PROFORMA FOR COLLECTION OF DATA OF RESEARCH PROJECTS IN SERICULTURE

PART- I: GENERAL INFORMATION

1	Name of the Institute / University / Organization submitting the Project proposal	:	Central Sericultural Research & Training Institute, Berhampore, West Bengal
2	Status of the Institute (s)	:	N.A.
3	Name (s) and designation (s) of the Executive Authority of the institute / University forwarding the application	:	Dr. A.K.Saha, Director (I/C)
4	Project Title	:	Evaluation of bacterial leaf spot resistant improved progenies of mulberry for field utilization
5	Category of the Project	:	Applied
6	Specific Area	:	P – Plant; I – Improvement; T - Biotechnology
7	Duration	:	3 years
8	Total Cost	:	7.70Lakh
9	Is the Project single or multi-Institutional	:	Single Institutional
10	If the Project is multi-institutional, please furnish the Name, Designation and Address of the Project Coordinator.	:	N.A.

11. (a) Project summery:

Foliar diseases limit quality leaf production in mulberry during monsoon in Eastern India to a considerable extent. Bacterial leaf spot (BLS) caused by *Xanthomonas campestris* pv. *mori* predominant in monsoon (June-September) months. The disease is responsible for ~15%foliage loss during their peak season of incidence; while quality of foliage and the cocoon crop losses are even more (Kore et al.1985). Though host resistance is considered as the most economic and sustainable control strategy still BLS resistant commercial cultivar of mulberry is lacking. In our previous initiative, we identified the sources of BLS resistance and determined the inheritance pattern of the resistant trait(s) through development of useful disease specific pseudoF_2 (=F_1) segregating population (~440) (resistant source: *M.multicaulis*,*M.rotundiloba*; susceptible sources: KPG-1, S-1 and C-2028) . Subsequently, we have established clonal sets (5 clones for each line) of altogether 175 mapping populations (*M.multicaulis* x KPG-1:65nos; *M.multicaulis* x S-1:55nos and *M.multicaulis* x C-2028:55nos) along with respective parents and a suitable spreader in ARBD. The disease reaction of the segregating populations indicated continuous frequency distribution suggesting quantitative nature of BLS resistance and possible involvement of at least three genes with additive

effect. Moreover, evaluation of the population for agronomic traits indicated that segregation was transgressive for ~ 12 F_1 (pseudo-F_2) lines towards more leaf biomass over the high yielding parent S-1. More importantly, DNA analysis with ~20 mulberry specific microsatellite markers (developed by CCMB and some others) were also tested on the segregating progenies to link with BLS resistance trait(s). Altogether 6 of tested SSR primers indicated polymorphism among 4 parental lines and two of them generated DNA amplification products with unique band of 200 and 250 bps under 3% agarose gel for five resistance and seven susceptible progenies, respectively. Similarly, another SSR primer also indicated identical band for susceptible lines. All these SSRs showed good association with transgressively segregating high yielding progenies. So these SSRs seem to be good candidates to search for marker-BLS responsive trait-association and have immense value in MAS based utilization. In this project we have a plan to build upon these leads into its logical end by:

1. Thorough evaluation of foliage biomass production potential and associated agronomic traits of identified promising resistance lines (F_1 / pseudo-F_2) to BLS under replicated on-farm trials and assessment of selected lines further through silkworm bioassay prior to recommend for direct field utilization.
2. Establishment of advanced breeding lines (~200 nos; F_2 / pseudo-F_3) obtained through sib-mating of 1-2 promising lines and assessment of the seggregants for important agronomic traits and BLS resistant trait for trait refinement.
3. Validation of identified DNA tags through three breeding generations to establish marker - trait association for MAS based utilization in mulberry.

(b) Aims and Objectives

Development of host resistance to BLS with better foliage yield potential than ruling cultivar(s) is the need for quality cocoon production in sericulture. Moreover, validation of molecular marker-trait association through three breeding generations and MAS based application for disease resistant line selection is still elusive in mulberry. Therefore, obtained leads of our previous studies will be searched further for a) the development of suitable BLS resistance line(s) with higher foliage yield and better silkworm rearing efficiency for direct commercial exploitation, and b) validation of identified SSRs (2-4) for BLS resistance using three breeding generations [parental, pseudo F_2 (=F_1) and pseudoF_3 (=sib-mating; between two pseudo F_2 lines)] with a aim to utilize in MAS based selection breeding in mulberry.

PART-II: PARTICULARS OF INVESTIGATORS

12	a) Name		**DR RITA BANERJEE**
	Date of Birth		05-04-1961
	Sex		F
	Indicate whether Principal Investigator/ Co-investigator		**PI**
	Designation		Scientist-D
	Department		Biotechnology
	Institute/University: Address		CSR&TI, Berhampore, West Bengal
	b) Name		**DR SOUMEN CHATTOPADHYAY**
	Date of Birth		15-07-1961
	Sex		M
	Indicate whether Principal Investigator/ Co-investigator		**CI-1**
	Designation		Scientist - D
	Department		Biotechnology
	Institute/University: Address		CSR&TI, Berhampore, West Bengal
	c) Name		**DR A K SAHA**
	Date of Birth		27-08-1957
	Sex		M
	Indicate whether Principal Investigator/ Co-investigator		**CI-2**
	Designation		Scientist-D
	Department		Sericulture Division
	Institute/University: Address		CSR&TI, Berhampore, West Bengal
	d) Name		**DR AK SAHA**
	Date of Birth		
	Sex		M
	Indicate whether Principal Investigator/ Co-investigator		**Executive Authority**
	Designation		Director(I/C)
	Institute/University: Address		CSR&TI, Berhampore, West Bengal
13	No. of Projects being handled by each investigator at present		
a	Dr R Banerjee		PROJECTS: 1 (AS CO-I); PROGRAMME: 1 (AS CO-I)
b	Dr. S Chattopadhyay		PROJECTS: 1 (AS PI-1), PROGRAMME: 1(AS PI)
c	Dr A K Saha		PROJECTS: 1 (AS PI-II)

14. Proposed Research Fellows [justification with work sharing is a must]:

NIL

PART-III: TECHNICAL DETAILS OF THE PROJECT

15. **Introduction**

15.1 **Definition of the Problem**:

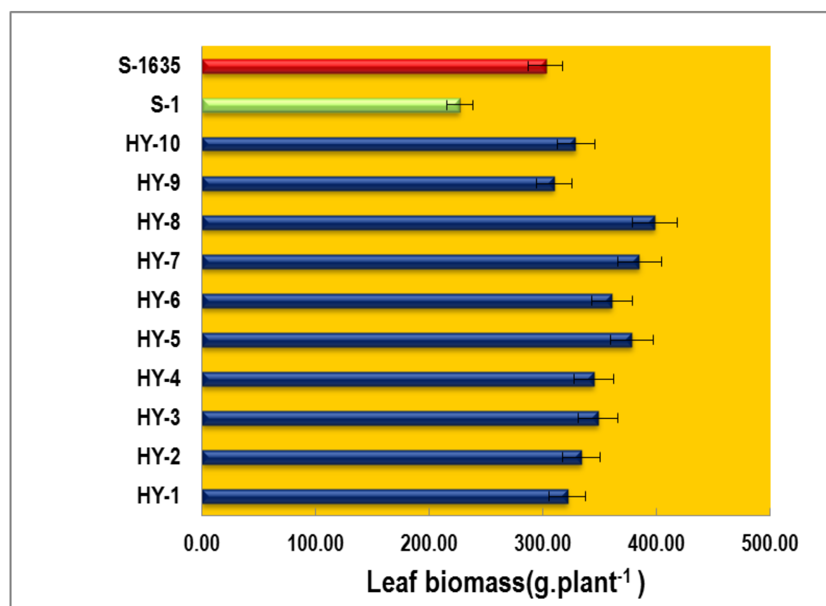
(a) Origin of the project:

Mulberry (*Morus* spp.) is the exclusive food plant of domesticated silkworm (*Bombyx mori* L.) and is mainly practiced in five states namely, Karnataka, Andhra Pradesh, West Bengal, Tamil Nadu and Jammu & Kashmir which jointly account for about 96% of the total mulberry silk production in the country. India is the second largest producer of silk in the world and contributes ~ 15.49 % share in global raw silk production. However, the production of bivoltine silk is still very limited in our country (~ 3870mt), and paucity of quality mulberry foliage availability is considered as one of the major bottlenecks for international grade silk production (www.csb.gov.in). In the present climate changing scenario, pathogen–induced foliage loss (both qualitative and quantitative) is considered as a suitable area of improvement. Indeed, the foliage loss due to major pathogens and pests in mulberry goes upto 25%; productivity and quality loss of silkworm cocoon is even more (Shree and Nataraja 1993; Gupta, 2001). Moreover, all of the presently available commercial cultivars are prone to various diseases. BLS is an important disease in Indian context.

BLS caused by *Xanthomonas campestris* cv. *mori* is prevailing in all mulberry growing areas of the country. The disease is responsible for considerable foliage loss spanning over June to September months covering two commercial and a seed cocoon crop seasons (Kore et al 1985). In order to develop host resistance in mulberry against BLS, we have assessed ~ 82 germplasm under field and inoculum based condition (2005-2010) and identified *M.multicaulis* and *M.rotundiloba* as useful sources of resistance against BLS. By the control crossing among identified resources, we further developed ~440 segregating BLS specific pseudo F_2 (=F_1) population (2013-2014) and determined that BLS resistance in mulberry is polygenic and controlled by additive genes. Subsequently, clonal set of ~175 segregating population obtained from crosses of *M.multicaulis* as resistant source, one susceptible line (KPG-1) and two otherwise improved cultivars, but susceptible to BLS (S-1, C-2028) were evaluated phenotypically for BLS responsiveness as well as for foliage yield and associated traits. Quantitative BLS assessment indicated involvement at least 3 genes for BLS resistance in mulberry. Most importantly, among the tested population ~12 lines indicated transgressive segregation for foliage biomass. Besides, these promising lines also showed

resistant/moderately resistant BLS response under natural field condition with expected incremental foliage of about ~6.4 -31.8% (based on projected value) over the ruling cultivar S-1635 (Figure 1).

Figure1. Leaf biomass of identified promising lines of the segregating population



Data are mean of five seasonal evaluation of leaf biomass; n=20
**Considered leaf biomass potential of S-1635 =43000 kg* ha^{-1} year^{-1} [source: Doss et al (2011) & Ghosh et al (2009)]

Moreover, parental DNA was analyzed with ~ 20 mulberry specific SSR primers (generously contributed by Dr R K Aggarwal, CCMB). Six of the tested SSRs were polymorphic to parental lines and two of them produced unique amplicon at 200/250bp in susceptible lines (parent, susceptible bulk and progeny). Besides one SSR (MUL_NGS_281) showed partial association with resistant lines.

Despite the aforementioned valuable information, the presently proposed works are the immediate need to focus followings:

- ✓ *Evaluation of the promising improved progenies through 'final yield trial (FYT)' for direct field utilization*
- ✓ *Thorough characterization of the developed lines for important agronomic traits, foliar diseases and rooting traits*
- ✓ *Trait refinement through sib-mating and assessment of inheritance pattern of BLS resistance in mulberry*
- ✓ *Validation of identified DNA tags for BLS resistance using three breeding generations*

- ✓ *Sequencing, characterization of DNA tags along with linkage analysis to identify major QTLs linked to BLS resistance traits will be searched (if feasible).*

b) Expected outcome:

a. Identification of improved mulberry line(s) with BLS resistance for commercial utilization.

b. Elucidation of DNA-marker based analysis for BLS resistance for effective utilization in MAS breeding in mulberry.

15.2 **Rationale of the Study:**

BLS caused by the soilborne bacterium *Xanthomonas campestris* pv*.mori* is endemic to much of tropical and subtropical countries. The disease leads to considerable foliage loss of mulberry (*Morus* spp.) by premature leaf fall, decreased leaf quality and impairing the feeding value of the leaves to silkworm (*Bombyx mori*) (Sharma et al. 2005). In our previous works, the sources of BLS resistance have been identified under field condition (Banerjee et al. 2009), and we found that BLS resistance in mulberry is polygenic and quantitatively inherited (Banerjee et al. 2012). This quantitative nature coupled with high heterozygous plant behavior and sex-incompatibility is the major impediments for targeted BLS improvement in mulberry. Moreover, classical breeding for resistance is time consuming and most importantly, not always been appears durable in many plants (Review: Amil-Ruiz et al. 2011). There is an increasing demand to generate alternative approaches for control of BLS in mulberry. In this backdrop, knowledge of stable markers for BLS resistance could help breeders in the selection and consequently sustainable development of mulberry with enhanced resistance to BLS.

In our recently concluded project we identified promising lines with higher foliage yield than the ruling cultivar. Besides, a few of the tested SSR markers were found polymorphic to parental clones and generated unique amplicons in resistant/susceptible progeny lines. Moreover all these markers showed good association with transgressive segregating high yielding progenies. So these markers are revealed to be a good candidate for use as molecular tag for BLS resistance and would be good source to test the potential for forward selection with the generated advance breeding lines, which is considered as an important step in MAS based application.

15.3 **Relevance to the current issues and expected outcome:**

The present project would address some current problems for targeted improvement in mulberry using DNA marker based breeding approach. The goals of our study are: thorough evaluation of identified high yielding lines(~10 nos) for leaf biomass and associated traits including silkworm bioassay for direct field utilization; validation of SSRs polymorphic to parents to ascertain the link between trait: marker; establishment of ~200 advanced breeding lines derived through crossing between identified promising lines for specific trait refinement/marker validation. Successful implementation of the above steps would be the first ever endeavor in mulberry for targeted breeding in BLS resistance and would provide a platform of possible MAS based molecular approach in genetic improvement of mulberry. The expected deliverables from this study are:

- ✓ Identification of suitable progeny with BLS resistance and better leaf biomass potential over the ruling cultivars S-1/S-1635/C-2038 for direct field use.
- ✓ Successful identification of DNA tags for BLS resistant/susceptible trait(s) would provide efficient screening tools and possibilities of MAS based utility breeding for development of improved elite lines. This in turn, would lead to considerable gains in time, need for reducing physical and financial resources.
- ✓ Generation of advanced breeding lines would effectively utilize for refinement of specific trait (s) and identification of good candidate progeny lines (i.e. foliage yield/ BLS resistance) for further exploitation in mulberry improvement programme.

15.4 Objectives:

- ***Evaluation of bacterial leaf spot resistant improved lines for foliage biomass and associated agronomic traits under RBD***
- ***Silkworm bioassay of the promising lines for prospective commercial utilization***
- ***Development of advanced breeding generation(F_2) through sib-mating of identified promising lines(F_1)***
- ***Evaluation for bacterial leaf spot resistance among the developed progeny (F_2) using identified SSRs to establish marker-trait link for MAS based utilization***

16. **Review of status of Research and Development on the subject**

16.1 **National Status**:

Mulberry is highly heterozygous perennial tree. It is the exclusive food source of monophagous silkworm. However, pathogen induced loss in mulberry leaf quality and

quantity is of major concern in mulberry improvement programme. Since mulberry foliage yield has direct bearing on silk production, therefore efforts have been initiated to develop host resistance in mulberry against major foliar diseases.

Bacterial leaf spot

BLS incited by *Xanthomonas campestris* pv. *mori* is one of the major foliar diseases of mulberry prevailing in almost all mulberry cultivating areas of the country (Maji et al. 2008; Ravikumar et al. 2004; Nagaraj et al. 2000). The symptom of this soil-borne bacterium emerges as numerous small water-soaked brown spots in the adaxial leaf surface, and then spots gradually spread to the abaxial side and form large irregular necrotic areas. Finally the leaves become crinkled, rotten and fall prematurely. The disease causes foliage loss up to ~15% and impairs the feeding value of leaves to silkworm (Kore et al. 1985; Sato and Takasahi, 1973).

Mulberry is usually cultivated in monoculture cropping system for 60-65 days and maintained by bottom pruning in concurrence with silkworm rearing seasons. Bacterial leaf spot in mulberry is predominant during June to September months and affects three important silkworm rearing seasons. Although 'Streptomycin' and Mancozeb (75% WP) are general recommendations for BLS control (Dandin and Giridhar 2010); however, these chemicals are mostly unaffordable by the small and marginal stakeholders and also injurious to soil micro-flora/fauna.

Host plant resistance to BLS would be a sustainable and economic control strategy, but yet to get proper impetus in mulberry. No cultivars presently available are resistant to *X. campestris* for commercial exploitation. Inadequacy of information regarding genetics of resistant trait(s), long juvenile period and highly heterozygous nature of mulberry are reported as the major limiting factors for development of BLS resistant mulberry.

Durability of resistant source/genetic features

Attempts were made to identify sources of resistance at national and international level through visual symptomic observation (Maji et al, 2006; Pathan,1987) and some of the potential resistant mulberry accessions were : S-146, Punjab local, *M.lambong*, *M.multicaulis*, Shini-Ichinose (Pandey and Singh,1989; Shikata et al,1985). However, information regarding durability /stability of resistance and the genetic nature of the resistant trait are still very scant.

In order to identify durable source(s) for BLS resistance in mulberry, as a first step we have evaluated ~ 82 germplasm accessions under field environment, tested through inoculum based analysis and identified useful sources of resistance and determined high

heritability estimates (broad sense) of resistance based on variance components (Banerjee et al. 2009).

Interrelationship between BLS resistance and morphological/biochemical traits/genetic component determination

The constitutive defense mechanisms are preformed physical and chemical barriers to pathogen establishment. Unlike fungal pathogens, bacteria can not directly penetrate the leaf epidermis. Alternatively, stomata is one the most important routes for the entry of foliar bacterial pathogens (Melotto et al. 2008). Furthermore, movement toward natural openings would possibly occur when the leaf surface is wet, thus outbreaks of foliar bacterial diseases often follow rain and high humidity (Nino-Liu et al. 2006). Graham et al. (1992) reported that stomata and water congestion in leaf tissue effect penetration of *Xanthomonas campestris* in citrus cultivars. In mulberry *Xanthomonas campestris* pv.*mori* enters host tissue through stomata (Gupta et al. 1995). Similarly, reports indicated trichome density on leaf surface can reduce bacterial multiplication (Nicks and Rubiales 2002). Because, for getting access the available nutrients in the host plant, the pathogens need to breach the natural barriers present in the healthy plants. Moreover, Djocgoue et al. (2007) has observed reduction in soluble sugar contents in parents/hybrids of *Theobroma cacao* under infection with *Phytopthora megakarya*. Reports also indicated a significant decrease of photosynthetic pigment in *Phaseolus vulgaris* inoculated with *Xanthomonas campestris* (Berova et al. 2007). Besides, higher levels of total soluble phenols were observed in tuber tissues of resistant varieties of potato to soft rot (Nagadze et al. 2012).

Our findings also revealed that stomata frequency and trichome density as well as concentration of total soluble sugar and polyphenol were significantly different in resistant and susceptible groups. Among morphological features, stomata frequency and trichome density were significantly higher in the susceptible and resistant lines respectively. There is an increase of the mean values of phenol (63.1 %) and TSS (78%) in resistant group over susceptible group. Moreover, high broad sense heritability coupled with genetic gain of those parameters indicated that they are under the influence of both additive and non-additive gene action which suggests hybridization followed by selection for these traits would be useful for generation of advanced breeding lines of BLS resistance in the mulberry improvement program (Banerjee et al.2014).

Development of segregating population and estimation of inheritance pattern for BLS resistance in mulberry

Inadequacy of information regarding genetics of resistant trait(s), long juvenile period and highly heterozygous nature of mulberry are the major limiting factors for development of BLS resistant mulberry. To develop full-sib pseudo-F_2 (F_1) progeny in out-bred trees, parental clones were used as male and female (non-inbred parent) based on their sexual compatibility. Like other woody angiosperms, the pedigree of mulberry normally involves only two parents and their full-sib, or half-sib progenies. Mulberry, being highly heterozygous may be considered as F_1 while exploited in controlled crosses (Ben Hui Leu 1998). Progeny derived from these crosses are F_1's but are considered pseudo-F_2 because the parents are most likely heterozygous at many loci due to cross-pollinated nature (Allard 1999) of mulberry. Tulsieram et al. (1992) stated that crossing between outbred tree parents can be assimilated to that of F_2 or BC_1 like self -pollinated crops.

We developed ~440 segregating progeny utilizing two resistant (Morus rotundiloba and M. multicaulis) and four susceptible sources (S-1, C-2016, C-2028 and KPG-1) selected from previous studies. Evaluation of the segregating population indicated high narrow sense heritability estimates (≥0.76) and non-segregating major genes suggested additive gene effects may control BLS resistance and the trait is most likely to be inherited quantitatively in mulberry. Heterosis results suggest parent with less disease severity (M.multicaulis) may subsequently be exploited for mulberry improvement program (Banerjee et al. 2012).

Foliage biomass, agronomic traits and BLS resistance

Leaf yield is a complex trait and strongly correlated with associated parameters like primary shoot length, intermodal distance, shoot number, petiole length, petiole width and above ground biomass in mulberry (Tikadar & Roy 2001). The heterotic effect for foliage biomass once fixed in early generation can be maintained for long period as clonal propagation is the main multiplication practice in mulberry (Dandin and Giridhar, 2010). Significant positive estimates of better-parent heterosis for leaf yield have been reported in mulberry (Ghosh et al.2009). Besides, high yielding progenies obtained either through open pollinated hybrids or full-sib mating are routinely undergone through evaluation for important agronomic traits/leaf yield as a selection/evaluation process in mulberry (Doss et al. 2011).

Our findings of ~ 10 promising F_1 lines indicated significant superiority for leaf yield over the better parent i.e., ruling cultivar S-1. Besides, these promising lines also showed resistant/moderately resistance BLS response under natural field condition with expected incremental foliage of about ~6.4 -31.8% over the National Check S-1635 and warrants further evaluation before recommendation for field utilization .

16.2 **International Status**

Genetics of bacterial leaf spot resistance

According to Johnson (1981) a cultivar with durable resistance remains effective for a long period in an environment favorable to the disease. Reports on quantitative resistance in common bean against *Xanthomonas campestris* pv. *phaseoli* (Duncun et al. 2011) and in cotton against and *X. axonopodis* pv. *malvacearum* (Wallace and El-Zik 1989) indicated that cumulative effect of multiple genes has a broad spectrum of resistance to many races of a pathogen. Bonos et al. (2003) reported continuous population distribution for dollar spot resistance of phenotypes for clones and progeny in perennial creeping bentgrass which suggested that the resistance may be quantitatively inherited.

For open pollinated species, the additive component is the most useful in phenotypic recurrent selection breeding program. General combining ability (GCA) and specific combining ability (SCA) provides measure of additive and non-additive variations, respectively (Ahuja and Dhayal 2007). Estimate of the GCA and SCA effect for understanding disease resistance genetics has been reported in several crop plants (Bus et al.2005; Acharya et al.2011).Moreover, narrow sense heritability provides a more accurate estimate of gain in selection (Poehlman and Sleper, 1995).The number of genes segregating for the resistance has also been estimated in many crops (Zhang et al. 2010; Hazica et al.2004).

Our findings indicated that BLS resistance may be controlled by additive gene effect with quantitative inheritance in mulberry. Combining ability of mulberry clones for BLS resistance was estimated following line x tester mating design (Banerjee et al. 2014). *Moreover, the χ^2 analysis of observed frequencies of F_1 lines expected probable involvement of 3-5 genes for the resistant trait (Banerjee et al unpublished).*

Targeted improvement in mulberry for BLS resistance, identification of molecular tags for utilization in MAS based utility breeding.

Targeted improvement in mulberry through conventional breeding program involves crossing followed by selection of the superior lines from the several segregated progenies (Review: Vijayan et al.2012). Such a procedure is laborious and time consuming, involving several years, several generations, and careful phenotypic selection like other tree species (Bianco et al 2014; WU et al. 2014). In addition, tight linkage of the desired loci with undesired loci may make it difficult to achieve the desired objective through this approach. Moreover, heterozygous plant behavior, the long juvenile period, together with inadequate genetic information regarding desirable traits, have promoted interest in marker-assisted selection (MAS) to accelerate breeding through early selection. MAS relies on identifying DNA markers, which explain a high proportion of variation in phenotypic traits (Review: Collard and Mackill

2008).Owing to genetic linkage, DNA markers can be used to detect the presence of allelic variation in the genes underlying these traits. Use of DNA markers to assist in plant breeding, efficiency and precision could be greatly increased (Tan et al 2012). Once tightly linked markers that reliably predict a trait phenotype have been identified; they may be used for MAS. Genetic linkage maps have been developed for most commercial tree species and these can be used to locate chromosomal regions where DNA markers co-segregate with quantitative traits (quantitative trait loci, QTL) (Wu etal.2014; Lee et al 2015).This markers may be useful for disease resistant traits, which are expressed at later developmental stages. Utilizing this technique undesirable plant genotypes can be quickly eliminated from seedling nursery. This may have tremendous benefits in mulberry breeding because the primary evaluation trial (primary yield trial) practices involve sowing of seeds and transplanting seedlings into nursery, making it easy to transplant only selected seedlings for final yield trial.

Over the last few decades, the use of molecular markers has played an increasing role in plant breeding and genetics. Of the different types of molecular markers, the most widely used markers are called simple sequence repeats (SSRs) or microsatellites (Gupta & Varshney 2000). Microsatellites have been utilized most extensively, because they can be readily amplified by PCR and the large amount of allelic variation at each locus. Microsatellites are also known as simple sequence repeats (SSR), and they are typically composed of 1–6 nucleotide repeats. They are highly reliable (i.e. reproducible), co-dominant inheritance, relatively simple and cheap to use and generally highly polymorphic. Microsatellites are successfully used in areas like selection and diagnostics in segregating population, genome selection during gene introgression, genome mapping, gene cloning, MAS, parentage analysis, fingerprinting, and phylogenetic and taxonomic studies. SSR markers have been reported to be associated with genes conferring resistance to various diseases of like leaf spot and rust resistance of groundnut (Sujay et al.2012), scab resistance in cucumber (Zhang et al.2010), blast resistance in rice (Askhani et al, 2011) and many others. In addition, molecular marker techniques provide efficient and powerful tools for linkage map construction and identification of putative molecular tags for utilization in advance breeding generations.

In mulberry RAPDs (Chatterjee et al 2004; Zhao and Pan 2004), ISSRs (Vijayan and Chatterjee, 2003; Awashti et al 2004; Vijayan et al. 2006) and AFLPs (Kafkas et al 2008) have been the commonly used marker systems for analysis of mulberry resources. The reports on specific PCR based codominant markers like SSRs are rare (Aggarwal et al 2005;

Zhao et al 2005; Balachandran et al 2013) and no reports on sequence based markers such as single nucleotide polymorphism (SNPs) have been available from mulberry.

In order to identify putative linked marker(s) for BLS resistance we tested about 20 mulberry specific microsatellite markers on the parental lines. Altogether 6 of the tested SSR primers indicated polymorphism among 4 parental lines (M.multicaulis : resistant parent; KPG-1, S-1 and C-2028: susceptible parents). Two SSR primers generated DNA amplification products showed unique band of 200 and 250 bps under 3% agarose gel for five resistance and seven susceptible progenies (out of 9 each of contrasting progenies derived from the cross of M. multicaulis x S-1). Similarly, one SSR indicated identical band for susceptible lines among resistant and susceptible progeny derived from cross of M.multicaulis x KPG-1 and M. multicaulis x C-2028 respectively at 3% agarose gel. Moreover all these markers showed good association with putative transgressive segregating high yielding progenies. So these markers are revealed to be a good candidate for use as molecular tag for BLS resistance and would be good source to test the potential for forward selection with the generated advance breeding lines, which is an important step in MAS based utility breeding in mulberry.

16.3 **Importance of the proposed project in the context of current status**:

BLS is one of the major limiting biotic factors for mulberry production throughout the country. The use of resistant cultivars is the most effective and economical way to control BLS, and therefore, breeding efforts to develop new resistant cultivar continue to be a priority for mulberry breeding programs. In our recently concluded study, we developed BLS specific segregating progeny derived from controlled cross of *M.multicaulis* (resistant source) and KPG-1 (susceptible line); horizontal transfer of resistant trait to otherwise improved cultivars (S-1,C-2028). DNA analysis has identified a few SSR markers for possible use as molecular tags for BLS disease reaction in mulberry plants. Our preliminary investigation based on clonal trial (=5 clone/progeny) have shown transgressive segregation in some promising lines for foliage biomass over S-1(high yielding parent) with increased BLS resistance and a few of them projected better leaf yield potential than the ruling cultivar S1635. The identified SSR markers (3) showed good association with the segregating high yielding progenies. Utilizing the project leads in the present project we proposed to:

- Thorough evaluation of promising (8-10) identified lines from the segregating population for further on-farm trial before field utilization.
- Validation of identified DNA tags through three breeding generations to establish trait /molecular marker association for MAS based utilization.

16.4 **Anticipated Products, processes/Technology, Packages/ Information or other outcome from the project and their expected utility**:

Anticipated product: Thoroughly assessed promising progeny for better foliage with durable BLS resistance than the existing ruling cultivar S-1/S-1635/C-2038 for direct field use.

Generation of useful information: Validation of DNA markers/tags for BLS resistant trait(s) would provide efficient screening tools and possibilities of MAS based accelerated breeding for developing improved elite mulberry genotypes. This in turn, would lead to considerable gains in time, need for reducing physical and financial resources.

16.5 **Expertise available with proposed investigation group/ institution on the subject of the project*:**

Name of the Scientists	Designation	Experience
Dr R Banerjee	Scientist-D	As a principal Investigator of the proposal, she has about 25 years research experience in the field of plant breeding and genetics. She has expertise in the areas of a) characterization of mulberry germplasm materials using anatomical, morphological and molecular tools as well as b) various aspects of conventional/targeted breeding in crop plants and C) disease resistance breeding of mulberry for BLS and other diseases. She has more than 40 publications in International and National peer review journals. She is instrumentally associated as PIs with two DBT supported project (collaborative with CCMB) on development of mulberry linkage map and powdery mildew SCAR marker development and one in-house project on development of bacterial leaf spot resistant mulberry.
Dr S Chattopadhyay	Scientist-D	As a Co-Investigator of the proposal, he has 21 years of research experience in various field of mulberry crop improvement. He has expertise in the areas of- a) screening of mulberry genetic resources using random molecular markers, b) analysis of various enzymes and cellular constituents of mulberry, and c) pathometrical analysis of various mulberry diseases. He has >49 publications in different International and National level peer review journals. He had concluded a DBT funded network project on-Identification of DNA markers associated with powdery mildew disease in mulberry as PI and instrumentally associated with two DBT supported projects (collaboratively with CCMB) to identify SCAR-markers associated with powdery mildew resistance and development of linkage map of mulberry.
Dr A K Saha	Scientist-D	As a co-investigator of the proposal, he has 30 years of research experience in various fields of silkworm breeding genetics, physiology and rearing technology. He has expertise (relevant to the proposal) in the areas of- bioassay of various silkworm breeds / hybrids, He has >100 publications in different International and National level peer review journals.

** Dr R K Aggarwal, Chief scientist (Director grade), CCMB, agreed to provide all kind of technical supports / inputs for the work.*

17. **Work Plan**:

17.1 **Methodology**:

I) Activity-1: ***Evaluation of identified promising lines for important above and below ground agronomic traits with silkworm bioassay:***

a) *Plant material/ Field layout*

Establishment of identified promising progeny lines along with high yielding parent S-1, ruling cultivar S-1635, pipe-line strain C2038 and suitable spreader to offer equal germ load in RBD with three replications at Institute Farm. Forty nine clonal cuttings of all the strains will be planted in 60x60cm spacing in each replication. Recommended intercultural operations will be followed and observation will be recorded from 25 entries per line.

b) Trait evaluation

i) Phenotyping of various traits would be done at the foliage harvest maturity stage coinciding with five important commercial rearing seasons of West Bengal (in total about 10 season data would be collected during the project period).

ii) The evaluation of foliage biomass, aboveground ancillary traits and assessment of BLS disease reaction will be done as follows:

- ✓ Foliage biomass and ancillary morphological traits will be measured according to the method of Matchii et al. (2001) with suitable modifications for tropical environment suggested in the mulberry descriptor (Thangavelu et al. 2000).
- ✓ The parameters like number of tillers, plant height, total shoot length, nodal distance, leaf fall percent, weight of 100 mature leaves, leaf biomass, shoot biomass, single leaf area and above ground biomass will be estimated after 60 ± 5 days of bottom pruning of the plants in each season (Banerjee et al 2014).
- ✓ Foliage constituents like chlorophyll, total soluble protein, total soluble sugar and total phenol will be estimated following standard procedures (Chattopadhyay et al.1992). The experiment will be repeated twice.
- ✓ The propagation efficiency and below ground traits, an identical clonal sets of all progenies (5 plant / progeny) along with parents will be raised in polybag filled with sand : FYM: soil (1: 1: 1; w/v). The survival, root volume, root biomass, root length will be evaluated after 60 ± 5 days of plantation. The experiment will be repeated at least thrice.

iii) Disease assessment

- ✓ ***Natural inoculums based assessment in field:*** Disease reaction of segregating populations will be scored using Horsefall-Cowling,1978 (0 to 10 points) scale giving equal importance on Disease severity index (DSI), Disease incidence (DI), area under disease progression curve (AUDPC) and apparent infection rate (r) according to Chattopadhyay et al. (2010). BLS reaction will be assessed in two important seasons of BLS occurrence in West Bengal during June-July and September-October of two consecutive years coinciding with 'Shravani' (June - July) and 'Aswina' (September - October) commercial silkworm crops.

- ✓ ***Artificial inoculums based assessment:*** Degree of resistance of each selected lines will be tested for BLS disease reaction through artificial inoculation study in potted plant assay following completely randomized design (CRD). Visual symptomatic disease assessment will be done at weekly intervals along with susceptible line. The experiment will be repeated twice.

iv) Silkworm bioassay

Silkworm bioassay of the promising lines will be done with multivoltine x bivoltine silkworm races before recommendation for field use following standard procedure suggested by Saha et al (2013).

II) Activity- 2: ***Development of advanced breeding lines***

Controlled crossing program will be carried out through sib-mating of contrasting progeny lines for BLS resistance during mulberry flowering season 2016-2017.

Establishment of advanced breeding lines/Experiment design:

i) ~ 200 advanced breeding lines would be generated and established in field in ARBD with checks

ii) BLS disease scoring under field condition across the segregating population

iii) Testing of progeny lines with identified SSR markers for marker validation and to establish link between marker-trait(s) as well as for inheritance study.

III) ***Activity-3: SSRs validation***

DNA isolation, purification and quantification

Genomic DNA will be isolated from the leaves of parental lines, contrast responsive F_1 (pseudoF_2) and F_2 (pseudo F3) segregating progenies following the method of Kang et al (1998). DNA concentration and quality will be evaluated by spectrophotometric observation and electrophoresis in 0.8% agarose gel before PCR amplification.

PCR amplification with SSR markers

Identified SSR primer pairs (~2/3 nos) would be validated with contrast responsive F1 and F2 progeny lines for BLS resistance. The homology of the SSR markers will be compared with Phenotyping disease reaction information for inheritance analysis.

PCR amplifications of all primers will be performed on a Palmcycler Gradient System (Corbett Inc, Sydney, Australia) in 20µL reaction volume consisting of 1x PCR buffer, 2mM $MgCl_2$, 0.2mM each DNTPs, 0.2U Taq DNA polymerase, 15-µM each of forward and reverse primers and 15-25ng of template DNA. The PCR amplification conditions will be as: 94°C for 7min, followed by 35cycles of 30s at 94°C, 30s at 57°C and 45s at 72°C with a final elongation step at 72°C for 5min.

The amplification products will be resolved initially on 3% agarose gel in Tris-borate-EDTA (TBE) buffer pH 8.0, to be stained on ethidium bromide and analysed on Bio-Print Mega imaging system (Viber Lourmat, Cedex, France). The result will be confirmed with at least two replicated assay. For better resolution, prospective SSRs will be amplified on 6% denaturing polyacrylamide gel and band will be scored after silver staining according to the method of Zeng et al (2010). All methods have already been standardized in our laboratory.

V) ***Statistical analysis***

- ✓ Quantitative estimation of leaf yield and associated parameters will be determined using genetic model for 'Statistica' version 8.0 software (Statsoft Inc., Tulsa, OK, USA)
- ✓ Frequency distribution for BLS severity across the population will be estimated by SPSS version 20 base. The number of genes controlling BLS resistance will be assessed using chi-square analyses (Lillemo and Skinnes, 2005) by classifying the pseudo-F_2 (F_1) lines.
- ✓ Establishment of correlation between *planta* traits and silkworm bioassay findings by Pearson's correlation coefficients and clustering will be done for selection of superior

progeny using genetic model for 'Statistica' version 8.0 software (Statsoft Inc., Tulsa, OK, USA)

- ✓ Forward selection will be done using SSRs to select for improved resistance to bacterial spot in segregating progeny. Polymorphic bands will be scored for the presence (1) or absence (0) of amplified products and will be analyzed using NTSYS-pc version 2.11 package (Rohlf 2004).

17.2 **Organization of Work Elements**

Name of Scientists	Designation	Time	Organization of work elements
1. Dr R Banerjee	PI	55%	• DNA isolation and SSR analysis (65%) • Disease reaction study • Phenotypic characterization for yield and associated parameters • Analysis of data using NTSYSPc softwares. • Compilation of analyzed data and all sorts of report writing
2. Dr S Chattopadhyay	CI	35%	• SSR analysis (35%) • Characterization for biochemical parameters • Propagation efficiency and associated root-traits • Analysis of data using 'Statistica' & 'SPSS' base 20 softwares.
3. Dr A K Saha	CI	5%	• Silkworm bioassay with suitable races.

17.3 **Proprietary / Patented items, if any, expected to be used for this Project**:

Nil

17.4 **Suggested plan of action for utilization of the expected outcome from the project:**

- Selected BLS resistant improved genotype(s) will be recommended for direct field utilization by the competent authority
- Sequencing, characterization of DNA tags along with linkage analysis to identify major QTLs linked to BLS resistance traits and deposit the information for web enabled database for future use.

17.5 **Time Schedule of activities giving milestones**:

#	Activity	Milestones	Expected date of	
			Starting	**Completion**
1.	Evaluation of identified promising lines for important agronomic traits /leaf yield/root parameters/silkworm bioassay	Establishment of promising lines along with ruling cultivars in RBD	Beginning of 1st quarter	End of 4th quarter
		Disease scoring phenotyping	Beginning of 5th quarter	End of 10th quarter
		Evaluation for foliage biomass and associated traits	Beginning of 5th quarter	End of 12th quarter
		Evaluation for survival and propagation parameters	Beginning of 5th quarter	End of 10th quarter
		Testing of genotypes through silkworm bioassay with improved crossbreds	Beginning of 9th quarter	End of 12th quarter
2	Development of advanced breeding lines	Sib-mating between identified lines; development of advanced breeding generation/establishment of plantation	Beginning of 1st quarter	End of 8th quarter
3	SSRs validation	Molecular marker validation with identified SSRs using three generations	Beginning of 3rd quarter	End of 12th quarter
		Forward selection in segregating population(s) using identified / validated marker(s)	Beginning of 9th quarter	End of 12th quarter
4	*Data analysis, compilation of results, report preparation and submission of report*		Beginning of 12th quarter	End of 12th quarter

17.6 **Project Implementing Agency /Agencies**:

Name of the agency	Address of the agency	Proposed Research Aspects	Proposed Amount	Cost Sharing %
Central Silk Board	Central Silk Board, Ministry of Textiles, Govt. of India, Bangalore		7.70	100%

Reference

Acharya, A.R., Wooford, D.S., Kenworthy, K,and Quesenberry, K.H.(2011). *Crop Sci*. 51:1928-1934.

Aggarwal R, Udaykumar D, Hendre PS, Sarkar A, Singh L (2004) *Mol Ecol Notes* 4: 477 – 479.

Ahuja. S.L. and Dhyal, L.S. (2007).*Euphytica*. 153:87-98.

Allard RW (1999) Priciples of Plant Breeding.John Wiley &Sons,New York.

Ashkani Sadegh,Mohd Yusop Rafii,Ibrahim Rusli,Meon Sariah, Siti Nor Akmar Abdullah , Harun Abdul Rahim and M. A. Latif (2012)*Plant Mol Biol Rep* DOI 10.1007/s11105-011-0315-4

Amil-Ruiz Francisco, Rosario Blanco-Portales, Juan Mun˜oz-Blanco , Jose´ L. Caballero.(2011). *Plant Cell Physiol*. 52: 1873–1903 .

Awasthi AK, Nagaraja GM, Naik GV, Kanginakudru S, Thangavelu K, Nagaraju J (2004) *BMC Genet* 5:1.

Balachandran MT, Kadam NN, Biradar J, Reddy SH, Ankaiah M,Narayanan MJ, Makarla U, Khurana P (2013) BMC Plant Biol 13:194.

Banerjee R, Chattopadhyay S, Saha AK and S. NK. (2014)*Archives Of Phytopathology And Plant Protection*, DOI: 10.1080/03235408.2013.868693

Banerjee, R., Das, N.K., Doss, S.G., Saha, A.K., Bajpai, A.K. and Bindroo, B.B.(2012).*Eur J of Plant Pathol*. 133:537-544.

Banerjee, R., Maji, M.D., Ghosh, P. and Sarkar, A.(2009). *Archives of Phytopathology and Plant Protection*. 42:291-297.

Ben Hui Leu(1998). In:Statistical Genomics Linkage, Mapping and QTL analysis.CRC press,LLCp59-60.

Bianco L, Cestaro A, Sargent DJ et al. (2014)*PLOS ONE*: 9(10) e110377.

Bonos, S. A., Casler, M. D., and Meyer, W. A. (2003). *Crop Sci*. 43:2189-2196.

Bus, V.G.M., Ranatunga, C., Alspach, P.A., Oraguzie, N.C. and Whitworth, C. (2005). *Euphytica*. 148:235-242.

Chatterjee SN, Nagaraja GM, Srivastava PP, Naik G (2004) Genetica 39: 1612- 1624.

Chattopadhyay S, Chattopadhay S, Ghosh B (1992) *Ind J Expt Biol.* 30: 231 – 234.

Chattopadhyay, S., Ali, A.K., Doss, S.G., Das, N.K., Aggarwal, R.K., Bandyopadhyay, T.K., Sarkar, A. and Bajpai, A.K.(2010).. *J Genl Plant Pathol*. 76: 87-93.

Collard BCY and Mackill DJ(2008) Phil. Trans. R. Soc. B 363, 557–572

Dandin, S.B. and Giridhar, K.(2010). In: Handbook of sericulture technologies. Central Silk Board, Bangalore, India, pp 157-158.

Govindaiah, Gupta VP,Sharma DD, Rajadurai S,Naik VN.(2005).In:Sampath J(ed.), Central Silk Board,pp 145-177.

Graham JH, Gottwald TR, Riley, TD, Achor D.(1992). *Phytopathology*. 82:1319-1325.

Horsefall, J.G. and Cowling, E.B.(1978). In: Horsefall JG and Cowling EB(ed), Plant disease, Academic Press, New York, USA,pp 120-136.

Kafkas S, Ozgen M, Dogan Y, Ozean B, Ercisli S, Ozean, Erccisli S, Seree S (2008) *J Am Soc Hortc Sci* 133: 593-597.

Kang HW, Cho YG, Yoon UH, Eun MY (1998) Plant Mol Biol Reporter 16: 1- 9.

Kore, S.S. and Patil, P.S.(1985). *Indian Phytopathology*. 38:622.

Lee May , Jun Hong Xia, Zhongwei Zou, Jian Ye, Rahmadsyah2 Yuzer Alfiko, Jingjing Jin,Jessica Virginia Lieandoaria Indah Purnamasari3, Chin Huat Lim2, Antonius Suwanto3, LimsoonWong,Nam-Hai Chua & Gen Hua Yue (2015) *SCIENTIFIC REPORTS* . 5 : 8232. DOI: 10.1038/srep08232

Lillemo, M., Skinnes, H., Singh, R. P., and van Ginkel, M. (2006). *Plant Dis*. 90:225-228

Machii H, Koyama A, Yamanouchi H, Matsumoto K, Kobayashi S, Katagiri K (2000) Misc. Publ. Natl. Inst. Seric. Entomol. Sci. 29: 1 – 307.

Maji MD, Banerjee R, Das NK,Chakrabarty S, Bajpai AK(2008). *Journal of Agrometeorology* (Special Issue-Part I): 193-196.

Melotto M, Underwood W, He SY.(2008). *Annu Rev Phytopathol*. 46:101-122.

Nagaraj MS, Srinivasachary,Khan ANA (2000) In:National Seminar on Tropical Sericulture.Chinnnaswamy KP, Govindan RK, Reddy DNR(ed).University of Agricultural Sciences,GKVK,Bangalore,India,pp124-126

Ngadze E, Icishahayo D, Coutinho TA, van der Waals JE.(2012). *Plant Dis*. 96: 186-192.

Nicks RE, Rubiales D.(2002). Euphytica. 124:201-216.

Nino-Liu DO, Ronald PC, Bogdanove AJ.(2006). *Molecular Plant Pathology*.7:303-324.

Saha AK, Suresh Kumar N, Chakrabarty S, Patnaik BB, Nayak SK, Roy S, Bindroo BB (2013a) Int J Indust Entomol. 26: 22-30

Sato M, Takahashi K (1973). *Ann Phytopathol Soc* Jpn 39:425-428.

Sharma, D.D., Chowdary, N.B., Naik, N., Rajan, M.V. and Dandin, S.B.(2005). *Sericologia*. 45:231-25

Sujay V. M. V. C. Gowda , M. K. Pandey ,R. S. Bhat,Y. P. Khedikar , H. L. Nadaf , B. Gautami , C. Sarvamangala , S. Lingaraju , T. Radhakrishan , S. J. Knapp ,R. K. Varshney (2012 *) Mol Breeding* : 30:773–788

Tan Huaqiang, Manman Tie, Qian Luo, Yongpeng Zhu, Jia Lai and Huanxiu L(2012). *Journal of Life Sciences* 6 : 1190-1199

Tulsieram LK,Glaubitz JC, Kiss G, Carlson JE(1992). *Nature Biotechnology (NY)* 10:686-690.

Vijayan K, Srivastava PP, Raju J and Saratchandra B. (2012) Czech J. *Genet. Plant Breed.* 48: 147–156

Wallace TP, El-Zik KM (1989). Crop Sci 29:1114–1119.

Wu J, Li L, Li M, Khan M. A, Li X, Chen H, Yin H and Zhang S.(2014)*Journal of Experimental Botany* doi:10.1093/jxb/eru311

Zeng S, Xiao G, Guo J, Fei Z, Xu Y, Roe BA, Wang Y (2010) *BMC Genomics* 11: 94 www. biomedcentral. com/1471-2164/11/94

Zhang S , Han Mi Xing-fang G, Yuhong Y, Bingyan X, Xiaowu W, Sanwen H, Yongchen D, and Rifei S (2010) *AMER. SOC. HORT. SCI*. 135(1):53–58

Zhao W, Miao X, Jia S, Pan Y, Huang Y (2005). *Plant Sci* 16:519-525.

PART-IV: BUDGET PARTICULARS

18. **BUDGET** (in Lakh): [In case of multi-institutional projects, the budget details should be provided separately for each of the Institute]

A) Non-Recurring (e.g. equipments, accessories, etc.): Nil

B) Recurring

B.1. Manpower: Nil

B.2. Consumables:

Sl. No.	Item	1st Year	2nd Year	3rd Year	Total (in lakh)
1.	Fine chemicals / chemicals / reagents/ others	1.60	2.00	1.60	3.30
	Sub Total B2:	**1.60**	**2.00**	**1.60**	**5.20**

Other Items:

Sl. No.	Item	1st Year	2nd Year	3rd Year	Total (in lakh)
B3	Travel	0.30	0.30	0.30	0.90
B4	Contingency	0.50	0.70	0.40	1.60
	Total	**0.80**	**1.00**	**0.70**	**2.50**
Sub-total (B1+B2+B3+B4 etc.)		**2.40**	**3.00**	**2.30**	**7.70**
Grand total (A+ B1+B2+B3+B4)		**2.40**	**3.00**	**2.30**	**7.70**

Justification:

Consumables:

The proposed work is experiment-intensive involving huge numbers of PCR reactions, DNA-amplicon separations, therefore needs relatively high consumable support.

Contingency

The proposed study requires intensive equipment support. The contingency amount may be utilized for repairing of equipment and other miscellaneous expenditure

PART-V: EXISTING FACILITIES

19. Available equipment and accessories to be utilized for the project:

SI. No.	Name of the Equipment/ Accessory	Make	Funding Agency	Year of Procurement
1	Palm Cycler	Genetix	CSB	2013
2	Uv-vis Spectrophometer	Eleico	DBT	2010
3	Ultra-freezer (-80°C)	Thermo	CSIR	2006
4	Medium speed cold centrifuge	Remi	CSB	2002
5.	Spinwin	Tarson	CSB	2015
5	Quick freezer	Remi	DBT	2011
6	Horizontal gel electrophoresis set with power pack	Atto Corporation	DBT	2007
7	Gel documentation system	Vilber Lourmat(France)	CSB	2012
8	Gel electrophoresis unit	Tarson	DBT	2011
8	Experimental filed of ~2 acre containing mapping populations specific for various diseases, leaf biomass and a core germplasm of ~130 resources raised and during implementation of four DBT supported projects since 2006. A portion of the resources will be utilized in the proposed study			

PART-VI: DECLARATION / CERTIFICATION

It is certified that

a. The research work proposed in the project does not in any way duplicate the work already done or being carried out elsewhere on the subject.

b. The same project has not been submitted to any other agencies for financial support.

c. The emoluments for the manpower proposed are those admissible to persons of corresponding status employed in the institute/ university or as per the Ministry of Science & technology guidelines (Annexure-III).

d. Necessary provision for the project will be made in the Institute in anticipation of the sanction of the scheme.

e. If the project involves the utilization of genetically engineered organism , it is agreed that we will ensure that an application will be submitted through our institutional bio-safety committee and we will declare that while conducting experiments, the bio-safety guidelines of the Department of Biotechnology would be followed in toto.

f. If the project involves field trials / experiments / exchange of specimens etc we will ensure that ethical clearances would be taken from the concerned ethical committees of Biotechnology before implementing the project.

g. It is agreed by us that any research outcome or intellectual property right(s)on the interven (s) arising out of the project shall be taken in accordance with the instructions issued with the approval of the Ministry of Finance . Department of Expenditure as contained in annexure-V

h.. We agree to accept the terms and conditions as enclosed in Annexure-IV. The same is signed and enclosed.

i. The institute agrees that the equipment, the basic facilities and such other administrative facilities as per terms and conditions of the grant will be extended investigators through out the duration of the project .

j. The institute assumes to undertake the financial and other management responsibilities of the project.

Signature of Executive Authority of
Institute with Seal & date

Signature of Project Co-ordinator with Seal & date
[Applicable for inter-institutional Projects only]

Signature of Principal Investigator & date	**Signature of Co-Investigator-I & date**	**Signature of Co-Investigator-II & date**