNTP's, *Taq* DNA polymerase and Mg Cl_2 in different combinations were tested. The different combinations are given in the Table-3. The template DNA used was Panniyur-1 and the primer was OPF-14. Among the twenty four different combinations tested, the eleventh combination i.e., 150 µM of dNTP's, 1.5 U of Taq and 0.5 mM of $MgCl_2$ gave good amplification with clear bands without any nonspecific banding (Fig.2b). Hence, only this combination was used in further PCRs i.e., for primer screening and RAPD analysis. Hence, 45 ng of DNA, 1x assay buffer, 150 mM of dNTP's, 0.5 µM of additional $MgCl_2$ and 1.5 U of Taq polymerase are optimal for generating good and consistent amplification of black pepper genomic DNA.

Optimization of PCR Programming

The optimized reaction conditions for PCR that gave good amplification is as follows.

Cycles: 3	Ist cycle	94^{o}C, 2 minutes	
		37 ^{o}C, 1 minute	
		72 ^{o}C, 2 minutes	Cycle repeats: 1
	IInd cycle:	94 ^{o}C for 1 minute	
		37 ^{o}C for 1 minute	
		72 ^{o}C for 2 minutes	Cycle repeats: 33
	IIIrd cycle :	94 ^{o}C for 1 minute	
		37 ^{o}C for 1 minute	
		72 ^{o}C for 15 minutes	Cycle repeats: 1

Preparation of Reaction Mixture

The reaction mixture was prepared as follows.

Assay buffer	- 2.5 0 µl (10 X)
Taq DNA polymerase	- 1.5 0 µl (1U/µl)
d NTP's	- 3.75 µl (150µM)
$MgCl_2$	- 2.50 µl (0.5 mM)
DNA	- 3.00 µl (45 ng / µl)
Primer	-2.00µl(10 picomoles)
Sterile distilled water	- 9.75 µl
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TOTAL	- 25.00 µl
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Screening of Primers

After optimization of PCR conditions and variables, one hundred decamer Operon primers i.e., OPA- 01 to 20; OPB- 01 to 20; OPC- 01 to 20; OPD- 01,02,03,05,07,08,10,12,13,15,16,19 and 20; OPE- 01, 02,03,04, 05, 07, 10, 11, 13, 14, 15 and 19; OPF – 01, 02, 03, 04, 05, 09, 10, 12, 13, 14, 15, 17, 18, 19 and 20 were screened using Panniyur-1 and Karimunda DNA as templates using optimized PCR components i.e., 45 ng of DNA, 1x assay buffer, 150 mM of dNTP's, 0.5 µM $MgCl_2$ and 1.5 U of Taq polymerase (Fig. 3, 4) The optimized PCR programming as mentioned above was taken.

Out of one hundred Operon primers tested, around 62 primers i.e., OPA – 01, 02, 03, 04, 05, 07, 09, 11, 12, 14, 15, 16, 19, 20; OPB – 02, 04, 05, 07, 10, 14, 15, 19; OPC – 01, 02, 05, 07, 08, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20; OPD – 02, 03, 05, 07, 08, 13, 15, 16, 20; OPE – 01, 02, 04, 07, 11, 15, 19; OPF – 01, 02, 03, 05, 09 , 13, 14, 15, 20 showed polymorphism (Fig. 3, 4).

Developing RAPD profiles

RAPD profiles were developed by using polymorphic primers to estimate the polymorphism between species, released varieties and some important cultivars.

Studies on RAPD polymorphism in **Piper** *species*

Developing RAPD profiles in Piper *species* – RAPD profiles were developed using twenty five polymorphic operon primers viz OPA-09, OPA-=15, OPB-07, OPC-07, OPC-13, OPD-02, OPD-03, OPE-11, OPF-09 and 24 on *Piper* species. Species of *Piper* namely *P. longum, P. hapnium, P. mullesua, P. attenuatum, P. argyrophyllum, P. hymenophyllum, P. babbudani, P. trichostachyon, P. galeatum, P. sugandhi, P. psuedonigrum, P. nigrum, P. schmidtii, P. wightii, P. barberi, P. betel,* karimunda, *P. chaba-1, P. chaba-2, P. magnificum, P.colubrinum-1, P.colubrinum-2, P. arboreum* and *P. ornatum* were selected for the study (Fig. 5 -16). This RAPD data was analyzed by NTSYS software to study species interrelationships and the dendro grammes were drawn (Fig. 17).

Species interrelationships between the male and female lines of *Piper*

The dendrogram grouped *P. longum, P. hapnium* and *P. mullesua* in one group and *P. attenuatum* and *P.argyrophyllum* in another group. *P. pseudonigrum*, *P. nigrum* and *P. galeatum* are clustered together. Two collections of *P. chaba* are placed in the same group. These are in accordance with earlier findings of Ravindran and Nirmal Babu. (1992; 1996). However, grouping of *P. magnificum* and *P. arboreum* with *P. hapnium* and *P. mullesua*, separate and unique clustering of *P. hymenophyllum*, unique clustering of karimunda are against the earlier observations and understanding. The anomalies observed may be due to failure of PCR reactions in certain genotypes, resulting in wrong clustering. More primers are need to be tested before we have abetter understanding of the interrelashionships of *Piper* species.

However the present study indicated that:

P. pseudonigrum, P. nigrum and P. sugandhi are distinct entities with clear cut differences in RAPD profiles amplified by certain primers. This indicates that theearlier understanding that *P. pseudonigrum* and *P. sugandhi* are same may not be true. The present study indicated *P. pseudonigrum* is more nearer to *P. nigrum* than *P. sugandhi*

P. attenuatum, *P. argyrophyllum* and *P. hymenophyllum* are in general similar to each other., morphologically also they differ from each other mainly by the amount and presence and absence of pubescence in the plant body. The two collections of *P. colubrinum* and *P.chaba* are nearest to each other indicating that they may be two collections of the same species.

The commonality of atleast two monomorphic bands in Indian collections of *Piper* may be indicating their common origin, though this was not represented in the dendrogram.

The present study also clearly indicates that distinct profile differences occur between male and female lines of same species and hence fingerprinting requires specific identification of the collection of the species and its sex. In general, similarities were noticed in male and female lines collected from same location but those collected from different locations significantly differ in their RAPD profiles.

Studies on RAPD polymorphism in black pepper cultivars

Developing RAPD profiles for major cultivars and released varieties of black pepper – RAPD profiles of fourteen major cultivars and ten released varieties viz., karimunda, kottandan, balancotta, neelamundi, kuthiravally, kalluvally, arakkulm munda, narayakodi, thomankodi, perambramunda, poonkjarn munda, valiakarimunda, cheriyakaniakadan, uthirancotta, panniyur-1, panniyur-2, panniyur-3, panniyur-4, panniyur-5, sreekara, subhakara, panchami, pournami and palode-2 were developed using twenty five polymorphic primers i.e., OPA-08,09,15; OPB-03,05,07,08,14,20; OPC-07,09,13; OPD-03,15; OPE-02,11, 13; OPF-09,10, 14, and 15 (Fig.18- 27). Similarity indexes were caluculated and dendrogrammes were drawn.

The study indicated that there are distinct differences between most of the cultivars ab nd varieties. Sreekara and subhakara are nearest to each other while Panniyur 1 is farthest from all the others as it formed a separate group. PLD 2 a selection from Kottanadan was very close to kottanadan. Though Panniyur 3 is seen closer to cheriakaniakkadan Panniyur 3 is seen very distent from it.

The RAPD profiles developed are being analysed to identify fingerprints.

Developing RAPD profiles for Phytophthora tolerant and susceptible varieties-

In an effort to identify RAPD markers linked to Phytophthora resistence, eleven lines each of *Phytophthora* susceptible and tolerant cultivars were used for the study. Phytophthora tolerent lines and suceptible lines were compared along with their bulks also. RAPD profiles were developed using ten polymorphic primers i.e., OPA-03, 09,13,14,18, OPC-02, 07,18; OPD-03; OPE-11, 14 and OPF-09 and their interrelationships were studied (Fig. 28- 31). The study indicated that in general the tolerant lines formed a cluster of their own and a few suscepts were grouped with tolerant lines. One marker at 700 bp region was found to be present in tolerant lines and absent in suscepts. Two of the lines did not follow the pattern. The most probable reason for this anomaly may be that so called tolerant or susceptible lines may be heterozygous for their traits thus having the other template also, which could be amplified in a PCR reaction. The study is being continued.

Standerdisation of AFLP

AFLP protocolwas standerdised for the first time in black pepper using 4 Eco R1 and Mse 1 primers based on the method developed by Vos et al 1995 (Fig 32). The AFLP data is to be developed and added to RAPD data.

2. ***To develop two mapping populations viz karimunda (subhakara) x Panniyur 1 and karimunda (subhakara) x P-24 (tolerant to Phytophthora) and to prepare preliminary molecular maps using polymorphisms expressed by RAPD and as well as AFLP markers***

Development of mapping population

For developing preliminary molecular maps, two mapping populations were developed. The first cross involves Panniyur-1 as female parent and subhakara as male parent as these varieties differ from each other in many morphological characters like wine growth, nature, canopy width, leaf size, leaf L/B, size of laterals, leaf laminar shape, leaf base, shoot tip color, spike length, protogynous nature and berry size in which they clearly differs .

A second cross was also made involving a resistance genotype P-24 as female and a suscept (karimunda) as a male as one of the main objectives is to tag resistance to Phytophthora capsici along with important agronomic characters. This population may help in tagging Phytophthora resistant gene (Fig 33).

Development of selfed progenies

Since black pepper is a perennial and most of the cultivars are known to give segregating populations when selfed. Indicating residual heterozygosity, selfed progenies of one hundred individuals of each parents, were also developed, to locate the 'traits' which are heterozygous (segregate in progenies) in black pepper.

Morphlogical charecterisation

Among the one thousand hybrids developed, two hundred and eighteen hybrids were transferred to the pots and morphological characters like seedling length, root length, number of roots, shoot tip color, cotyledon, second and third leaf shape, leaf shape, base, tip,growth and branching pattern were recorded (gene (Fig 34-37).One hundred randomly

selected progenies will be planted in the field for recording the segregation data on various yield, quality and resistence to pests and pathogens. (Since pepper flowers only after third year, this takes a few years and hence will be continued as an Institute project)

DNA isolation

Simultaneously DNA was isolated from these randomly selected one hundred progenies.

RAPD profiles

RAPD profiles were developed for all these progenies and their parents, using 30 polymorphic primers (Fig 38- 48) and the segregating data was recorded on polymorphic loci. This data will be used to develop preliminary molecular maps.

Preparation of molecular maps is a long term project. Hence this work is being continued as institute project.

Summery of level of completion of each objective

Molecular profiling of black pepper germplasm collections using RAPD and AFLP markers and estimation of genetic diversity.

S. No.	**Activity Name**	**Achievement made**	**Remarks**
1.	Collection of germplasm material for	Completed	100 lines including 24 species and 50 cultivars and varieties
2.	Standardization and isolation of DNA	Completed	Protocol standerdised
3.	Standardization of RAPD protocol	Completed	Protocol standerdised
4.	Isolation of DNA from pepper germplasm	Completed	Completed 100 lines
5.	Screening of random primers	Completed	100 primers were screened and 63 primers were polymorphic
6	Development of RAPD profiles for 24 related species of *Piper*	Completed	24 polymorphic primers were screened and species inter relation ships studied
7	Development of RAPD profiles for 40 cultivars and released varieties of pepper	Completed	24 polymorphic primers were screened and rapd profiles developed.
8	Screening of RAPD profiles between tolerant and susceptible cultivars	Partially Completed	A putative marker was identified to co segregate with toletent lines. More work need to bbe done.
9.	Standardization of AFLP protocol	Completed	With 4 primers
10	Development of AFLP profiles	Initiated	More time needed

To develop two mapping populations viz karimunda (subhakara) x Panniyur 1 and karimunda (subhakara) x P-24 (tolerant to Phytophthora) and to prepare preliminary molecular maps using polymorphisms expressed by RAPD and as well as AFLP markers

S. No.	Activity	Achievements made	Remarks
1.	Developing mapping population of Panniyur x Subhakara and P-24 x Subhakara	Completed	Over 1000 progenies were developed
2	Establishment of selfed progenies of Panniyur,Subhakara and P-24 in nursery	Completed	200 each wereestablished
2.	Isolation of DNA from mapping population	Completed	DNA was isolated from 100 progenies
3.	Screening of random primers	Completed	100 primers were screened of which 63 were polymorphic
4	Developing RAPD profiles for mapping population and scoring of data	Completed	RAPD profiles were developed with 30 polymorphic primers
5	Recording of juvenile characters in mapping population	Completed	Germination characters, Leaf, shoot and root characters were recorded for 200 progenies
6	Screening of AFLP primers	In progress	More time required
7.	Developing preliminary map	In progress	More time required
	Long term		
8.	Field evaluation to record segregation data on agronomically important characters for linking with molecular markers	Started	More time required

11. Outcome / Impact of the achievements and the project as a whole on the advancement of sciences / technology development / farmers:

a. The DNA profiles developed in this project for major species, varieties and cultivars of black pepper will be used in molecular profiling and registration of crop genotypes, especially in the post GATT scenario.
b. The standardized molecular profiling will be used to identifying duplicates in the germplasm and to short list core collections for conservation and maintenance.
c. The study of intra and inter relationships between species, varieties and cultivars will help molecular taxonomy and in selecting parental combinations for better hybrid vigor.

d. Analysis of pepper genome using molecular markers will help to tag the important agronomic characters and will assist in marker assisted breeding.

e. The linked markers can be used to locate and isolate useful genes from DNA libraries and for map based cloning.

f. The protocols for RAPD and AFLP were not standardized at the time of this project initiation and these easy to use protocols developed in the present study could used by many workers in molecular biology. There are no published reports of development of AFLP protocol for black pepper so far.

12. Foreign / national trainings obtained by the PI and CCPIs: Nil

13. **Trainings arranged, if any:** Nil

14. List the manuals / Instructional material / CDs / extension material developed:

a. Training Manual on Molecular Biology techniques for Horticultural Crops (27.12.02 to 04.0.103) was prepared by the institute in which the following topics were contributed

- *DNA isolation and developing RAPD profiles*
- *Mapping of QTLs in black pepper*

b. Summer Training Manual on Techniques in Biochemistry and Biotechnology (5.5.2003 to 4.6.2003), including the following topics

- *Development of Molecular Maps in plants*
- *DNA markers in plant improvement - AFLPs / SSRs*

Three students submitted M.Sc dissertations and one will be submitting Ph.D based on this work.

15. **Infrastructural development** (List only equipments and works costing more than Rs. 1.00 lakh each) :

a. Table top Centrifuge	3,27,374
b. Lyophiliser	3,86,305
c. Electrophoresis unit	4,30,751
d. Micropipettes	1,61,435
e. JOINMAP software	2,52,125

16. **Improvement in audio visual system (if any)** : Nil

17. **List the papers published based on work under NATP in referred journals**: Nill

But the following Manuscripts are under preparation

Studies on interrelationships of black pepper species and cultivars using RAPD polymorphism. - K. Nirmal Babu et al

Molecular charadterisation a few Black pepper cultivars and release varieties using RAPD polymorphism. - K. Nirmal Babu et al

18. **Honours & awards received based on work under NATP** : Nil

19. Technologies developed.

a. DNA isolation protocol for black pepper was developed
b. Standardized RAPD protocol for molecular characterization of black pepper cultivars and species
c. Standardized AFLP protocol for molecular characterization and preparation of molecular maps in black pepper
d. RAPD profiles were developed for 50 cultivars and released varieties of black pepper and 24 related species of piper.

20. **Patents applied for / obtained** : nil

21. Your suggestion regarding what has to be done in future as a continuation of your project or as a new project

Molecular characterization and molecular mapping is a long-term project. A good beginning was made and lot of data was generated.

RAPD profiles were developed and species and cultivar interrelationships were studied. This information will add up to the existing understanding on these aspects based on morphometrical, numerical, chemotaxonomical interrelationships.

The profiles developed for each of the collection and species will add up to the morphological and yield characterization while registering important genotypes and varieties.

Usually mapping projects take a long time especially in perennial crops. So far no work was done in spices. A very good beginning was made in generation of RAPD data in this project for preparation of molecular maps. More work need to be done to identify markers linked to important characters. This will help in marker linked selection and breeding. The data is still very preliminary. This has to be continued further with AFLP data and with second mapping population.

This programme is being continued as institute project at IISR, CALICUT. A second mapping population was already developed. And work is in progress

Signature Principal Investigator (K Nirmal Babu)

Date : 5-8 2003

Dr V A Parthasarathy
Director

Date : 5-8-2003

Name of PI : K. Nirmal Babu **Sanction no. 28 (1) /99 – NATP/CGP-I/32;dated 31.5.2000**

Financial (Consolidated) Statement of Expenditure

Head	Total sanctioned allocation (Rs. In lakhs) (A)	Funds received (Rs. In lakhs)			Total funds received (Rs. In lakhs) (B)	Difference between allocation and funds received (A-B)	Funds utilized (Rs. In lakhs)			Total fund utilized (Rs. In lakhs) (C)	Balance fund (Rs. In lakhs) as on 01.04.2003 (A-C)	Unspent balance (Rs. In lakhs) as on 01.04.2003 (B-C)
X. Recurring		2000-01	2001-02	2002-03			2000-01	2001-02	2002-03			
TA	1,50,000	50,000		31,420	81,420	68,580		31,420	-3,309	28,111	1,21,889	53,309
Contractual services	7,34,760	2,41,800		1,78,354	4,20,154	3,14,606		1,68,994	2,01,473	3,70,467	3,64,293	49687
Operational expenses	12,00,000	4,00,000		1,91,784	5,91,784	6,08,216		1,91,784	2,87,598	4,79,382	7,20,618	112402
Seminar												
Workshop	3,00,000				-	3,00,000				-	-	-
Total of X	23,84,760	6,91,800		4,01,558	10,93,358	12,91,402		3,92,198	4,85,762	8,77,960	15,06,800	215398
Y.Non recurring												
Equipment	20,95,000	20,95,000		-	20,95,000	0	85,144	89,497	15,57,990	17,32,631	3,62,369	362369
Civil works	-											
Total of Y	20,95,000	20,95,000			20,95,000	0	85,144	89,497	15,57,990	17,32,631	3,62,369	362369
Z. Institutional charges	2,38,476	69,180		27,043	96,223	1,42,253	-	26,107	70,116	96,223	1,42,253	0
Grand total X+Y+Z	**47,18,236**	28,55,980		4,28,601	**32,84,581**	**14,33,655**	85,144	5,07,802	21,13,868	**27,06,814**	20,11,422	**5,77,767**

Signature : Principal Investigator **Asst. Finance and Accounts Officer** **Director**

Date :