



INTERNATIONAL
SERICULTURAL
COMMISSION

UNITED NATIONS REG. NO. 10418

ISSN 0250-3980

Volume 55 | Number 4 | 2015

SERICOLOGIA

Journal of Silkworms
A Quarterly Publication of
International Sericultural Commission



SERICOLOGIA

Journal of Silkworms

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PRINTED AT

Ramya Reprographic Pvt. Ltd

No. 16/2B, Singasandra Village

Behind Dakshin Honda Show Room

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RNA INTERFERENCE - A POTENTIAL TOOL FOR CROP IMPROVEMENT IN SERICULTURE

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ABSTRACT

RNA interference is the process by which an organism degrades mRNA of a gene before it is translated into protein. The phenomenon was first reported in petunia in 1990, when a transgenic petunia plant harbouring the *chalcone synthase (chsA)* coding gene under the control of a 35S promoter seemed to have lost both endogene and transgene to produce variegated flowers with white sectors. This is quite different from the antisense mechanism as only a few dsRNA molecules are enough to prevent all the endogenous mRNA from expression. In simple RNAi mechanisms, the dsRNA intermediates are processed into RNA duplexes of 21-24 nucleotides by the enzyme Dicer, which are then incorporated into RNA induced silencing complex (RISC). One strand of the siRNA duplex, which is known as the guide strand acts as a guide to the degradation of complementary mRNAs. The RNAi gene silencing technique is now being used for induction of resistance against a number of viral diseases, improving quality of fruits, changing the colour of flowers *etc.* Since, intensive research is being carried out in RNA silencing, it is expected that new and exciting discoveries could be made to benefit both agriculture and medical fields. In silkworm, RNAi has already made its impact by incorporating viral resistance through knocking out genes which facilitate viral entry and its multiplication in the silkworm. Since most of the host plants of silkworms are trees with high heterozygosity and are recalcitrant even to many of the widely used modern biotechnological tools, application of RNAi for silencing of specific genes *via* transgenesis offers a tool of greater hope for improving specific traits especially those such as resistance to biotic and abiotic stresses, palatability to silkworm, propagation efficiency *etc.* This review highlights the attempts in this direction and emphasize the significance of gene silencing for crop improvements of both silkworms and their host plants along with major steps of gene silencing.

Key words: Genesilencing, mulberry, RNAi, silkworm.

INTRODUCTION

The central dogma of biology says that genes express themselves through proteins, which are produced through an intermediate guiding molecule called mRNA. The process of formation of the intermediate mRNA from the gene (DNA) is called transcription and formation of protein from mRNA is called translation. In fact, mRNA carries the essential information from the gene to decide the nature of the protein by translating itself into proteins through amino acid sequences. Any interruption in the

process of transcription and translation can stop the gene from proper functioning. Recently, it has been observed that translation of mRNA into protein is sometimes prevented by an endogenous production or artificial introduction of a small double strand RNA having nucleotide sequence complementary to the target gene to prevent expression of unwanted genes and this phenomenon is called RNA interference or gene silencing (Bosher and Labouesse, 2000). Subsequent research revealed that it is a natural phenomenon wherein the

transcription of gene is normal and the protein synthesis is prevented by destroying the mRNA before its translation. Napoli *et al.* (1990) first reported this phenomenon quite accidentally by working on a chalcone synthase (CHS) gene overexpression in petunia. They found that the levels of endogenous as well as introduced CHS were 50-fold lower in transgenic plants as compared to that of the wild-type due to “cosuppression” of the endogenous CHS gene by the introduced transgene. The phenomenon was later reported in *Neurospora crassa* (Romano and Macino 1992), and in *Caenorhabditis elegans* (Guo and Kemphues, 1995). Subsequently, Fire *et al.* (1998) provided an explanation for the previously reported silencing phenomenon by confirming the role of dsRNA in the silencing process. Subsequent studies proved that RNAi mechanism has evolved as a protectionary means against virus attacks (Bernstein *et al.*, 2001) as it is equally effective against both positive-strand and negative-strand RNA viruses (Muller *et al.*, 2010). Similarly, it is observed that introduction of antisense RNA of specific endogenous genes could phenocopy a null or hypomorphic mutation in the targeted gene (Fire *et al.*, 1998). Subsequent studies through introduction of RNAs without any DNA template further substantiated the role of RNAi in controlling the viral replication (Pooggin *et al.*, 2003). It is also presumed that cells employ RNAi to tightly regulate protein levels in response to various environmental stimuli (Milhavet *et al.*, 2003). It is apparently one of the ancient defence mechanism against invading viruses and deleterious transposons, for regulation of gene expression and chromatin modifications (Tamilarasan and Rajam, 2013).

Mechanism of RNA interference

Although different terms such as post translational gene silencing (PTGS) in plants, RNAi in animals, quelling in fungi, and virus-induced gene silencing have been used to term the process of gene regulation by RNA, the essence of the mechanism has similar components and functions. All RNAi processes begin with an RNA precursor that is completely or partially double-stranded (dsRNA). This precursor is then processed into active

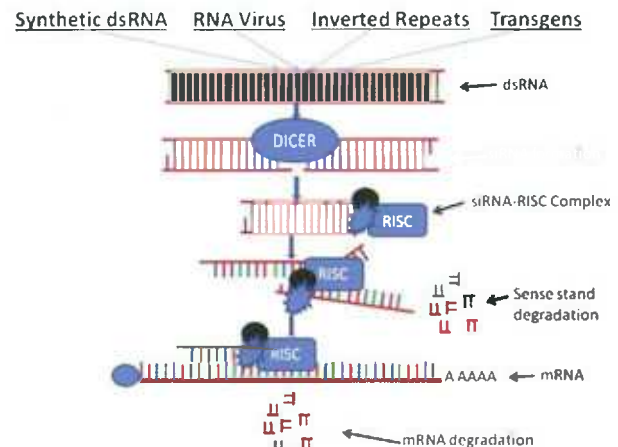


Figure 1: Mechanism of RNA interference

small interfering RNA (siRNA) by an enzyme called Dicer (Figure 1). Formation of siRNAs of 20-24 nt in length is one of the important components of RNAi mechanism, which are formed and accumulated as double-stranded RNA molecules of defined chemical structures that participate in the degradation of the target mRNA in a homology dependent fashion. To accomplish this degradation, a set of proteins similar in structure and function is essential. Similarly, the whole process of gene silencing starts with the formation of small interfering RNA (siRNA) or micro RNA (miRNA) of 21-25 nt in length with 2 base overhang at the 3' end and two different groups of which are produced by Dicer (Bender, 2001; Ghildiyal and Zamore, 2009; Asgari, 2013). siRNA mediates RNA-interference by cutting the target mRNAs into small pieces through endonucleolytic cleavage with the help of an enzyme with four distinct domains such as an aminoterminal helicase domain, dual RNase III motifs, a dsRNA binding domain, and a PAZ (Tabara *et al.*, 1999; Catalanotto *et al.*, 2000). The target mRNAs are, thus, not allowed to accumulate in the cytosol (Fagard and Vaucheret, 2000). Upon cleavage, one strand of the siRNA duplex, the guide strand, is loaded onto Argonaute proteins present at the core of the RNA-induced silencing complex (RISC). Argonaute proteins are members of a highly conserved family that have been implicated in RNAi across several species (Carmell *et al.*, 2002). Argonaute proteins have been found to be associated with RNAi in

various organisms to screen for RNAi-deficient mutants. These include *C. elegans* *rde-1*, *Arabidopsis* AGO1, and *Neurospora* QDE2 (Cogoni and Macino, 1997; Fagard *et al.*, 2000). There are eight human Argonaute proteins, of which four belong to the Argonaute subcategory (Ago1-4) and the remaining four belong to the Piwi subcategory (hPiwi 1-4). Although the Argonaute family members have been linked both to the gene-silencing phenomenon and to the control of development in diverse species, the exact component of it was found only later and it was found that Ago2 is the enzyme responsible for cleaving the passenger strand siRNA as well as target mRNA (Liu *et al.*, 2004). However, purified Ago2 cannot use double-stranded siRNAs as a substrate for targeting the mRNA cleavage (Liu *et al.*, 2004). The RISC is a ternary complex made up of the Argonaute protein, Dicer and the dsRNA-binding protein. The RISC/Ago2 complex determines the strand to cleave, based on the thermodynamic properties of functional and nonfunctional siRNAs, as the 5' antisense region of the functional siRNAs were less thermodynamically stable than the 5' sense regions, providing a basis for entry into RISC (Khvorova *et al.*, 2003). The non-guide strand also called the passenger strand is cleaved and ejected out of the complex. RISC, incorporates one strand of a small interfering RNA (siRNA) or microRNA (miRNA). RISC uses the siRNA or mRNA as a template for recognizing complementary mRNA (Tang *et al.*, 2003). The siRNA pathway is induced by invasive nucleic acids, such as viruses, transposons, or transgenes, and serves to defend the host plant against such invaders (Alvarado and Scholthof, 2009). In contrast, miRNAs are produced by an endogenous pathway that is used to control an organism's own gene expression (Vazquez *et al.*, 2010). Degradation of homologous mRNA transcripts can be induced by dsRNA while it is being processed into 21-23 base siRNAs by Dicer. The resulting siRNAs function as sequence specific guides to direct RISC to cleave the target (mRNA) to effect the silencing. Degradation of target mRNA happens in two different pathways on the basis of complementarities of guide strand. Perfect complementarities resulted in complete degradation of

target mRNA while partial complementarities resulted in suppression of genes. If the gene silencing is triggered by the production of dsRNA during viral replication, the process is called virus-induced gene silencing (VIGS) (Lu *et al.*, 2003). One interesting feature of RNA silencing in plants is that once it is triggered in a certain cell, a mobile signal is produced and it spreads through the whole plant causing the entire plant to respond to it (Dunoyer *et al.*, 2007). After triggering RNA silencing, the mobile signalling molecules can be relay-amplified by synthesis of dsRNAs on the primary cleavage of product templates or by their cleavage into secondary siRNAs. This amplification leads to the transitory nature of silencing reaction that may spread along the mRNA, though initiated by a locally targeted single siRNA and spreads in both the 5' and 3' directions. This bi-directional transitions further have been witnessed by a process where both the 5' and 3' cleavage products of the initial target RNA acts as an aberrant mRNA to trigger dsRNA synthesis, and induces secondary silencing reactions. This silencing process is also enhanced by the enzymatic activity of the RISC complex, mediating multiple turnover reactions (Hutvagner and Zamore, 2002). Furthermore, production of the secondary siRNAs leads to enrichment of silencing *via* its spread from the first activated cell to neighbouring cells, and systemically through the system. The cell-to-cell spread can be mediated as passive spread of the small RNAs *via* plasmodesmata or by the silencing signal complex (Kobayashi and Zambryski, 2007). The systemic spread in phloem is mediated by the 24 bp siRNA, and it seems to be mediated *via* plasmodesmata, since it does not spread into meristematic cells. The discovery of RNA binding protein (PSRP1) in the phloem and its ability to bind 25 bp ssRNA species add further to the argument that siRNAs are the key components for systemic silencing signal. The extent of cell-to-cell movement is dependent on the levels of siRNAs produced at the site of silencing initiation, but is independent of the presence of siRNA target transcripts in either source or recipient cells. However, such processes of spreading of the signal to all the cells are not universal, as these are not found in flies and mammals.

Another endonuclease enzyme of RNaseIII family such as Drosha, having a proline-rich region and an arginine- and serine-rich region at the N terminus, catalyses the initial processing of the primary-miRNA with hairpin structure in the nucleus, thereby yielding pre-miRNAs, which are hairpin structures with phosphorylated 5' ends and 3' dinucleotide overhangs. The purified Drosha cleaves dsRNA nonspecifically; however, specific cleavage of pri-miRNAs requires association with an RNA-binding protein called DGCR8, which assists the RNase III enzyme, Drosha, in the processing of micro RNAs (miRNAs). In human, the core region of DGCR8 is composed of a tandem pair of dsRBDs. This process occurs in the complex called the microprocessor. DGCR8 binds to the base of the pri-miRNA hairpin, positioning Drosha to cleave the pri-miRNA stem at a distance of 11 base pairs from the junction between the duplex stem and the flanking ssRNA regions. By its activity, DGCR8 is likely a trans-acting specificity determinant, analogous to the PAZ domain of Dicer. After its export to the cytoplasm, the hairpin undergoes another endonucleolytic cleavage, which is catalyzed by Dicer, generating an miRNA-miRNA duplex of 21-25 nucleotides (Fire *et al.*, 1998).

Potential applications of RNAi

RNAi has recently been extensively used in agriculture and medicine (Zhang *et al.*, 2013). It has been used to control viral, bacterial and fungal diseases and to rectify certain gene functions (Tables 1, 2 and 3). Chuang and Meyerowitz (2000) for the first time demonstrated silencing of a homeobox gene through introduction of an RNAi vector. The plant RNAi vector consisted of an inverted repeat harbouring target sequences under the control of a strong promoter. It is also pertinent to note that two different species of RNA such as siRNA and miRNAs are involved in RNA interference and, hence, the technology used for both these two species differs accordingly as in siRNA-based approach, the transgene is designed to produce a population of siRNAs that represents the entire dsRNA region and has complementarities to a chosen target messenger RNA.

Table 1: RNA interference in controlling some of the destructive pests

Inset	Target region	RNAi effect	Reference
<i>Ostrinia furnalis</i>	<i>DS10 & DS28</i>	Mortality increased up to 73-100 %	Wang <i>et al.</i> (2011)
<i>Phyllotreta striolata</i>	<i>PsOrl</i>	Block host-plant-seeking behaviour	Zhao <i>et al.</i> (2011)
<i>Nilaparvata lugens</i>	<i>TPS</i>	Decline expression of target gene	Chen <i>et al.</i> (2010)

Table 2: Effects of targeted region of RNAi in various plant virus systems

Host system	Virus	Target genes	Reference
Barley, Wheat	Barley stripe mosaic virus	<i>Pds</i>	Cakir <i>et al.</i> (2010)
Soybean	Bean pod mottle virus	<i>Pds, Actin</i>	Zhang and Ghabrial (2006)
Banana	Banana bract mosaic virus	<i>CP</i>	Williams <i>et al.</i> (2004)
Cassava	African cassava mosaic virus	<i>pds, su, cyp79d2</i>	Fofana <i>et al.</i> (2004)
Mung bean	Yellow mosaic virus	<i>YMV-Vig</i>	Pooggin <i>et al.</i> (2003)
<i>Arabidopsis</i>	Cabbage leaf curl virus	<i>gfp, CH42, pds</i>	Turange <i>et al.</i> (2002)

Table 3: RNA-mediated improvement of important traits in various crop plants

Host plant	Targeted genes	Nutritional value	Reference
Cotton	d-cadinene synthase	Reduction of toxic gossypol in seeds	Sunil kumar <i>et al.</i> (2006)
Wheat	Starch branching enzyme (SBEIIa & SBEIIb)	High amylase	Regina <i>et al.</i> (2006)
Coffee	Theobromine synthase	Decaffeinated coffee plant	Ogita <i>et al.</i> (2004)
White pea	Genes responsible for BOAA	Non-toxic	Williams <i>et al.</i> (2004)

The siRNA population is generally very effective at silencing the target gene because there are many different small RNAs all targeting the same transcript. Whereas, in miRNA based approach, a natural endogenous miRNA gene is genetically engineered to produce the endogenous miRNA that is complementary to the targeted messenger RNA. The modified precursor is then processed into a novel miRNA called artificial miRNA (Ossowski *et al.*, 2008). This approach produces a single small RNA rather

than a population of small RNAs. The miRNA approach may be less effective in silencing the target gene (because only one small RNA is produced rather than a population), but it is more selective and less likely to result in off-target silencing. Artificial miRNAs thus provide an elegant method to silence genes in a very specific manner (Sablak *et al.*, 2011). Further, a number of factors can also influence the result of RNAi technology. Important among them are cellular uptake of the RNAi molecule (usually dsRNA), the production of secondary dsRNA molecules in the cell, and the transfer of these molecules to other cells. Where the RNAi-mediated silencing is transmitted widely throughout the treated organism, RNAi is described as systemic (Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010). Since RNAi is operative in fungi, nematodes, plants, flies, and animals, it is emerging as a powerful tool not only to be used as weapon against invading pathogens, but also to study the function of individual genes and proteins (Milhavet *et al.*, 2003). siRNA mediated gene knockdown of signal transduction has been successfully employed in cell proliferation studies (Ancellin *et al.*, 2002; Irie *et al.*, 2002; Kisielow *et al.*, 2002; Czauderna *et al.*, 2003) and cancer therapy (Milhavet *et al.*, 2003), to control diseases by interfering with expression of genes responsible for replication of the pathogen in the host cell (Capodici *et al.*, 2002; Jacque *et al.*, 2002) and even in control of Huntington's disease (Rubinshtein, 2002).

RNAi in insects

Much work has been done on insects with RNAi (Table 4). It is proved from these studies that RNAi contributes to insect immunity against viruses with a dsRNA genome or dsRNA replicative intermediates in the cytoplasm of infected cells (Blair, 2011; Schnettler *et al.*, 2012), though in some cases, it elicits low responsiveness to exogenously-applied dsRNA because of alternative anti-viral defences (Merkling and van Rij, 2013). Further, insects infected with asymptomatic, persistent virus coding an RNAi suppressor would show limited responsiveness to experimental RNAi (Berry *et al.*, 2009). Likewise, it is also found that the efficacy of RNAi

Table 4: Experimentations with RNAi in Lepidoptera
(adapted from Terenius *et al.* (2011))

Species	Family	Reference
<i>Bombyx mori</i>	Bombycidae	Dai <i>et al.</i> (2008), Gui <i>et al.</i> (2006), Huang <i>et al.</i> (2007), Masumoto <i>et al.</i> (2009), Onishi <i>et al.</i> (2006), Quan <i>et al.</i> (2002), Tabunoki <i>et al.</i> (2004), Zhang <i>et al.</i> (2006)
<i>Bombyx mandarina</i>	Bombycidae	Qian <i>et al.</i> (2015)
<i>Antheraea assama</i>	Saruniidae	Shukla and Nagaraju (2010)
<i>Antheraea mylitta</i>	Saruniidae	Gandhe <i>et al.</i> (2007)
<i>Antheraea pernyi</i>	Saruniidae	Hera <i>et al.</i> (2004); Liu <i>et al.</i> (2015)
<i>Hylophora cecropia</i>	Saruniidae	Bettencourt <i>et al.</i> (2002), Terenius <i>et al.</i> (2007)
<i>Sprodoptera exigua</i>	Noctuidae	Tian <i>et al.</i> (2009)
<i>Sprodoptera frugiperda</i>	Noctuidae	Griebler <i>et al.</i> (2008)
<i>Sprodoptera littoralis</i>	Noctuidae	Tian <i>et al.</i> (2009)
<i>Sprodoptera litura</i>	Noctuidae	Rajagopal <i>et al.</i> (2002)
<i>Mamestra brassica</i>	Noctuidae	Tsuzuki <i>et al.</i> (2005)
<i>Helicovera armigera</i>	Noctuidae	Sivakumar <i>et al.</i> (2007)
<i>Manduca sexta</i>	Sphingidae	Levin <i>et al.</i> (2005), Eleftherianos <i>et al.</i> (2009), Whyard <i>et al.</i> (2009)
<i>Plodia interpunctella</i>	Pyrilidae	Fabrick <i>et al.</i> (2004)
<i>Plutella xylostella</i>	Plutellidae	Yang <i>et al.</i> (2009)

is influenced by the mode of delivery of the RNAi. The most widely used methods of administering RNAi into insects are injection into the hemolymph and feeding. Microinjection was the first successful method that was used to knockdown a gene in *Drosophila melanogaster* (Kennerdell and Carthew, 1998). This method was quickly transferred to other insects (Brown *et al.*, 1999; Blandin *et al.*, 2002). Oral delivery, on the other hand, is a less-invasive and potentially a high-throughput method for RNAi delivery especially for those insects that are intolerant to injection. Oral delivery has successfully been used in honey bees, aphids, whiteflies and psyllids

(Aronstein *et al.*, 2006; Ghanim *et al.*, 2007; Whyard *et al.*, 2009; Wuriyanghan *et al.*, 2011). Xiong *et al.* (2013) used oral delivery of dsRNAs of a moult-regulating transcription factor gene, *HaHR3*, of cotton bollworm to control it. RNAi can also be employed against insects by engineering plants to express dsRNAs in plant systems. Stable transformation can be achieved by hairpin dsRNAs that target insect genes (Baum *et al.*, 2007) or transient virus-induced gene silencing (VIGS), in which engineered viral vectors carrying the gene sequence of interest are transformed into *Agrobacterium tumefaciens* and infiltrated into the plant tissue (Burch-Smith *et al.*, 2004; Baum *et al.*, 2007; Pitino *et al.*, 2011; Zha *et al.*, 2011; Kumar *et al.*, 2012). Soaking the organism into a dsRNA solution has also been adopted widely and is being considered as a convenient method to trigger RNAi response (Yu *et al.*, 2013).

RNAi for pest control

The potential of RNAi for the pest management has been exploited to protect agricultural crops from devastating pests such as western corn rootworm, cotton bollworm (Baum *et al.*, 2007; Mao *et al.*, 2007; Xue *et al.*, 2012). Transgenic cotton plants expressing CYP6AE14 dsRNA drastically reduced the growth of the cotton bollworm larvae, leading to less damage to the plants (Mao *et al.*, 2011). In a similar experiment, cotton plants engineered to express cysteine proteases attenuated the peritrophic matrix of cotton bollworm, resulting in increased uptake of the dsRNA (Mao *et al.*, 2013). Further, cotton plants expressing both the dsCYP6AE14 and cysteine protease were found more protected from bollworm than use of single gene (Mao *et al.*, 2013). The mechanism of dsRNA uptake through insect gut has been comprehensively reviewed by Huvenne and Smagghe (2010).

Bacterial disease resistance: Imani Fooladi *et al.* (2012) demonstrated that siRNAs can potently inhibit *P. aeruginosa* flagellin production *in vitro*, and that the number of viable bacteria was significantly decreased by pre-treatment with siRNA.

Viral disease resistance: Waterhouse *et al.* (1998) first used RNAi to develop a potato resistant to virus by developing a vector simultaneously expressing both the sense and antisense transcripts of the viral helper-component proteinase (HCPro) gene. Later, Missiou *et al.* (2004) transformed commercial variety of potato (cv Spunta) with a dsRNA developed from the 3' part of the coat protein gene of PVY. The transgenic lines were highly resistant to three strains of PVY. Likewise, Vanderschuren *et al.* (2007) developed a transgenic cassava resistant to cassava mosaic disease (CMD).

Control of Microsporidiosis: The potential of RNAi to control the microsporidian parasite was illustrated clearly in honey bee by controlling the parasite *Nosema*, which causes high morbidity and mortality of honey bees (Martin-Hernandez *et al.*, 2011). *Nosema* can easily be controlled using RNAi as it has the molecular machinery for RNAi and it inhabits in the midgut epithelial cells to which the ingested dsRNA has easy access. Paldi *et al.* (2010) fed honey bees with dsRNA specific to the *Nosema* ADP/ATP transporter gene, which is essential for its energy metabolism, and thereby reduced *Nosema* load and mortality.

Enhancing nutritional qualities: Davuluri *et al.* (2005) reported that RNAi-mediated suppression of endogenous photomorphogenesis regulatory gene *DET1* using fruit-specific promoters can significantly increase carotenoid and flavonoid content in the fruit without affecting the level of other components.

Value addition: A blue rose was developed by CSIRO, Australia through gene silencing in 1997. The gene silencing technology turned off the activity of the red pigment producing gene *DFR* and replaced it with a *DFR* gene from an iris producing blue pigment resulting in the production of blue rose flowers (Katsumoto *et al.*, 2007).

Abiotic stress: Production of miRNAs in response to abiotic stresses such as drought, cold, salinity, and oxidative stress was described by Sunkar and Zhu (2004) in *Arabidopsis* seedlings.

RNA interference (RNAi) in silkworm: RNAi has successfully been employed in a number of lepidopteran species (Table 4) and in the silkworm *Bombyx mori*, it was used to control the devastating Baculovirus, *Bombyx mori* Nucleo Polyhedro Virus (BmNPV), which accounts for nearly 50 % of the total crop loss, especially during the summer months (Khurad *et al.*, 2006). Prevention of the disease through disinfection of rearing house and rearing appliances are the only effective methods of controlling this disease and no effective cure is available due to its sturdy nature (Subbaiah *et al.*, 2013). Gene expression studies using both resistant and susceptible strains of silkworm revealed that 8 genes (gloverin-4, gloverin-3, lebocin, serpin-5, arylphorin, promoting protein, cathepsin B and actin A3) expressed in the midgut (Bao *et al.*, 2009) and 8 genes (gloverin-1, gloverin-2, gloverin-3, gloverin-4, hsp19.5, hsp70, hsp90 and HOP) expressed in fat body and haemocyte (Bao *et al.*, 2010) of the resistant strain were induced significantly by BmNPV. Further, it was observed that some genes were expressed at the initial stages of proliferation while others in the middle and late stages (Huh and Weaver, 1990 a, b; Hoopes and Rohrmann, 1991). Detailed studies identified a few proteins such as *IE-1*, a transregulator that activates a number of late expression factors, *LEF-1*, a DNA primase of the replication complex and *LEF-3* a single stranded DNA binding protein that are useful for controlling the proliferation of the virus inside the worm (Mikhailov and Rohrmann, 2002; Evans *et al.*, 1997). Accordingly, efforts were made to incorporate dsRNA-encoding genes of *LEF-1* or *IE-1* in the silkworm. However, it provided only short living moderate effects of RNA interference (Isobe *et al.*, 2004; Kanginakudru *et al.*, 2007) indicating the involvement of a number of factors in deciding resistance to the virus.

Later, attempts were made to enhance the strength and the duration of the interfering effect by incorporating a construct composed of several viral genes (Gandhe *et al.*, 2007; Mrinal and Nagaraju, 2010; Terenius *et al.*, 2011). These genes viz., *IE-1*, *LEF-1*, *LEF-3* and *P74*, were placed in the control of the silkworm cytoplasmic actin A3 gene promoter (Zhang *et al.*, 2014). As a reporter gene,

green fluorescent protein (gfp) was introduced along with the construct. The whole construct composed of sense, antisense and inverted repeat vectors in a piggyBac vector backbone was introduced into the silkworm through germline transgenesis using a helper plasmid pHA3PIG. In order to test the efficacy of the gene insert, both transgenic and control silkworms were infected with BmNPV and the viral accumulation was assessed on 4th day of the V instar larvae. It was found that 75 % mortality occurred in the control while only 20 % mortality was reported in the transgenic silkworms (Subbaiah *et al.*, 2013). The less viral load in the transgenic silkworm was also confirmed through qPCR and Western blot analyses. This study has demonstrated conclusively that blocking the expression of genes crucial to the accomplishment of the viral cycle through RNA interference is an efficient method to control deadly diseases in silkworm to enhance the productivity (Tan and Yin, 2004; Muller *et al.*, 2010). It has also shown that the construct incorporated into the silkworm breed "Nistari" can be transferred to high yielding breeds viz., CSR2 and CSR4 through conventional breeding methods with the help of selective markers, opening new areas of research to develop disease resistant silkworms suitable to rear in tropical humid regions. Earlier, inhibition of the proliferation of the virus *Autographa californica* NPV in *Trichoplusia ni* larvae through delivery of dsRNAs of *GP-64*, *IE-1* and *P74* was demonstrated (Valdes *et al.*, 2003; Haas-Stapleton *et al.*, 2004). These studies clearly showcased the potential of RNAi in controlling the viral diseases in silkworms.

Further, RNA interference was used to investigate the functional role of a number of genes in silkworms. For instance, RNAi mediated down-regulation of cathepsin D of *Bombyx mori* (*BmCatD*) was found to have significant effect on the larval-pupal transformation (Gui *et al.*, 2006). Similarly, RNAi-mediated silencing of BmSoxE expression suppressed cell proliferation in BmN4-SID1 cells due to cell cycle arrest in G1 phase (Wei *et al.*, 2014), silencing of bursicon genes affected wing expansion in adults silkworm (Huang *et al.*, 2007) and RNAi down regulated expression of *Ap-ApoLp-III* gene in *Antheraea pernyi* (Liu *et al.*, 2015). These studies have explicitly

proved the worth of RNAi in elucidating the functional roles of key enzymes and proteins in silkworms.

Limitations of RNAi technology: Although RNAi has remarkable potential to be used to control gene expression, its use does have limitations. One of the major limitations is the suppression of RNAi as siRNA-based RNAi is an antiviral defence mechanism, hence many plant viruses encode proteins that suppress silencing (Burgyn, 2008; Alvarado and Scholthof, 2009). Likewise, since post-transcriptional silencing in plants is mobile and it spreads throughout the plant, it might not be suitable for those applications that require tissue-specific silencing of genes. Prediction of potential off-target effects is very difficult as there are only a few nucleotides of sequence complementarity between a siRNA and mRNA (Escobar *et al.*, 2001). Further, the vast genetic variation that exists within and among insect populations may throw new challenges for pest control, depending on the amount of mismatch present between the dsRNA and the target transcript. RNAi inhibition can have widely varying effects depending on the target gene, the region of the transcript that is targeted and even between sibling plants carrying identical RNAi constructs (Wang *et al.*, 2005). Lastly, instability can also result from silencing of the transgene as long hairpin transgenes appear to be particularly sensitive to transcriptional silencing leading to a loss of RNAi phenotypes over several generations.

CONCLUSION

Thus, it is clear that RNAi technology has emerged recently as one of the most potential technology to both investigate and control gene functions in organisms where RNAi mechanism is present. Recent advances have shown that RNAi-mediated resistance in crops would be one of the best choices to avoid excess use of pesticides and fungicides. However, one should always keep in mind that RNAi is no panacea for all the problems, but provides a range of new conceptual and technological solutions for the challenges posed by organisms *viz.*, viruses, insects and pests. In sericulture, RNAi has not made much advances, though the RNAi based virus resistant silkworm is all set to go to field testing. More and

more efforts have to be made to employ this highly potential tool in sericulture especially in the field of host plant improvement as most of the host plants of silkworms are highly heterozygous and recalcitrant to conventional way of manipulations. Since, most of the breeding plans end with development of F1 hybrids and their subsequent selections, introgression of genes from wild relatives and weeds cannot be done without compromising the better quality of the recipient parent. Here, RNAi can do a lot either by way of silencing unwanted genes of the host plants or their attackers.

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L'INTERFERENCE D'ARN- UN OUTIL POTENTIEL POUR L'AMELIORATION DES RECOLTES EN SERICICULTURE

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RÉSUMÉ

L'ARN interférence est le processus par lequel les organismes dégradent les ARNm d'un gène avant qu'il ne soit traduit en protéine. Le phénomène a été découvert chez le pétunia en 1990, lorsqu'un pétunia transgénique contenant le gène de la chalcone synthase (*chsA*) sous le contrôle d'un promoteur 35S semblait avoir perdu aussi bien le transgène que le gène endogène pour produire des fleurs avec des parties blanches. Ceci est très différent du mécanisme anti-sens où seulement quelques molécules d'ARNdb sont suffisantes pour abolir l'expression de tout l'ARNm endogène. Dans les mécanismes simples d'ARNi, les intermédiaires ARNdb sont convertis en duplexes de 21-24 nucléotides par l'enzyme Dicer, puis ils sont ensuite intégrés dans un complexe d'extinction ARN (RISC). Un brin du duplexe ARNsi, connu comme brin guide agit comme organisateur de la dégradation des ARNm complémentaires. La technique de mutisme des gènes par ARNi est aujourd'hui utilisée pour induire la résistance à de nombreuses maladies virales, améliorant la qualité des fruits, changeant la couleur des fleurs *etc.* D'intenses recherches sont menées sur l'extinction ARN desquelles on espère de nouvelles découvertes bénéfiques en agriculture et en médecine. Chez le ver à soie l'ARNi a déjà permis d'introduire une résistance virale à travers l'extinction de gènes qui facilitent l'entrée et la multiplication chez le ver à soie. Alors que la plupart des plantes hôtes des vers à soie sont des arbres présentant une forte hétérozygotie et qui sont réfractaires à beaucoup d'outils biotechnologiques modernes largement utilisés, l'application d'ARNi pour rendre silencieux des gènes spécifiques via la transgénèse offre un outil très prometteur pour améliorer certains caractères, particulièrement ceux de la résistance aux stress biotique et abiotique, de la palatabilité pour le ver à soie, de l'efficacité de propagation *etc.* Notre revue met en lumière les tentatives dans cette direction et souligne la signification de l'extinction de gènes pour l'amélioration des récoltes de vers à soie et de leurs plantes hôtes ainsi que les étapes majeures de l'extinction de gène.

Mots-clés: Extinction de gène, mûrier, ARNi, ver à soie.



THE FRONTIERS IN SILKWORM REPRODUCTION OFFERING HOPE FOR QUALITY SEED PRODUCTION – A REVIEW

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ABSTRACT

Production of good quality silkworm seed is a pre-requisite for stabilizing the silkworm crops to produce superior quality cocoons. Silkworm reproduction which involves two major physiological events, oogenesis and spermatogenesis has to be well understood by seed producers for successful operation of seed production activities. This review is done in this backdrop focusing on the main physiological and developmental events that fall under the reproductive process, such as egg maturation, sperm production and maturation, sperm hatching in achieving fertilization and the functional aspects of different genital organs.

Key words: Fertilization, oogenesis, silkworm reproduction, spermatogenesis, sperm dimorphism, sperm motility.

INTRODUCTION

It is intended to initiate this topic with two exalted and exciting quotations on reproduction.

- 1) "Reproduction is a luxury function of the body not physiologically necessary for life of the individual and usually not performed until the animal reaches nearly adult size and nutritive supplies are ample for both dam and the fetus".

Robberts, 1971

- 2) Ganguli (1981) inclined to delineate Reproduction as follows: "Reproduction is a unique phenomenon which recognizes the living from the non-living. This physiological event is endowed with myriad functions like carrying the genetic message, ensuring the appearance of new generation, bestowing the inheritance to the offspring and proliferation as the living species. Above all, reproduction is a sine qua non for the living organisms and more so to the mankind as an endowment of inheritance".

If one closely examines the 'scene' of this reproductive function, one would observe that most of these events are discharged by the female counterpart. One can therefore see how fortunate is the male component whose dialogue

is over just after the deposition of a 'good' sperm in the female.

The major athletes

In the game of reproduction which calls for the participation of male and female, the two major athletes are the sperm and ovum. The purpose of the present paper is to present the current developments in this arena of physiology of reproduction in the silkworm, *Bombyx mori* L. The prelude on the major physiological events in the reproduction *i.e.*, oogenesis and spermatogenesis is very pertinent along with a brief account on both male and female reproductive systems.

Growth and development of ovary and testes

The oogenesis and spermatogenesis are physiological processes in silkworm which eventually produce ovum and sperm, respectively. In the larva, the sexual phase begins in the late instars when it is possible to see a pair of triangular ovaries (gonads) in the female and kidney shaped testes in the male situated on the dorsolateral side of the 5th abdominal segment. It is difficult to discriminate between ovary and testes in the newly hatched larvae. In 2 days old

pupa, ovarioles break open from the ovarian capsule and project into the body cavity. There are 4 ovarian tubes on each side of the ovary. From three days after pupation till eclosion/emergence, the ovarioles show a drastic growth in length and volume by the occupation of matured eggs, about 80 in number in each ovariole.

Oogenesis

The ovary is divided into four compartments in the younger stage of the larva. In the course of time, they elongate and assume a tubular shape and are called the ovarioles. At the anterior end of each ovariole, the oogonia are formed. The oogonia increase in number by direct division. There are about 400 oogonial cells at the anterior end of each side of the ovary. The oogonia differentiate into oocytes in the course of development. One oocyte and 7 nurse cells together with some 5,000 follicle cells aggregate into one group called as ovarian follicle. The oocyte continues to grow and later becomes the egg, while nurse cells degenerate completely.

Critical stages to monitor for egg production

During the life cycle of silkworm, certain critical stages have to be keenly observed and taken care for the production of superior quality seed. They are:

- 1) During spinning/cocooning stage, the abiotic factors should be maintained at optimum *i.e.*, 23-25 °C temperature, 65-75 % relative humidity and proper aeration (1m/sec) which can improve quality of cocoon and in turn egg production.
- 2) After mounting of larvae for cocooning up to moth emergence, it exhibits a 'U' pattern of oxygen consumption wherein on 6/7th day, it consumes the least O₂ and is the most suitable period for cocoon transportation. During the beginning stage of cocooning and the preceding stage of moth emergence, it demands high O₂ levels for histolysis and histogenesis, respectively. This explains why early and late cocoon transportation during this developmental period affects the health of pupa and in turn egg production. Hence, cocoons should be transported invariably on 6/7th day so as to avoid

pupal mortality

- 3) Prior to emergence: The adult is formed beneath the pupal skin which is covered with moulting fluid. This is a very vulnerable time and if the prevailing RH is low, it affects both moth emergence and also egg maturation (oogenesis) followed by fecundity due to body water loss. This underlines the need for maintaining 70-75 % RH invariably during this stage for easy moth emergence with high fertility rate. Further, just before choriogenesis, each oocyte deposits about 65 % water content individually during vitellogenesis. In each pupa, 500 – 600 eggs mature by the time of moth emergence. Hence, the role of adequate water content remains very crucial for this phase of development.

Spermatogenesis

Spermatogenesis is a physiological event which starts from the day of larval hatching and continues to almost till the end of moth's life. Results of autoradiographic studies of spermatogenesis using tritiated thymidine helped in the estimation of approximate duration of each stage of spermatogenesis (Sado, 1965). Three days after hatching, germ cells of larvae begin to differentiate into spermatogonial cells in the testes. In subsequent instars, differentiation of spermatogonia into primary spermatocytes and secondary spermatocytes occurs. During mid 5th instar (within a day after metaphase-I) phase, the secondary spermatocytes and spermatids appear in the testes. One day prior to spinning stage, appearance of spermatozoa are noticed. By the 9th day after pupation, no. of spermatozoa seem to increase continuously. The total duration of an entire sequence of spermatogenesis is about 26 days as following:

Spermatogenesis

Spermatogenic stage	Duration
Primary spermatogonia	34 h
Secondary spermatogonia (sum of 6 synchronous divisions)	5-6 days
Spermatocytes	
I) Resting stage	< 1 day
II) Meiotic prophase – I	9 days
III) Metaphase-I to Anaphase – II	<1 day
Spermatid	9 days
Spermatozoa (stay within the testis)	1-14 days

(Source: Sado, 1965)

Sperm dimorphism

The silkworm is a lepidopteran insect which produces dimorphic sperms, eupyrene (nucleate) and apyrene (anucleate). The eupyrene sperms develop from the spermatocytes during the fifth larval instar and apyrene sperms develop after pupation (Machida, 1929). Immediately after moth emergence, some of the eupyrene sperm bundles (ESB) and considerable no. of apyrene sperm bundles (ASB) were noticed in the testes. The males have to be allowed for some time to dry up the body after emergence before they are used either for mating or cold storage at 7-10 °C to exhibit normal potency. Katsuno (1987) also reported the origin of the eupyrene sperms (ES) and apyrene sperms (AS) as a result of spermatogenesis through histological observations of testes and cytological observations of germ cells.

The time of differentiation and determination of both types of spermatozoa occurred between the stage of the spermatogonia and leptotene stage of the spermatocytes (Katsuno, 1987). In the silkworm, formations of nucleated eupyrene spermatozoa and anucleated apyrene spermatozoa do not occur simultaneously in the testes (Katsuno, 1977 a). Control of dimorphic spermatogenesis is due to commitment of spermatogenesis for the production of apyrene sperm consequent to the reduction in juvenile hormone (JH) titre, immediately after pupation. In silkworm, ES and AS were first observed by Toyama (1894), with subsequent confirmation by several investigators. Spermatocytes which perform meiotic division during the larval period develop into ES, but those that divide during the late pupal period transform into AS (Machida, 1929). Iriki (1941) and Katsuno (1977 a, c, d) observed the behaviour of both types of spermatozoa in the internal reproductive organs of the adult male and female that the AS played an important role for the ES in releasing from ESB and transportation up to spermatheca from bursa copulatrix (BC).

Katsuno (1987) reported that high temperature injury to meiotic spermatocytes leads to development of apyrene sperms due to abnormal meiotic division, i.e., non-reduction of chromosomes from $2n$ to n . Hence, it is anucleated sperm. The beginning of abnormal meiotic division is observed on 3rd day after spinning in a small

number of spermatocytes and this attains a maximum on the 3rd and 4th day after pupation. The AS exist even in high temperature exposed pupae, but they cannot fertilize eggs. Subsequently, sterility incidence occurs in males. Sugai and Kiguchi (1967) have reported that when young pupae of the male silkworm were treated with high temperature (32°C), male sterility was induced and the apyrene spermatogenesis was markedly impaired. Induced male sterility seems to be caused by high temperature injury occurring at the time of meiotic division of the spermatocytes which ultimately transform to AS (Katsuno, 1987).

Insemination: The ESB and AS are placed in the bursacopulatrix of the female through an opening which is independent of the female genital tract. The sperm leave the spermatophore and reach the seminal receptacle (spermatheca) via the ductus seminis, vestibulum and ductus tortuosus. In the bursa, the ESB and apyrene sperms inter-mingle (Friedlander and Gitay, 1972).

The detailed process of insemination is interesting which begins with the starting of mating. Within 30 minutes of mating, the ejaculation process transports ESB, AS, seminal fluid and secretions of male accessory glands which is collectively called as semen into BC.

Sperm maturation: In all animals, the physiological phenomena which are accompanied by various metabolic activities often correspond with the morphological changes in the structures of cells and tissues. A typical case can be seen in the male reproductive system of the silkworm wherein, immature spermatozoa in males transform into motile spermatozoa just prior to reaching the fertilization site in the female system. Sperm after its birth in the testes, gets transported through the male tract, vas deferens and mixes with seminal fluid from male accessory glands and gets discharged into female system bursa copulatrix. During the mating, all the reproductive glands and their various secretions spontaneously change from a static to a dynamic state on ejaculation. These secretions collectively initiate many biochemical reactions which are essential for completion of sperm maturation. The flow of semen into bursa copulatrix of female system leads to form a "spermatophore". Osanai (1987) and Osanai *et al.* (1990) analyzed the physiological changes occurring in

spermatophore in the course of development to understand the process of sperm maturation.

In the seminal vesicle, apyrene sperms get separated, while eupyrenes exist as bundles. Spermatophore of silkworm is regarded as an important site for the maturation of spermatozoa. Sperm maturation in the spermatophore involves the acquisition of motility of apyrene spermatozoa, the dissociation of eupyrene bundles for separation of eupyrene spermatozoa and acquisition of their motility (Kasuga and Osanai, 1984; Osanai *et al.*, 1987 c).

Biochemical changes during sperm maturation

The spermatophore of the silkworm is a metabolic reactor, in which the various processes necessary for sperm maturation occur. Glycogen is converted to pyruvate by the action of phosphorylase. Protein is degraded by proteases (Aigaki *et al.*, 1985) and urea & alanine accumulate (Osanai *et al.*, 1985, 1986). The others include, a specific pathway coupled with glycolysis for conversion of arginine to 2 – oxoglutarate (Osanai *et al.*, 1985, 1986) which acts as a preferred substrate for spermatozoa and as a regulator of their respiration (Osanai *et al.*, 1985, 1987 a, b) and also a specific arginine supply system composed of an endopeptidase (Aigaki *et al.*, 1987) that cleaves proteins and exopeptidases (Kasuga *et al.*, 1987). The secretions of the various glands of male reproductive system have been found to contain the following components necessary for eupyrene maturation separately. The secretion of the glandula lacteola contains glycogen (Kasuga *et al.*, 1985 a), the seminal fluid in the seminal vesicle contains arginase (Aigaki *et al.*, 1985) which determines the irreversible reaction series of the 2 – oxoglutarate producing pathway and exopeptidase and glycogen granules (Kasuga *et al.*, 1985 b). Proline and succinate were effective substrates for the respiration of silkworm spermatozoa (Aigaki and Osanai, 1985).

Stimuli such as presence of spermatozoa in the BC or spermatheca did not elicit oviposition in *Bombyx mori*. Vestibulum is the organ sensing the oviposition activating stimulus by the presence of matured eupyrene spermatozoa (Karube and Kobayashi, 1999). Eupyrene sperms are released from bundles in the spermatophore

and acquire slight motility and then enter into the spermatheca. Here, they mature and acquire active motility and fertility after which the mature eupyrene spermatozoa enter the vestibulum (Miya, 1984; Osanai *et al.*, 1987 a, b; Sugai, 1991). Hence, the maturation of eupyrene sperm which occurs in female tract is a critical step for acceleration of egg laying. The quantity of spermatozoa may affect the acceleration of oviposition. Sperm count / mating ranged from 82,000 – 135,000. Minimum count was recorded in the batch exposed to high temperature for 96 h during cocooning resulting in significant decrease in egg laying when crossed with males having low sperm count. To obtain normal egg laying, the sperm count per mating must be at least 120,000 – 135,000 (Vemananda Reddy and Venkatachalapathy, 2003-2004).

Fugo and Arisawa (1992) confirmed that the mechanical stimulus by mating with sterile males and oviposition stimulating substance in semen (OSS) exerted a limited effect on oviposition. Hence, the candidate for the oviposition stimulation factor is the active eupyrene spermatozoa in the vestibulum. Apyrene spermatozoa do not activate oviposition (Sugai and Sugita, 1976).

The function of apyrene sperm is transportation of eupyrene sperm through the female genital duct (Riemann, 1970; Kinefuchi, 1978). The eusperms are immotile until activated by alkaline phosphatase in the female bursa copulatrix. Whereas, the apyrene sperms are active in the semen (Kinefuchi, 1978). Moreover, oviposition does not occur actively, until a large number of the parasperms (apyrene sperms) have undergone the degradation and it is deduced that the apyrenes play a beneficial role in assisting fertilization by eusperm, presumably by providing nourishment or other physiological stimulation (Katsuno, 1977 a, b).

Sperm hatching: Generally, it is well known that the eggs hatch by 10 days of incubation. In *Bombyx mori*, the eupyrene sperm sheath is discarded before fertilization, is a process termed “hatching” of eupyrene sperm. The hatching of sperm takes place while the sperms pass from the ductus seminalis to the seminal receptacle to release free sperms (Riemann and Thorson, 1971) and only naked eupyrene sperm can enter the canaliculus to reach the vestibulum (Friedlander and Gitay, 1972; Gaber and

Danilova, 1979).

Apyrene sperm in the bursa copulatrix, ductus seminalis and spermatheca resemble those in the ejaculatory duct and unlike the eusperm, do not “hatch” from their sheaths, apyrene do not enter the canaliculus fecundans or vestibulum where the eggs are fertilized in *Bombyx mori* (Friedlander and Gitay, 1972). There is an amazing synchronization in the sperm maturation processes in the female system to achieve the sole goal of fertilization of ovum/egg. To achieve this goal, apyrenes sacrifice themselves for the sake of eupyrenes. However, unless the sperms hatch, there may not happen any fertilization of the eggs.

Sterility in silkworm (Infertility)

Male sterility: In *Bombyx mori* silkworms, incidence of male sterility occurs due to high temperature mainly during spinning, pre-pupal and pupal stages (Sugai and Kiguchi, 1967; Sugai and Takahashi, 1981). The reasons for male sterility are: failure of reduction division to form haploid (n) sperms (Katsuno, 1987) and change in the ratio of eupyrene sperms and apyrenes from 1:1.84 to 1:0.85. Actually, high temperature reduces eupyrene to 67.2% of that in 25°C (100%) and the apyrene sperms to 31.2% indicating that apyrene sperm production gets affected maximum (Osanai *et al.*, 1989 b). Even starvation (or) malnutrition at crucial stages cause formation of degenerative sperm bundles resulting in reduction in sperm count (Sugai, 1966).

Studies conducted on the occurrence of sterility in silkworms due to high temperature (33°C) during cocooning, revealed that the female of multivoltine also showed sterility by laying unfertilized eggs (>50 %) even after mating with normal males (Vemananda Reddy *et al.*, 2003).

Sterility in females: The mating experiments proved that the sterility was due to failure of oviposition of the females (Suzuki, 1951). On examining the genital system, it was clarified that the vestibulum of the vagina was distinctly constricted and other sexual organs were normal and ovarian tubes were filled with eggs. The failure of egg laying was due to vaginal constriction. Female sterility

was also noticed in cases wherein the egg changed to horizontal position in ovarian tubes and not able to descend down during oviposition (Vemananda Reddy *et al.*, 1992).

Motility of sperm: For isolated eupyrene sperm bundles and apyrene sperms, addition of the prostatic secretion containing initiatorin (Osanai *et al.*, 1987 b; 1988) or trypsin to the seminal fluid causes activation of apyrene spermatozoa *in vitro* (Osanai *et al.*, 1987 c; 1989 a). When oxoglutarate which is a preferred substrate for energy supply to the silkworm sperm (Osanai *et al.*, 1986, 1987 a), was also added, the apyrene spermatozoa showed enhanced motility. Acquisition of vigorous motility *i.e.*, capacitation of eupyrene spermatozoa occurs after their decapsulation (hatching) in the spermatheca (Friedlander and Gitay, 1972; Miya, 1982). The sperm motility is very much dependant on the chemical quality of the seminal fluid. Future prosperity of sperms depend on both temperature and nutritive value of semen secreted from seminal vesicle, different male accessory glands and active secretions from the female genital system (spermathecal gland). The spermatozoa while in female reproductive system have to perform several biological assignments with a view to achieve fertilization with the ovum / egg. The major events which the sperm has to undergo/perform are capacitation and fertilization of the egg.

In recent years, regulation of reproduction has attained a pivotal role in the control and improvement of the mankind. Although reproduction is a well known event, for the living species, the mechanism involved in this complex operation is hitherto relatively unknown to a common man. Lack of knowledge in this regard is responsible for the inadequacies in the production of high quality seed in all egg production units of silk industry. The information on the sperm contents and its reaction with the various components of semen is limited.

In the female, seminal fluid from the seminal vesicle and secretions from exocrine glands of the male reproductive system become enclosed in a spermatophore formed inside the bursa copulatrix (Omura, 1938; Mann, 1984). In the silkworm, eupyrene spermatozoa mature in spermatophore (Kasuga *et al.*, 1985), apyrene spermatozoa acquire motility (Shepherd, 1974; Katsuno, 1977 c) and then the matrix of eupyrene sperm bundle disappears and

the individual eupyrene spermatozoa migrate into the receptaculum siminis (spermatheca) (Katsuno, 1977 a, b, c). Active apyrenes have been shown to be necessary for separation of eupyrenes from their bundles (Katsuno, 1977 a, b, c, d).

In the seminal vesicle, the individual apyrene spermatozoa are immotile, while eupyrene bundles contain immotile spermatozoa surrounded by a slightly electron – dense matrix. After transfer by ejaculation to the BC, the apyrene spermatozoa in spermatophore are activated by the prostatic fluid and begin to move, then both the envelope and matrix of the eupyrene cyst/bundle soon disappear. Initially, apyrene sperms must be activated in the spermatophore to stir the contents and promote dissociation of eupyrene bundles and separation of individual eupyrene sperms both mechanically and by biochemical reactions (Osanai *et al.*, 1987 c, d). Actually, mixture of semen in the spermatophore offers metabolic security to the spermatozoa in the new environment. There were more apyrene spermatozoa noticed in the seminal fluid than eupyrene spermatozoa. They constitute over 65% of the total spermatozoa. Both the enzymes and substrates remain intact before ejaculation, but react together after ejaculation resulting in sperm maturation and motility (Osanai *et al.*, 1987 b, c, d; 1989 a). The eupyrene bundles dissociate progressively and almost complete the process in 80 min post mating. Then the eupyrene spermatozoa also acquire motility (Osanai *et al.*, 1987 c). In each testis, there are 3000 ESB and 256 individual eupyrene sperms in each ESB. Hence, the total sperm count per male ($256 \times 3000 \times 2$) is about 15,00,000. The weight of BC increased with spermatophore enlargement and reached a constant level on completion of spermatophore formation *i.e.*, 20 to 30 min after the beginning of copulation. At this time, the weight of BC including the spermatophore was six times that of before mating. The spermatophore weighed 8.4 mg which was 86 % the maximal weight of the BC (Osanai *et al.*, 1986).

Mechanism of oviposition

The female *Bombyx mori* senses mechanical stimulation and chemical stimulation at mating, which sets into play a series of complex behaviours that stimulate oviposition. Karube and Kobayashi (1999) reported that

the presence of matured spermatozoa in the vestibulum stimulates oviposition and neither mechanical stimulation nor a chemical factor is responsible for the induction of oviposition. Males provide a mechanical stimulation during mating as follows: the physical act of copulation, insertion of penis and the stimulus of internal reproductive organs accompanied with semen progression. None of these features stimulate oviposition. Pr-males (penis removed) caused only the physical act of copulation and failed to stimulate oviposition. Insertion of penis and distention of the bursa copulatrix did not accelerate egg laying because neither tr-males (testes removed) nor hss – males (heat shock sterilized) elicit oviposition. Karube and Kobayashi (1999) and Yamaoka and Hirao (1977) suggested that an oviposition stimulating substance (OSS) present in the male genital system (semen) was transferred to the female, diffused into haemocoel and then activated oviposition. If so, females with a spermatophore or spermatheca filled with semen would be induced to oviposit. However, results of experimental transplantation of the BC and ligation of spermathecal duct showed semen in the spermatophore and spermatheca did not elicit oviposition (Karube and Kobayashi, 1999). Thus, the above results and concepts disagree with those of Yamaoka and Hirao (1977).

Later, a new model was proposed for the mechanism of acceleration of egg laying (Karube and Kobayashi, 1999) that a new substance is synthesized in the females through an unknown metabolic pathway and functions as an oviposition stimulating factor. However, this possibility was ruled out because, the injection of semen derived from the spermatophore and spermatheca into the oviduct showed no effect, even when females had normal eggs. Alternatively, the candidate for the oviposition stimulation factor is the spermatozoa. Apyrene spermatozoa did not activate oviposition (Sugai and Sugita, 1976). After conducting different studies involving mating with hss – males, transplantation and removal surgeries, it became clear that the presence of normal eupyrene spermatozoa in the BC did not elicit oviposition. Sasaki and Riddiford (1984) reported that the presence of sperm or testicular fluids in the BC contributed to a change from the virgin to mated state. Studies indicated that the vestibulum would be the organ sensing the oviposition activating stimulus in the presence of matured eupyrene spermatozoa

which move from the spermatheca into vestibulum.

An endocrinological mechanism involved is responsible for the initiation and activation of oviposition in mated female moths in which the male factor, OSS present in the semen which has to cross the female genital organ into haemocoel to stimulate neuro-secretory cells present in the brain and / or eusperms investibulum stimulate central nervous system (CNS) for induction of ovulation and oviposition (Yamaoka and Hirao, 1977).

But how females recognize the presence of eupyrene spermatozoa in the vestibulum and the neural pathway to CNS used for the acceleration of egg laying remains to be resolved. Also, the maturation mechanism of spermatozoa is a subject needed for understanding the mechanism of oviposition stimulation.

A better understanding on the diverse events in the process of reproduction in male and female silkworm *Bombyx mori* lays the foundation for the production of good quality silkworm seed. The major phases as described in this comprehensive account play a crucial role and are highly sensitive to the prevailing temperature and humidity conditions and hence also emphasize the inevitable need of maintenance of optimum ambience in the silkworm seed production centers to raise high quality seed.

ACKNOWLEDGEMENT

Authors express their gratitude to the Scientist D & Head, Silkworm Seed Technology Laboratory, Bengaluru, for extending co-operation in preparation of the manuscript. Thanks are also due to Mrs. Jayammal, for the help rendered in preparation of manuscript for publication.

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Revue

A LA POINTE DE LA REPRODUCTION DU VER A SOIE DANS L'ESPOIR D'UNE PRODUCTION DE GRAINES DE QUALITE - REVUE

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R É S U M É

La production d'une graine de ver à soie de qualité est un pré-requis pour stabiliser les récoltes et obtenir des cocons de qualité. La reproduction des vers à soie qui nécessite deux événements physiologiques majeurs que sont l'ovogénèse et la spermatogénèse nécessite d'être bien comprise par les producteurs de graines pour des activités de grainage fructueuses. Cette revue a été réalisée en se concentrant sur les événements physiologiques et développementaux principaux comprenant les processus de reproduction tels que la maturation des œufs, la production de sperme, le dimorphisme des spermatozoïdes, la stérilité, la motilité et la maturation du sperme, le rôle du liquide séminal dans l'accélération des spermatozoïdes et la réalisation de la fécondation ainsi que les aspects fonctionnels des différents organes génitaux.

Mots-clés: Fécondation, ovogénèse, reproduction du ver à soie, spermatogénèse, dimorphisme spermatique, mobilité des spermatozoïdes.



DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISM (SNP) MARKERS LINKED TO DIGESTIVE AMYLASE GENES IN THE MULBERRY SILKWORM, *BOMBYX MORI* L.

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ABSTRACT

Using the productive bivoltine silkworm strains with low activity digestive amylase gene (Amy dⁿ allele) as recurrent parents (RPs), near isogenic lines (NILs) have been developed by introgressing high activity amylase genes (Amy d^{iv} or Amy d^v alleles) from the indigenous polyvoltine donor parents (DPs), which have shown significant improvement in the survival of the NILs. To know whether the amylase gene itself is responsible for improved viability or other genes flanking the amylase locus are involved, DNA profiling of the DPs, RPs and NILs was carried out using five SNP markers of 8th linkage group having the Amy d locus. Polymorphic PCR products specific to Amy d^{iv}, Amy d^v and Amy dⁿ alleles have been detected. The results have shown the prospects of characterising the chromosome segments conferring viability traits.

Key words: Digestive amylase, near isogenic lines, silkworm, SNPs.

INTRODUCTION

The indigenous polyvoltine silkworm strains are reared throughout the year in the tropical climate of India, which are characterized by low cocoon and post-cocoon traits and poor silk quality but with high survival potential. On the contrary, the exotic bivoltine silkworm strains of temperate origin of mostly Japan and China, are endowed with high yield and superior silk quality, but their viability is poor under the tropical environment. In view of superior quantitative traits, rearing of these bivoltine strains needs more care and attention, as they are susceptible to the high germload and unfavourable climatic conditions of tropics leading to crop failures and low yield. To identify the biochemical markers linked to robustness and disease resistance, a number of enzymes in the haemolymph and digestive juice were screened and their correlation with yield and survival was analysed among 54 silkworm strains of diverse origin (Chatterjee *et al.*, 1992, 1993), which showed that digestive amylase has

a close association with survival. Previously, Hara *et al.* (1984, 1986) reported that digestive amylase gene is located on the eighth linkage group with three multiple alleles, namely, Amy d^v (with five active bands), Amy d^{iv} (with four bands) and Amy dⁿ (null type) due to lack of cathodal isozymes. The silkworm strains with Amy d^v and d^{iv} alleles had many fold higher activity than the Amy dⁿ strains. Studies on Indian silkworm strains has also shown that the indigenous polyvoltine strains are carrying the high activity alleles of either Amy d^v and d^{iv}, while the exotic bivoltine strains are of Amy dⁿ type with negligible amylase activity (Patnaik and Datta, 1995). Abraham *et al.* (1992) have analysed the starch content in the fecal matter and reported that low amylase activity strains excreted five fold more starch than the high activity strains. These studies clearly showed the prospects of using digestive amylase as a marker for improving the viability in silkworm as it contributes for better digestibility and has close association with the survival (Datta and Ashwath, 2000). To confirm these findings, a breeding scheme was

designed and high activity amylase genes from the indigenous low yielding polyvoltine donor parents (DP) were introgressed into the productive bivoltine strains used as recurrent parents (RP) and near isogenic lines (NIL) of RPs were developed. These NILs showed significant improvement in their survival without any deterioration in yield traits (Ashwath *et al.*, 2001). Large scale field testing of hybrid of the NILs has clearly shown its consistent performance both during favourable and unfavourable seasons (Sharmila *et al.*, 2010) indicating its suitability for small and marginal farmers who are presently rearing cross breeds of indigenous and exotic strains. Recently, through DNA profiling of RPs, DPs and NILs, linkage of six RAPD markers with digestive amylase genes in the silkworm has been established (Ashwath *et al.*, 2010).

In the light of the above, a new breeding programme was initiated using the productive strains, namely, CSR3 (oval cocoons) and CSR6 (dumb-bell cocoons) which were authorised in 1999 for commercial use in India. These strains are characterised by "null" type of amylase with low digestive amylase activity. To improve their digestibility and survival, high activity amylase genes from the polyvoltine donors, namely, Pure Mysore, Nistari, Cnichi and Daizo were introgressed by backcross breeding employing amylase marker assisted selection. As the evolved NILs have shown higher survival than the RPs, it is believed that the amylase gene itself may confer higher survival by improving digestibility or some other closely linked genes flanking the amylase locus may be responsible for better viability of the NILs. Against this background, SNP profiles among the DPs, RPs and NILs were analysed with the objective of identification of DNA markers linked to and bordering the amylase locus paving the way for further characterization of chromosome segments conferring higher survival in the evolved lines.

MATERIALS AND METHODS

Silkworm strains

Two productive bivoltine strains, *viz.*, CSR3 (oval) and CSR6 (dumb-bell) both with 'null' type of amylase (Amy d⁺ allele) with very low amylase activity were used as recurrent parents (RPs). Three low yielding indigenous

polyvoltines, namely, Pure Mysore (PM), Nistari and Cnichi and one low yielding hibernating strain, Daizo with high amylase activity genes were taken as Donor Parents (DPs). PM, Cnichi and Daizo are having '4 band' type of digestive amylase (Amy d⁺ allele), while Nistari has '5 band' amylase pattern (Amy d⁺ allele). In addition, bivoltine strains previously used as RPs (Ashwath *et al.*, 2010) for developing NILs, namely CSR2, CSR5 and their NILs, GEN1 and GEN2 were used for the analysis.

Development of Near Isogenic Lines

For improving the survival potential of the RPs with low amylase activity, the DPs (PM, Nistari, Cnichi, Daizo) with high amylase activity were crossed with the RPs (CSR3, CSR6) and F1 progeny was raised. The F1s were backcrossed to their respective RPs and BC1 progeny was raised. BC1 generation onwards, the progenies were individually screened for amylase and the larvae with 'null' type were rejected and BC1 larvae with DP type of amylase gene were selected and backcrossed to the RPs to raise BC2. The procedure was repeated up to BC6. BC6 larvae with DP type of amylase gene were inbred and BC6S1 was raised. BC6S1 progeny was screened by amylase assay and those with DP type were selected and BC6S2 progeny was raised. As we cannot distinguish the homozygous and heterozygous progeny for '4 band' or '5 band' amylase types, the BC6S2 progeny was testcrossed with the 'null' type to identify the homozygous families. The resultant lines are called as Near Isogenic lines (NILs), which are nearly isogenic to the RPs for all the productivity traits, while they have been introgressed with the high activity amylase genes of the DPs. Thus, four oval NILs of CSR3, namely 3P (PM-CSR3), 3N (Nistari-CSR3), 3C (Cnichi-CSR3) and 3D (Daizo-CSR3) and four dumb-bell NILs of CSR6, *viz.*, 6P (PM-CSR6), 6N (Nistari-CSR6), 6C (Cnichi-CSR6) and 6D (Daizo-CSR6) were evolved.

Quantitative and qualitative assay for amylase

Digestive juice samples from the DPs, RPs and NILs were collected on the IV day of V instar by brief exposure of larva to chloroform vapours resulting in instantaneous vomiting. Amylase activity was assayed following the method of Chatterjee *et al.* (1993) by incubating 10 µl of digestive juice in 0.2 % starch as the substrate for 30 min at

37°C and the reaction was stopped using 1 % dinitrosalicylic acid followed by heating in boiling water for 5 min. Amylase assay was carried out in 5 replications for each silkworm strain. Optical Density (OD) values were recorded at 525 nm and the enzyme activity was expressed as mg of maltose released per ml for 30 min. Mean and Standard Deviations were estimated for the 5 replications. For qualitative analysis, vertical Polyacrylamide gel electrophoresis (PAGE) was carried out adopting the procedure of Hara *et al.* (1986) with minor modifications and cathodic fractions were separated by reversing the polarity of the electrodes. After the run for 6-7 hours, the gels were incubated in 1 % starch solution for 30 min and subsequently stained with iodine solution for 5 min.

DNA extraction, primers and PCR

High molecular weight genomic DNA from the DPs, RPs and NILs was extracted from the whole larval body of V instar larvae using the phenol-chloroform method and purified using the standard procedure of Sambrook and Russel (2001). The purified DNA was diluted to a final concentration of 20 ng/μl.

A total of five SNP primers (forward and reverse) belonging to 8th linkage group, namely 08023, 08034, 08047, 08050 and 08058 (Yamamoto *et al.*, 2006, 2008) generously supplied by the Insect Genome Research Group, National Institute of Agrobiological Sciences, Tsukuba, Japan, were used to amplify the DNA of the parents and NILs.

PCR reaction consisted of 20 ng of template DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Fermentas Inc, Maryland, USA), 1 μM of SNP primer and 0.45 U of *Taq* DNA polymerase (Fermentas Inc, Maryland, USA) in a 10 μl volume. Thirty five cycles of PCR were performed on a PTC 200 Thermocycler Enginee (Bio Rad Laboratories, CA, USA) as follows: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min with a final extension of 72 °C for 1 min. PCR products were analyzed by electrophoresis on 2% agarose gels and stained with ethidium bromide and visualised under UV light.

RESULTS

Yield status and digestive amylase activity in the DPs, RPs and NILs

The donor parents, the recurrent parents and the evolved Near Isogenic Lines were reared under standard rearing conditions. The survival of the DPs represented by yield/10000 larvae by number (Table 1) ranged from 9257 to 9571, while in the RP, it was only 8098, while in the NILs, the values were more than 9000. The yield denoted by the weight of cocoons harvested out of 10000 larvae ranged from 8 to 9 kg in the DPs, about 12 kg in RP and 13-14 kg in the NILs. The cocoon weight was below 1 g in the DPs, 1.5 g in the RP and 1.5 to 1.6 g in the NILs. Likewise, the shell weight was below 0.13 g in the DPs, above 0.3 g in the RP and NILs. The shell % ranged from 12 to 13 in the DPs, above 22 in the RP and nearly 22 in the NILs. The filament length was below 350 m in the DPs, above 1000 m in the RP and ranged between 888 to 1062 m in the NILs.

Digestive amylase activity in the parents and NILs

The amylase activity in the DPs expressed as mg/ml ranged from 213.4 in Nistari to 271.4 in the case of Daizo (Table 1). The enzyme activity in the RP was found to be 70 mg/ml. The amylase activity among the NILs of the RP was found to be the highest in 3D (230.7) followed by 3P (200.3). In the case of 3N and 3C, the activity was marginally less than 200 mg/ml.

Isozyme profile of parents and NILs

The qualitative assay of the digestive juice samples collected on the 4th day of V instar was carried out by PAGE and the cathodic digestive amylase isozyme pattern is shown in Figure 1. The DPs *viz.*, PM, Cnichi and Daizo show '4 band' pattern (Amy d^{iv} allele), while in Nistari, it is of '5 band' type (Amy d^v allele). The RP, *i.e.*, CSR3 is of the 'null' type (Amy dⁿ allele). The oval NILs of CSR3, namely 3P, 3C and 3D show the corresponding Amy d^{iv} allele introgressed from their respective DPs, while 3N has Amy d^v pattern derived from its DP, Nistari.

Table 1: Yield traits and digestive amylase activity in the Donor parents (DP), Recurrent parent (RP) and Near Isogenic lines (NILs)

DPs/RP/ NILs	Yield/10000 larvae By No.*	By wt. (kg)**	Cocoon weight (g)	Shell weight (g)	Shell (%)	Filament length (m)	Amylase type	Amylase activity (mg/ml)
DPs								
PM	9571 ± 212	9.22 ± 1.6	0.994 ± 0.12	0.132 ± 0.02	13.3 ± 0.6	346 ± 23	Amy d ^{iv}	218.6 ± 18.4
Nistari	9257 ± 189	8.54 ± 1.3	0.953 ± 0.13	0.122 ± 0.01	12.9 ± 1.1	326 ± 15	Amy d ^v	214.1 ± 17.1
Cnichi	9423 ± 293	9.02 ± 1.2	0.912 ± 0.09	0.111 ± 0.01	12.2 ± 0.2	317 ± 32	Amy d ^{iv}	213.4 ± 31.9
Daizo	9297 ± 197	8.67 ± 1.2	0.988 ± 0.12	0.133 ± 0.02	13.5 ± 0.7	344 ± 43	Amy d ^{iv}	271.4 ± 12.7
RP								
CSR3	8098 ± 650	11.87 ± 2.2	1.516 ± 0.21	0.340 ± 0.05	22.4 ± 0.9	1022 ± 80	Amy d ^o	70.3 ± 3.4
CSR6	8125 ± 350	12.44 ± 1.3	1.585 ± 0.15	0.339 ± 0.04	21.4 ± 0.8	886 ± 108	Amy d ^o	79.6 ± 1.6
NILs								
3P	9020 ± 283	13.84 ± 1.2	1.531 ± 0.14	0.336 ± 0.04	21.9 ± 0.7	1062 ± 160	Amy d ^{iv}	203.3 ± 19.3
3N	9016 ± 431	13.73 ± 1.5	1.566 ± 0.14	0.343 ± 0.05	21.9 ± 1.4	888 ± 23	Amy d ^v	191.7 ± 17.0
3C	9113 ± 212	14.75 ± 1.2	1.634 ± 0.16	0.354 ± 0.03	21.7 ± 1.1	1001 ± 104	Amy d ^{iv}	188.1 ± 24.2
3D	9049 ± 386	13.84 ± 1.2	1.500 ± 0.15	0.329 ± 0.04	21.9 ± 1.2	923 ± 40	Amy d ^{iv}	230.7 ± 13.5
6P	8961 ± 248	13.92 ± 1.2	1.571 ± 0.21	0.335 ± 0.05	21.3 ± 1.1	855 ± 120	Amy d ^{iv}	190.4 ± 23.6
6N	8980 ± 341	13.68 ± 1.3	1.532 ± 0.19	0.322 ± 0.03	21.0 ± 0.9	827 ± 98	Amy d ^v	207.4 ± 40.2
6C	9103 ± 401	14.60 ± 1.4	1.527 ± 0.18	0.329 ± 0.04	21.6 ± 1.2	856 ± 104	Amy d ^{iv}	204.1 ± 30.2
6D	8906 ± 356	13.84 ± 1.2	1.513 ± 0.16	0.320 ± 0.03	21.1 ± 0.8	861 ± 76	Amy d ^{iv}	216.7 ± 30.4

* Denotes number of pupated cocoons harvested out of 10000 larvae retained after III moult, which is a measure of survival.

** Denotes weight of cocoons harvested out of 10000 larvae retained after III moult, which is a measure of yield.

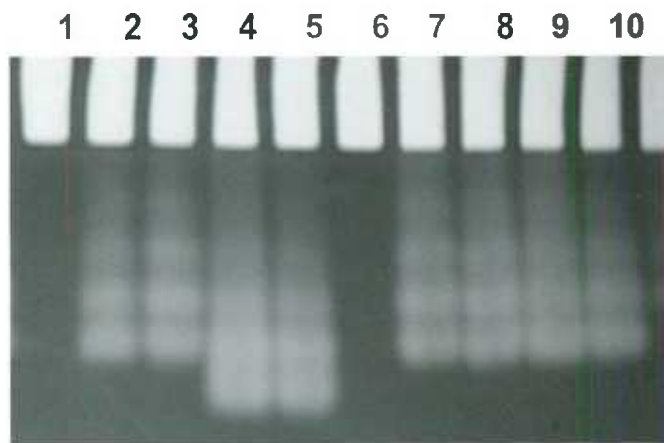


Figure 1: Polyacrylamide gel electrophoresis (PAGE) profiles of donor parents (DPs), recurrent parents (RPs) and evolved Near Isogenic Lines (NILs) showing digestive amylase polymorphism with 4 band type (Amy d^{iv}), 5 band type (Amy d^v) and null type (Amy d^o) alleles.

Lanes 1: CSR3 (DP-Amy d^o), 2: PM (DP-Amy d^{iv}), 3: 3P (NIL-Amy d^{iv}), 4: Nistari (DP-Amy d^v), 5: 3N (NIL-Amy d^v), 6: CSR3 (RP-Amy d^{iv}), 7: Cnichi (DP-Amy d^{iv}), 8: 3C (NIL-Amy d^{iv}), 9: Daizo (DP-Amy d^{iv}), 10: 3D (NIL-Amy d^{iv}).

Polymorphism of SNP markers of 8th linkage group

The genomic DNA of the DPs, RPs and NILs were amplified with five SNP primers of 8th linkage group, namely 08023, 03034, 08047, 08050 and 08058. The sequences of the forward and reverse primers are given in Table 1.

Initially, six polyvoltline strains with high activity digestive amylase alleles, namely Amy d^{iv} expressed by PM, Cnichi and Daizo, Amy d^v carried by Nistari, Moria and Sarupat as well as the low activity allele, Amy d^o (null) found in the bivoltline strains viz., CSR2, CSR3, CSR5, CSR6 and CSR12 were selected for screening with the SNP primers 08047 and 08058 of the 8th linkage group (Figure 2 a & b, respectively). The genomic DNA of these strains when amplified with the primer 08047, showed the PCR product of about 0.4 kb size found in all the polyvoltlines, while the bivoltlines showed a heavier

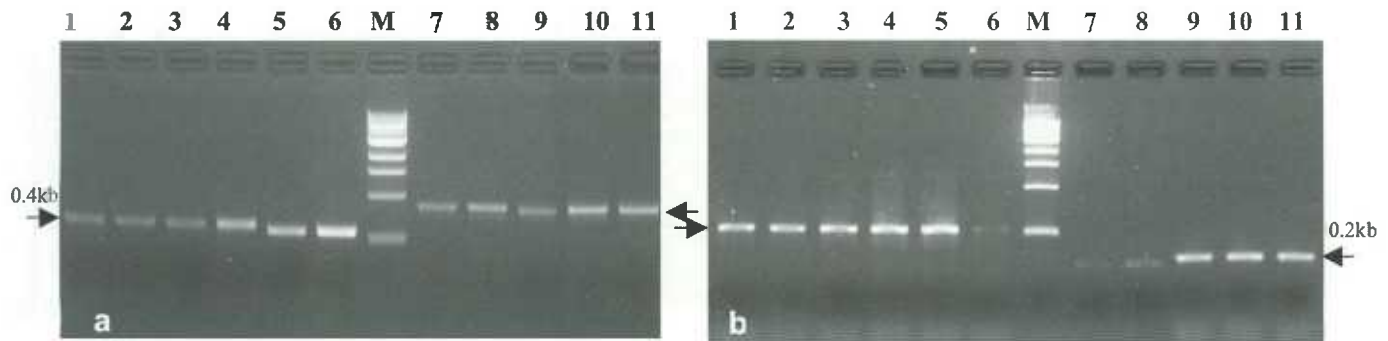


Figure 2: Polymorphic SNP profiles of a few polyvoltines (1-6) and bivoltines (7-11).

Lanes : PM, 2: Nistari, 3: Cnichi, 4: Daizo, 5: Moria, 6: Sarupat, M= Marker DNA of 1 kb DNA ladder, 7: CSR2, 8: CSR3, 9: CSR5, 10: CSR6, 11: CSR12. Lanes 1 to 6: polyvoltines with Amy d^{iv} (1, 3 & 4) and Amy d^v (2, 5 & 6) alleles. Lanes 7 to 11: bivoltines with Amy d^v (null type). a) amplified with SNP primer 08047 showing 0.5 band in polyvoltines and 0.9 kb band in bivoltines. b) amplified with SNP primer 08058 with 0.5 kb band in polyvoltines and 0.2 kb band in bivoltines.

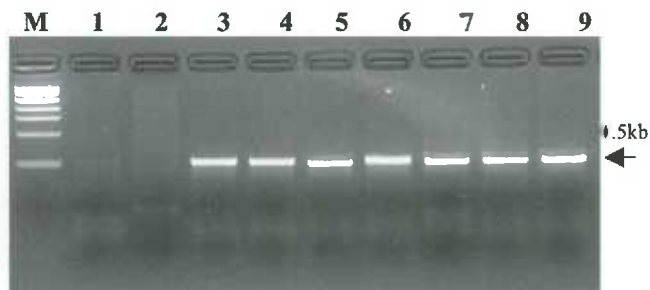


Figure 3: SNP profile of RPs, (1 & 2), DPs and NILs (3-9) amplified with the SNP primer 08023 showing polymorphism between Amy d^{*} and Amy d^v alleles.

Lanes : 1: CSR2 (RP), 2: CSR3 (RP), 3: PM (DP), 4: GEN1 (NIL of CSR2), 5: 3P (NIL of CSR3), 6: Daizo (DP), 7: 3D (NIL of CSR3), 8: Cnichi (DP), 9: 3C (NIL of CSR3). Note the absence (null type) of amplified product in RPs and 0.5 kb band in the DPs (3, 6 & 8) and NILs (4, 5, 7 & 9). The RPs are of Amy d^{*} (null type of amylase) and the DPs and NILs are of Amy d^v (4 band type of amylase) type.

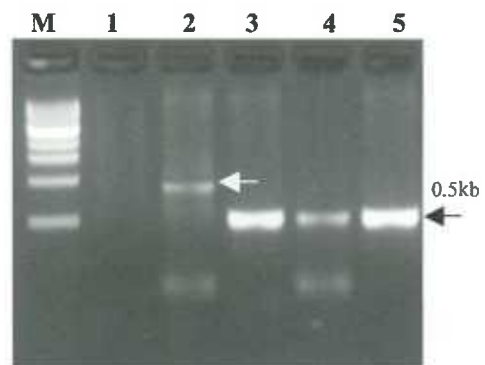


Figure 5: SNP profile of RPs, (1 & 2), DPs (3) and NILs (4 & 5) amplified with the SNP primer 08050 showing polymorphism between Amy d^{*} and Amy d^v alleles.

Lanes 1: CSR3 (RP), 2: CSR5 (RP), 3: Nistari (DP), 4: 3N (NIL of CSR3), 5: GEN2 (NIL of CSR5). Note the absence (null type) of amplified product in the RP, CSR3 and polymorphic product of 0.9 kb (white arrow) in the RP, CSR5 and 0.5 band in the DPs and NILs. The RPs are of Amy d^{*} (null type of amylase) and the DPs and NILs are of Amy d^v (5 band type of amylase) type.

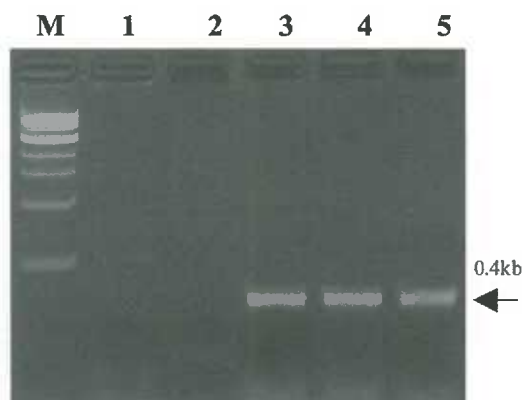


Figure 4: SNP profile of RPs, (1 & 2), DP (3) and NILs (4 & 5) amplified with the SNP primer 08034 showing polymorphism between Amy d^{*} and Amy d^v alleles.

Lanes 1: CSR3 (RP), 2: CSR5 (RP), 3: Nistari (DP), 4: 3N (NIL of CSR3), 5: GEN2 (NIL of CSR5). Note the absence (null type) of amplified product in the RPs and 0.4 kb band in the DPs and NILs. The RPs are of Amy d^{*} (null type of amylase) and the DPs and NILs are of Amy d^v (5 band type of amylase) type.

product of 0.9 kb (Figure 2 a). In the case of primer 08058, all the polyvoltines showed an amplified product of 0.5 kb and the bivoltines had a polymorphic PCR product of about 0.2 kb.

Further, to detect the SNP markers linked to 4 band amylase (Amy d^v allele) pattern, the DNA profile of RP DPs and NILs was analysed with the SNP primer 08023 (Figure 3). The RP, CSR3; DPs, PM, Cnichi and Daizo; NILs, 3P, 3C and 3D were used. In addition, the RP, CSR2 and the NIL, GEN1 evolved previously (Ashwath *et al.*, 2010) using PM as DP were also screened. It was observed that the RPs failed to show any amplified product

indicating 'null' type, while in the case of all the DPs and NILs having the Amy d^{iv} allele, PCR product of 0.5 kb was observed.

For identifying SNP markers linked to the 5 band amylase (Amy d^{iv} allele) pattern, the SNP profile of RPs, DPs and NILs was screened with the primer 08034 (Figure 4). In addition to the RP, CSR3; DP, Nistari and NIL, 3N; the RP and NIL generated earlier (Ashwath *et al.*, 2010) viz., CSR5 (RP) and GEN2 (NIL) were also included. Here also, absence (null type) of amplified products were noticed in the RPs, while the DPs and NILs with Amy d^{iv} alleles showed 0.4 kb product.

In the case of the primer 08050 (Figure 5), the RP, CSR3 showed 'null' type without any amplified product, however, the RP, CSR5 had shown a polymorphic PCR product of 0.9 kb, while in the DP, Nistari and NILs, 3N and GEN2 which had Amy d^{iv} alleles, amplified product of 0.5 kb was seen.

DISCUSSION

To detect DNA markers tightly linked to the target gene or genomic region, generally two strategies are used. One is by the bulked segregant analysis (BSA) in which the DNA profile is compared between the parents, F1 and pooled F2 samples segregating for the target trait. The DNA markers that are polymorphic between the F2 bulks are likely to be linked to the target gene (Michelmore *et al.*, 1991).

The other strategy is by using the near isogenic lines (NILs) which have been developed by transferring the target gene from the donor parent (DP) into the recurrent parent (RP) by repeated backcrossing of DP for 5-6 generations, simultaneously selecting the target gene in every generation. The resultant line is known as NIL, which is nearly isogenic to the RP having most of the genome derived from RP along with a small portion of the genome of DP flanking the target gene. The strategy of identification of DNA markers linked to the target gene involves screening of the DNA markers between the DP and RP and identification of polymorphic markers. These informative primers will be analysed using the

DP/NIL/RP trio sets. The sets showing RP/NIL allelic contrast and corresponding NIL/DP allelic equality for the informative DNA marker will establish the linkage of the marker with the target trait (Young and Tanksley, 1989).

The NILs have been successfully used for identification of DNA markers that are closely linked to the gene of interest like powdery mildew resistance genes (Ma *et al.*, 2004) and leaf rust resistance genes in wheat (Gupta *et al.*, 2005), QTLs for root traits in rice (Steele *et al.*, 2006), bacterial blight resistance genes in barley (Nduulu *et al.*, 2007) and rice (Gao *et al.*, 2008). Recently, using the NILs, RAPD markers linked to the high activity digestive amylase genes in silkworm have been identified (Ashwath *et al.*, 2010).

Similarly, in the present investigation also, the new set of NILs developed in silkworm has been used for detecting SNP markers associated with high activity digestive amylase genes.

The yield status of the DPs, RPs and NILs (Table 1) shows that the DPs have 12 to 15 % higher survival than the RP, represented by yield/10000 larvae by number and the NILs after the introgression of the high activity amylase genes from the DPs have 10 % higher survival than the RP. This indicates that amylase genes are contributing to better digestibility indirectly resulting in higher survival. The yield denoted by the weight of cocoons harvested out of 10000 larvae has also shown 2-3 kg higher in the NILs than the RP. The cocoon traits show large differences between the DPs and RP, which is about two folds higher in the RP for cocoon weight, shell weight and shell % and about three folds higher for filament length. These traits in the NILs are almost on par with those of the RP, suggesting that there is no significant deterioration of these traits in the NILs in spite of using low yielding polyvoltine strains as DPs. It clearly shows that most of the high productive genetic background of the RP has been recovered in the NILs through the strategy of amylase marker assisted backcross breeding.

The digestive amylase activity in the DPs are more than 200 mg/ml (Table 1), which was almost three fold higher than that of the RP, where it was quite low (70 mg/ml). It was observed that the enzyme in the NILs of the RP have

Table 2: Details of SNP primers of 8th linkage group used

Primer ID	Forward	Sequence	Reverse
08023	CGGTTTCAGTAGTTTCGGAGC	CTTCGCGAATGCAATGTTTA	
08034	TAGCGGGTTTGGTCACTGTT	ATTTCTGGTTTCAGTGTGCG	
08047	CATGATAGGAGAGTTATAGCGTCG	CCTCCCTTCAAGCCGAAAC	
08050	CTCAGGTACACACCGGGATT	CCTAGAGGAAACAAGTCTAGCA	
08058	GGACCGAGTCAGCAGAACAT	ATTGAGGCGTTTGGTTGAG	

almost regained the high activity profiles of their respective DPs with over 200 mg/ml, except in 3N and 3C, where the activity is marginally less than 200 mg/ml.

The cathodic digestive amylase isozyme profiles in Figure 1 shows 4 band pattern (Amy d^{iv} allele) in the DPs, PM, Cnichi and Daizo and also in the NILs, viz., 3P, 3C and 3D showing the corresponding Amy d^{iv} allele introgressed from their respective DPs. In the DP, Nistari, it is cf' 5 band 'type' (Amy d^v allele), which is also found in the NIL, 3N, derived from its DP, Nistari. The RP, CSR3 is of the 'null' type (Amy dⁿ allele), where no bands are seen. It is interesting to note that digestive amylase gene produces 4-5 bands and occurrence of such multiple bands in PAGE has also been reported previously in the human salivary amylase gene where a pattern of 5-6 isozymes are observed and these multiple bands occur due to post-translational modifications of the primary gene product through deamidation and glycosylation (Bank *et al.*, 1991).

To detect the SNP markers linked to the amylase genes, 5 SNP primers of 8th linkage group, namely 08023, 08034, 08047, 08050 and 08058 were used to amplify the genomic DNA of the DPs, RPs and NILs. Initially, representative polyvoltline strains with high activity digestive amylase alleles, namely, Amy d^{iv} and Amy d^v and bivoltines carrying low activity allele, Amy dⁿ (null) were screened with the SNP primers 08047 and 08058 of the 8th linkage group. The results revealed the presence of 0.4 kb PCR product in all the polyvoltines, while the bivoltines showed a heavier product of 0.9 kb when the SNP primer 08047 was used. In the case of primer 08058, all the polyvoltines showed an amplified product of 0.5 kb and the bivoltines had a polymorphic PCR product of

about 0.2 kb.

Screening with the SNP primer 08023, showed linkage of 0.5 kb product with the 4 band amylase (Amy d^{iv}) allele, while the RPs failed to show any amplified product indicating 'null' type. Further, the SNP primers 08034 and 08050 were found to be linked to the 5 band amylase (Amy d^v) allele. The primer 08034 generated PCR product of 0.4 kb for Amy d^v allele and no amplification was observed for the null type (Amy dⁿ) allele. Interestingly, in the case of the primer 08050, the RP, CSR3 did not show any amplified product, but in the RP, CSR5, a PCR product of 0.9 kb was seen, while the DP, Nistari and NILs, 3N and GEN2 which had Amy d^v alleles, showed the polymorphic amplified product of 0.5 kb. This apparently indicates that the RPs CSR3 and CSR5 both of which have Amy dⁿ alleles are having different genetic backgrounds acquired during the course of breeding.

Yamamoto *et al.* (2008) have mapped 60 SNPs in the 8th linkage group which harbours the Amy d locus. In the present study, we have used only 5 markers, namely, 08023, 08034, 08047, 08050 and 08058, out of which only the first four have been mapped so far and the map positions (cM) of these SNPs are 37.1, 54.0, 29.2, and 34.9, respectively. The SNP marker 08058 has not been mapped. It may be observed that the primers 0823 and 08034 which are located at the distal end of the 8th linkage group have not generated PCR products for the Amy dⁿ alleles, while the primers 08047 and 08050, whose locations are relatively proximal in the linkage group and 08058 (which is yet to be mapped) have shown amplified products for the Amy dⁿ alleles, suggesting the possibility that these three markers could be closely linked to the amylase locus. The results of this preliminary investigation underlines the need for detailed analysis using a large number of SNP markers of the 8th linkage group to identify SNPs tightly associated with the amylase locus. This will pave the way for further characterisation of the chromosome segments flanking the high activity amylase gene which confers better digestibility and higher viability.

ACKNOWLEDGEMENT

The authors are grateful to the research group of National Institute of Agrobiological Sciences, Japan, especially Dr. K. Yamamoto and Dr. K. Mita, for their

support. The authors are thankful to the Central Silk Board, Ministry of Textiles, Govt. of India for financial support to carry out the present investigation.

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Article scientifique

DETECTION DE MARQUEURS DE POLYMORPHISME D'UN SEUL NUCLEOTIDE (SNP) LIES AUX GENES D'AMYLASE DIGESTIVE CHEZ LE VER A SOIE *BOMBYX MORI* L.

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RÉSUMÉ

En utilisant la souche de vers à soie bivoltins productive ayant un gène à faible activité amylase digestive (allèle Amy dⁿ) comme parent récurrent (RPs), une lignée presque isogénique (NILs) a été obtenue par introgression de gènes d'amylase très actifs (allèles Amy d^{iv} ou Amy^v) provenant de parents indigènes polyvoltins (DPs) qui confèrent une amélioration significative de la survie chez les NILs. Pour savoir si le gène de l'amylase lui-même est responsable de l'amélioration de la viabilité ou si d'autres gènes flanquant le locus de l'amylase sont impliqués, le profilage de l'ADN des DPs, RPs et NILs a été mené en utilisant cinq marqueurs SNP du 8^{ème} groupe de liaison comportant le locus Amy d. Les produits de PCR polymorphiques spécifiques de Amy d^{iv}, Amy d^v et Amy dⁿ ont été détectés. Les résultats montrent les perspectives de caractérisation des segments de chromosome conférant des caractères de viabilité.

Mots-clés: Amylase digestive, lignées presque isogéniques, ver à soie, SNPs.



CHARACTERIZATION AND PATHOGENICITY OF *FUSARIUM* SPP. CAUSING MULBERRY (*MORUS* SPP.) ROOT ROT IN REGION 1, PHILIPPINES

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ABSTRACT

Mulberry (*Morus* spp.) is extensively grown as food for silkworm (*Bombyx mori* L.). The plant has been attacked by diseases caused by fungi, viruses and bacteria. The present investigation spanning from April 2013 to February 2014 was conducted to survey, identify, and characterize the pathogen which causes root rot in mulberry. The different aspects viz., symptomatology, identification and characterization of pathogen, assessment of root rot incidence and yield loss were covered after surveying 23 mulberry farms in Region 1, Philippines. Samples isolated from roots and surrounding rhizosphere of mulberry plants of 23 plantations revealed 115 isolates of *Fusarium* spp. Pathogenicity tests showed development of typical symptoms of mulberry root rot for isolates from infected tissues of mulberry root. Incubation period was reported between 12 and 14 days at 24-29 °C during rainy season, and 5-9 days at 32- 35 °C during summer under greenhouse (open) condition. The pathogens identified to be associated with mulberry root rot belong to seven *Fusarium* species, i.e., *Fusarium oxysporum*, *F. solani*, *F. moniliforme*, *F. pseudocircicatum*, *F. phaseoli*, *F. graminearum* and *F. verticillioides*. An average level of mulberry root rot severity of 32 % can cause reduction in leaf yield from 9.84 t. ha⁻¹ to 7.57 t. ha⁻¹ in mulberry cv. Batac and thus emphasize that *Fusarium* spp. plays a crucial role in adversely affecting the biomass production of mulberry per unit area and hence pose a serious threat on its growth and foliage production.

Key words: Batac cultivar, *Fusarium* species, mulberry root rot, pathogenicity, rhizosphere, symptomatology.

INTRODUCTION

Mulberry (*Morus* spp., Family: Moraceae) is widely distributed around the world (Yoshida *et al.*, 2002). It is extensively grown as food plant for silkworm, *Bombyx mori* L. that produces silk. Like any other important crop, it has been attacked by diseases caused by fungi, bacteria, viruses, mycoplasma and nematodes. At present, in mulberry, soil borne diseases are widely prevalent and pose a serious constraint for the cocoon production. Among the soil borne diseases of mulberry, root rot caused by species of *Fusarium* is considered the most destructive and are observed both in nursery and established fields (Philip *et al.*, 1997). It is more alarming in view of the potential of the fungus to thrive well in soil

and its ability to spread fast once occurred. The absence of disease resistant varieties and inadequate control measures against this disease are also important points of concern.

In the Philippines, mulberry root rot (MRR), caused by a soil born fungus, *Fusarium* spp. was first recorded in the mulberry plantations of Sericulture Research and Development Institute (SRDI) and Sta Maria, Ilocos Sur (Telan and Gonzales, 1998). In India too, the disease is rampant and alarming. (Sengupta *et al.*, 1990; Biswas, 1992; Teotia and Sen, 1994). In 2008, the disease caused 30-40 % field infestation in the experimental area of SRDI, that led to immediate uprooting and burning of infected plants as a measure to avoid widespread outbreaks and to save the healthy plants (Gonzales and Telan, 2008). At

present, the institute recourse to more hazardous chemical control measures, such as sapling treatment with Dithane M 45 and Benlate drenching of soil before planting at recommended rate to control the disease. Preliminary *in vitro* studies to control the disease using phyto-extracts, biological agents (Telan and Gonzales, 1998) and greenhouse trial of developed pelletized bio-con agents were undertaken. Detailed study on the symptoms of the pathogen (Gonzales, 2005) and varietal screening to identify resistant genes under nursery condition for sapling production were reported by Angeles (2006).

So far, no attempt has been undertaken to investigate the impact of incidence of mulberry root rot on leaf output and cocoon yield losses. Likewise, there has not been any effort to identify, characterize and document the role of the *Fusarium* spp. in the development of root rot in mulberry field. The identification and characterization of this pathogen can lead to more effective management of the disease, thus enabling ample feed supply for silkworm during rearing.

MATERIALS AND METHODS

Symptomatology: Infected cuttings, saplings and fully grown mulberry plants were collected from different project sites in region I and brought to the laboratory for examination. The disease symptoms were recorded. The fungal structures from young or advancing rotting lesions were picked carefully with a dissecting needle, transferred into a drop of plain lactophenol on a clean glass slide and covered with a cover slip. The morphology of the fungal structure was examined and documented. The fate of infection of the disease was carefully monitored and observed under nursery or sapling production as well as in grown up mulberry plantation. The different cultural practices were recorded to correlate with the disease cycle.

Isolation and identification on the basis of morphological characteristics: Isolation trials were carried out on different non-diseased and diseased mulberry plants showing symptoms of root rot and wilt,

collected from 23 mulberry plantations in Region I. Mulberry roots and soil around the rhizosphere of mulberry trees with or without rotting and wilting symptoms from the experimental sites were also used to isolate associated pathogens during the period from April 2013 to February 2014.

The roots from infected and non-infected plants were cut into pieces (approximately 3 to 5 mm long) and were washed in running tap water, surface disinfected with 0.6% sodium hypochlorite for 5 min, rinsed twice in sterile distilled water, blotted dry on sterilized tissue paper, and then plated on potato dextrose agar (PDA, Merck Laboratories, Germany) added with 5 drops of 25 % lactic acid per 250 ml PDA to avoid bacterial growth. Dishes were incubated at 28 °C and examined periodically. After 3-5 days, emerging fungal mycelia were transferred onto new PDA plates.

Simultaneously, soil samples were collected from the stem base to a depth of 15 cm at 3 points around the plant (100 g per plant from infected and non-infected field in all of the 23 mulberry plantations in Region I). In order to assay the organisms in the soil, each sample from infected field was thoroughly mixed. Ten grams of each mixed sample was transferred to 90 ml of sterilized distilled water in a 250 ml Erlenmeyer flask, was agitated by continuously shaking the flask for 30 min and 10-fold dilutions were made serially from the original suspension. From each of the dilution, 100 µl aliquot was spread over the surface of potato dextrose agar in 9 cm diameter Petri plates, (with 3 plates per dilution), and incubated at 28 °C for daily observation. Emerging colonies were cut and transferred individually onto new PDA plates. Further purification of cultures was done through single spore techniques, *i.e.*, sub-culturing of isolates into SNA and WA and then into PDA plates for microscopic examination. Same procedure was done for samples collected from non - infected area. Identification was carried out on the basis of morphological characteristics described by Burgess and Summerell (2005). The pathogens were identified based on growth and morphological characteristics, such as color and structure in culture media, size and types of

macroconidia, microconidia, conidiophores and conidiogenous cell, hyphae and chlamydospores formations.

Pathogenicity test: Cuttings of cv Batac with susceptible reactions to mulberry root rot were used in the experiment. Mature mulberry plants were dipped and incubated in ANAA rooting hormone to produce different rooting growth mass and planted in previously sterilized soil mixed with *Fusarium* in poly-bags. Each group was replicated 20 times. All recommended cultural practices were followed viz., fertilization, watering etc., to give favorable conditions for the vigorous growth and development of the test plants. The methods of Philip *et al.* (1994) were adopted in conducting the experiment. Disease development, symptoms and incidence were observed and recorded daily as per cent of pre-emergence damping-off of seedlings throughout two weeks from planting. Post emergence damping-off was recorded and healthy plants were counted up to 45 days from planting according to Philip *et al.* (1994).

Root rot incidence and yield loss assessment: The survey for root rot incidence, disease severity, disease cycle and the resultant damage on yield biomass was conducted in 23 farmers' fields in Region 1 during the months of April, 2013 to February, 2014. Each farm, serving as experimental unit was planted with mulberry cv. Batac provided with recommended cultural practices. Data on total and infected areas, total number of mulberry trees, number of infected trees, age of mulberry trees and type of damage (collective or sporadic) were determined. The severity of the disease in the area was determined by counting the total number of healthy mulberry plants and infected plants. The yield was estimated based on average weight of harvested mulberry leaves randomly determined in the plantations using 20 plants in each farm at 55 days after planting (DAP). The yield loss was determined using the formula:

$$\text{Yield loss} = (Y1 - Y2) / Y1 \times 100$$

where: Y1 is the ideal yield in an area without

disease; Y2 is the yield in MRR infected area.

The data on disease severity, yield and yield loss were subjected to Analysis of Variance (ANOVA); and the means were separated using the DMRT at 5 % level of significance.

RESULTS AND DISCUSSION

Symptoms caused by mulberry root rot: During the survey, incidence of MRR was noticed throughout the year. In mulberry, the first symptom of *Fusarium* root rot is usually observed as leaf blighting in the upper half of the branch, or sometimes in the whole branch, followed by sudden withering and burnt appearance in 2-3 days (Figure 1). The symptoms progress downwards. Sudden wilting of buds occur, thus stopping of apical growth. Infected roots show rotting of barks and cortex of both primary and secondary roots. The root appearance varies, some turns into black color, while in some, totally brownish-violet peeled-off roots appear.

Mulberry root rot disease cycle: Mulberry root rot is caused by several fungal species in the genus *Fusarium*. *Fusarium oxysporum* is the most common pathogen causing MRR of mulberry in the sericulture farms in Region 1 of the country, but other *Fusarium* species are also known to cause the disease particularly, *F. solani*. *Fusarium* spp. are common in most soils where mulberry are grown and can survive as resistant spores free in the soil for very long periods of time. There are two main opportunities in the mulberry crop cycle for *Fusarium* spp. to infect mulberry: (1) during transplanting of infected saplings raised from stem cuttings that were affected by various soil borne pathogens, i.e., *Fusarium* spp. (Gupta *et al.*, 1997; Singh and Cohan, 1984; Sukumar *et al.*, 1991) and (2) the spread of the disease from the standing infected plant to nearby healthy plants in the field (Philip *et al.*, 1995; Gupta *et al.*, 1997) (Figure 2).

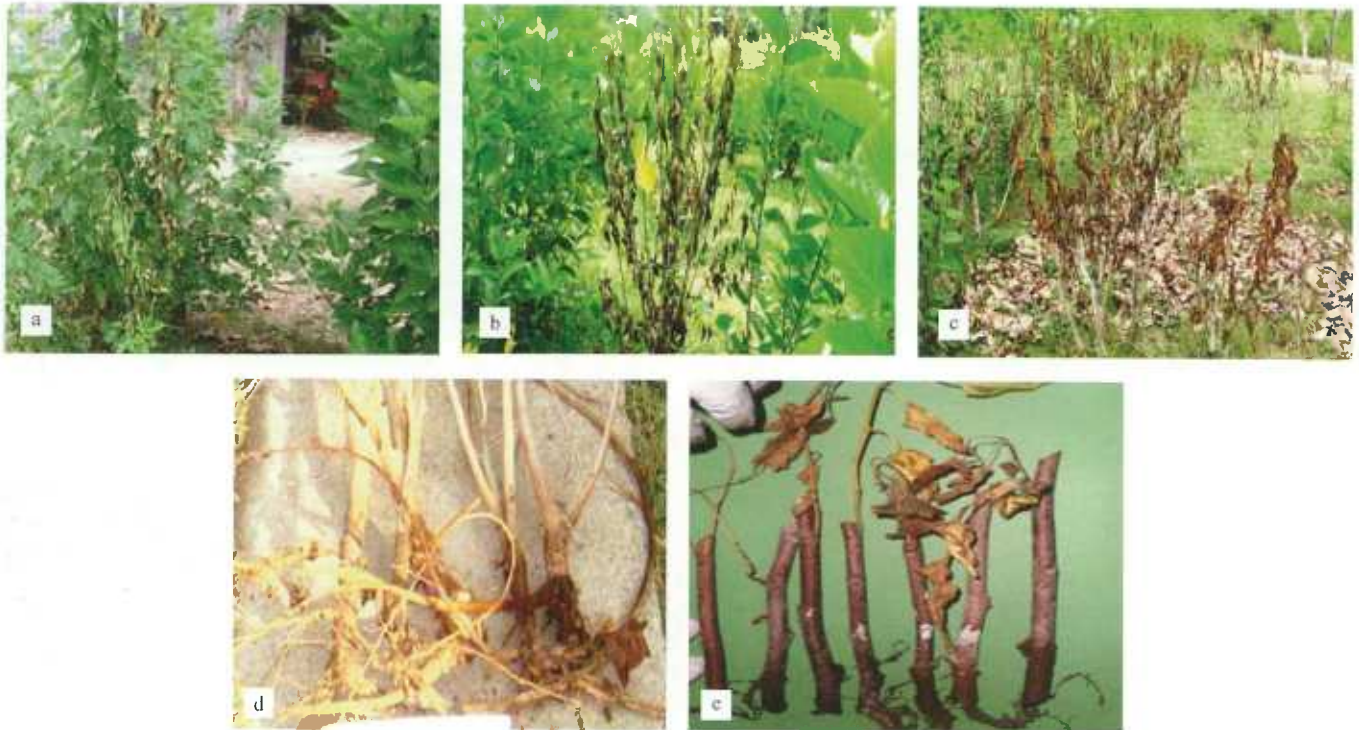


Figure 1: Typical symptoms of mulberry root rot infected tree in an established garden:

(a) early symptoms with some branches still looking healthy; (b) sudden wilting of the whole plant; (c) scattered appearance of dead plants in mulberry plantation; (d) roots of infected plants; (e) infected saplings.

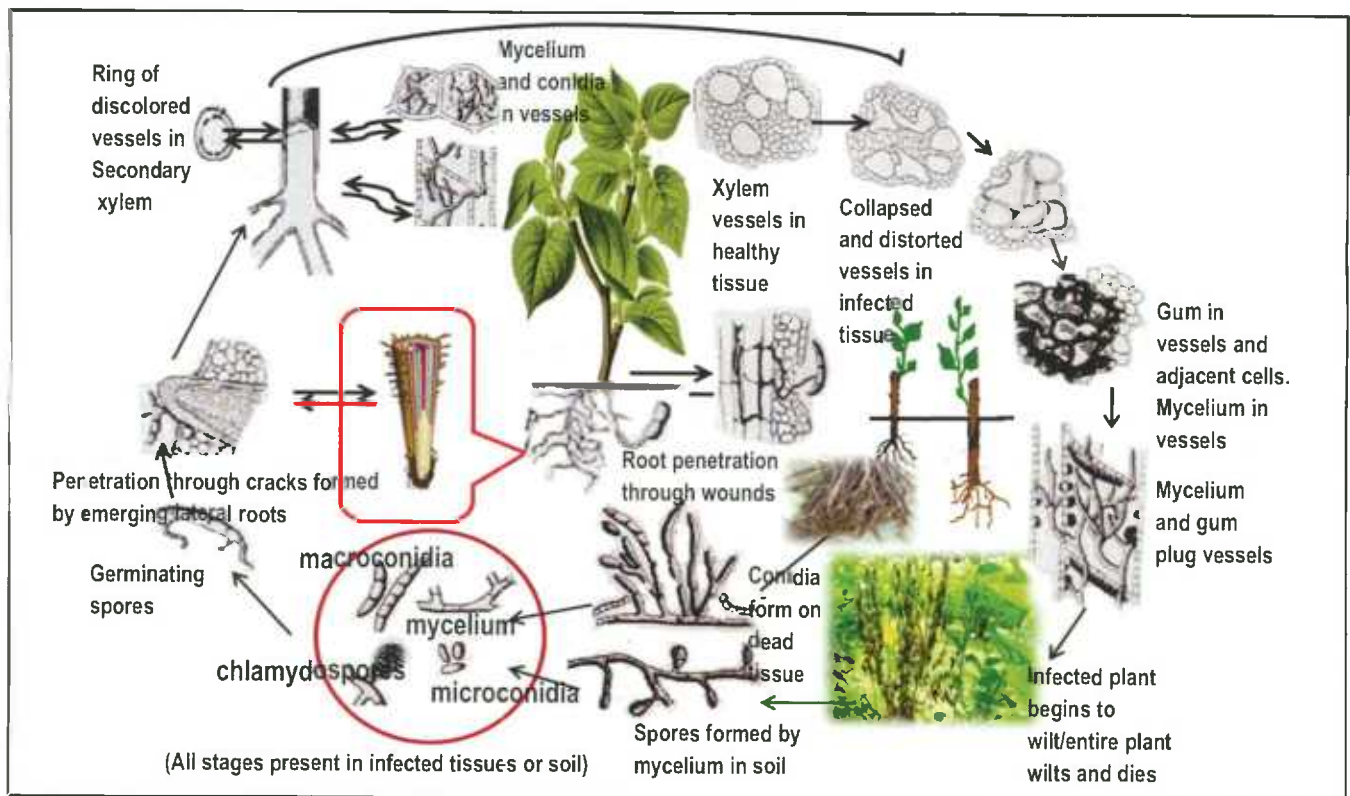


Figure 2: The life cycle of *Fusarium* sp. and spread of mulberry root rot in the field

Fusarium species are commonly found on roots of saplings. When saplings from nursery are grown and maintained at infected nursery beds, some level of MRR is always present in transplanted saplings. During the transplanting phase of mulberry establishment and production, it is a practice that roots of the saplings are normally trimmed prior to planting in the sericulture farms in the Philippines, resulting in weakening of sapling and sometimes the whole plant in the case of infected ones may rotten or dry up completely before planting.

Alternatively, after planting, infected saplings may become diseased and killed out right before recovery. Damage at this stage results in dying of saplings and is usually expressed as poor and uneven stands with weakened plants. Reduction in crop vigor occurs due to expenditure of saplings' vegetative energy to produce secondary or tertiary sprouts to compensate for damage to primary sprouts of buds or root development as the case maybe. Adjacent healthy saplings may too become contaminated with *Fusarium* spores as they develop in the field. However, most species of *Fusarium* remain dormant (asexual chlamydospores) in the soil or decaying mulberry root (Philip *et al.*, 1995). On stimulus achieved by suitable soil temperature and moisture, they germinate mycelia on which new chlamydospores and conidia are formed. Wounds formed during intercultivation and handling provide dormant spores on the root surfaces with multiple points of entry into the plant.

Fusarium secretes pectolytic enzymes that catalyze the hydrolytic reactions and partly destroy the middle lamellae of parenchymatous cells and degrade pectic compounds in the walls of vessels and tracheids. Parenchymatous cells are killed and turned brown due to the oxidation of polyphenols into dark melanin pigments. The fungus also produces extracellular toxins that move to the leaves where they induce loss of turgor in the parenchymatous cells. According to Philip *et al.* (1995), once the pathogen has penetrated the roots it begins to

grow in the root tissue, causing root rot lesions at the point of entry (Figure 2). In infected mulberry plantation, root rot develops most rapidly under conditions of high relative humidity and temperature. The penetrated fungus spread throughout the root by means of mycelia growth or conidia. The macroconidia produced on mycelia may get converted into chlamydospores through condensation. Since *Fusarium* are both soil and air borne in nature, movement of the fungus may be associated with soil irrigation or flood water, contaminated field tools, planting materials, wind and rain. In banana plantation for example, *F. oxysporum* spores have also been proven to live on non-host plants in the absence of a susceptible host. This provides a means of survival for the fungus, which remains virulent until it gets access to a host plant. When non-host plants become infected, they show a few symptoms if any, and become a carrier of the pathogen. When banana seedlings are planted, spores germinate in the soil and grow towards the nearby roots of banana plants in response to the chemical compounds exuded from the roots. In a farm devastated by *Fusarium* wilt, it was found that there were traces of *F. oxysporum* in three common types of grass, a low growing herb and the roots of common weeds (Daly, 2006).

Isolation and identification on the basis of morphological characteristics

Out of 115 isolates of *Fusarium* found associated with mulberry plants in 23 mulberry plantations in Region 1, Philippines, 93 and 28 isolates were from roots and soil, respectively (Table 1). The tentative species identified to be associated with mulberry roots belong to seven *Fusarium* species, i.e., *Fusarium oxysporum*, *F. solani*, *F. moniliforme*, *F. phaseoli*, *F. graminearum*, *F. verticillioides* and *F. pseudocircinatum* isolates (Table 2). Among these, *Fusarium oxysporum* was found to be the most commonly associated with mulberry garden followed by *Fusarium solani* with 64 and 20 % association, respectively.

Table 1: Occurrence of *Fusarium* spp. in mulberry roots

<i>Fusarium</i> spp.	Isolates recovered			
	Mulberry root		Soil	
	MRR symptoms	No MRR symptoms	MRR symptoms	No MRR symptoms
<i>F. oxysporum</i>	36	26	4	8
<i>F. solani</i>	14	2	5	2
<i>F. moniliforme</i>	2	-	-	-
<i>F. phaseoli</i>	-	-	1	2
<i>F. graminearum</i>	4	1	1	2
<i>F. pseudocircinatum</i>	2	-	-	2
<i>F. verticillioides</i>	4	2	1	-
Total	62	31	12	16

Data were collected after surveying 23 mulberry plantations in Region 1, Philippines.

Pathogenicity test: Pathogenicity tests showed development of typical symptoms of mulberry root rot for isolates from infected tissues of mulberry root. Incubation period was between 12 and 14 days at 24-29 °C during rainy season, and 5-9 days at 32-35 °C during summer under greenhouse (open) condition (Table 3). The result showed that the number of wilted saplings increased during 5-14 days after planting (DAP) in rainy and summer seasons. One to four weeks-old saplings were highly susceptible to *Fusarium* spp. Both Ilocos and La Union isolates readily infected the mulberry cv. Batac but La Union isolates produced slightly higher infection than Ilocos Sur isolate. The result only shows that the latter is more virulent than the former isolate.

Root rot incidence and yield loss assessment

Assessment of yield loss of mulberry cv. Batac due to MRR indicated that the amount of harvestable mulberry leaves was determined by the severity of the mulberry root rot infection (Table 4). Mulberry yields (t.ha^{-1}) was consistently higher in healthier plantations. Data showed that average disease severity of 30.77 % can result in 2.30 t.ha^{-1} of harvestable leaf biomass. The average yield obtained from MRR infected field in sericulture farm in Region 1 with 7-61 % disease severity, was $0.517 - 4.558 \text{ t.ha}^{-1}$. This was significantly lower compared with the yield obtained from MRR free field which was 7.466 t.ha^{-1} . Under the condition of the sericulture farms, thus the effect of MRR infection on mulberry can be translated into 30 % reduction in the harvestable leaves. In another location, a similar result was obtained using the same experimental assessment. Results confirmed that when mulberry cv Batac planted farm was infected with MRR with a disease severity of 30 % (a paralleled), the same percentage of leaf biomass was reduced when compared with the similar cultivar devoid of MRR infection. The results of the yield loss assessment of mulberry cv. Batac against MRR corroborate with the findings of Sukumar *et al.* (1991); Philip *et al.* (1995) and Gupta *et al.* (1997) in that MRR disease causes 15 % reduction in yield in established mulberry gardens. Philip *et al.* (1995) reported 55 % incidence in Anantapur (Andhra Pradesh) and Chamarajanagar (Karnataka, India). In addition, Kazempour and Kamran (2005) reported that severe root

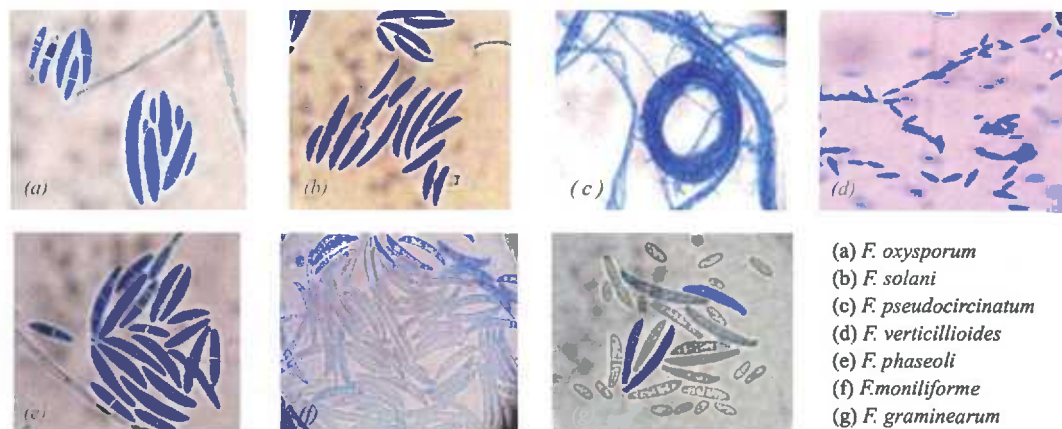


Figure 3: *Fusarium* species associated with mulberry plantations in Region 1, Philippines: a-g: scale bar = 20 μm

rot of mulberry trees were associated with *F. solani*, *F. oxysporum*, *L. theobromae*, *Rhizoctonia solani* and *Rosellinia necatrix*.

30 % leaf yield losses, respectively (Gunasekhar and Govindiah, 1993). The yield loss in mulberry plants attacked by *Fusarium* was observed to be 10-12 % (Sikdar

Table 2: Morphological characteristics of *Fusarium* spp. found associated with mulberry roots in 23 mulberry plantations in Region 1, Philippines

<i>Fusarium</i> spp.	Morphological characteristics		
	Macroconidia	Macroconidia	Chlamydospores
<i>F. oxysporum</i>	Short to medium length, straight to slightly curved and slender in shape; usually 3 septate; Tapered and curved, sometimes with a slight hook apical cell; Foot shaped to pointed basal cell.	Oval, elliptical or kidney shaped and usually 0-septate; Short monophialides conidiogenous cells and abundant in the aerial mycelia.	Formed abundantly and quickly (2-4 weeks on PDA) by most isolates, but some isolates form chlamydospores slowly if they form them at all; Usually formed singly or in pairs, but also may be found in clusters or in short chains; Smooth or rough walled.
<i>F. solani</i>	Relatively wide, straight, stout and robust with 5 to 7 septate; Blunt and rounded apical cell; Distinct foot shaped basal cell, sometimes poorly developed, straight to almost cylindrical, usually with a notched or a rounded end basal cell.	Oval, ellipsoid, reni- form and fusiform with 0 or 1 to occasionally 2 septa; Monophialides, often quite long conidiogenous cells.	Commonly formed abundantly and rapidly, usually within 2-4 weeks on PDA; May be intercalary in the hyphae ; sometimes formed terminally on short lateral branches usually singly or in pairs; May be globose to oval in shape; Smooth or rough walled.
<i>F. moniliforme</i>	Usually narrow and straight, sparsely produced.	Produced in chains from simple phialices in the aerial mycelium.	
<i>F. pseudocircinatum</i>	Slender, slightly falcate and thin walled with predominantly 3 septate; Beaked apical cell; Foot shaped basal cell.	Oval to obovoid and 0 to 1, but usually 0 septate; Usually monophialides, but occasionally polyphialides conidiogenous cells.	Absent but circulating hyphae observed.
<i>F. phaseoli</i>	Falcate and formed on slender conidiophores arising from hyphae.	Ellipsoidal, obovate or naviculate produced from slender aerial conidiophores on aerial mycelium.	Formed frequently in mycelium and in conidia, mostly intercalary, single but sometimes in chains.
<i>F. graminearum</i>	Slender, thick-walled, and of medium length; Moderately curved to straight with 5 to 6 septate; the ventral surface straight and the dorsal side smoothly arched; Tapered and occasionally constricted to a snout-like apical cell; Well developed foot shaped basal cell.	Absent	Variable, but often very slow to form most commonly in the macroconidia, but may also form in the mycelia; Produced singly, in clusters, and in chains, globose with a finely roughened, but not varicose, appearance.
<i>F. verticillioides</i>	Relatively long and slender, slightly falcate or straight and thin walled with 3- to 5-septate; Curved and often with tapered apical cell; Notched or foot shaped basal cell.	Oval to club shaped with a flattened base and usually 0 septate; Long chains are common, but small aggregates of a few spores occur ; Monophialides, which are occasionally produced in pairs to give a "rabbit ear" conidiogenous cells.	Chlamydospores are not produced, although some isolates may produce swollen cells in the hyphae that can easily be mistaken for chlamydospores or pseudochlamydospores.

(source and guide for identification: after Summerell et al. (2003) and Nelson et al. (1990).

The reduction in yield is directly associated with the presence of diseases. Many diseases have been reported on mulberry causing 10-15 % leaf yield losses besides reducing its quality. These are leaf spot, powdery mildew, and leaf rust which inflict 10-15 %; 40 % and 10-

and Krishnaswami, 1980) and up to 10-15 % (Philip et al., 1995). In addition, mulberry being a perennial crop, soil borne diseases are widely prevalent and are serious constraints for the production of enough quantity of good quality leaf for silkworm feeding.

Table 3: Pathogenicity test of two *Fusarium* isolates on *Morus alba* cv. Batac saplings

Isolate No.	Source of isolates	Season ¹	Infection				Per cent mortality
			Days after planting (DAP) ²				
			5-7	10-14	15-21	20-28	
Rainy							
SM-13	Ilocos Sur		2	6	11	55	
SE-13 ¹	La Union		7	16	16	80	
Summer							
SM-13	Ilocos Sur		4	12	16	80	
SE-13 ¹	La Union		5	15	18	95	

¹Temperature ranged from 24-29 °C in rainy season and from 32-37 °C in summer; ²Average no. of dead cuttings from 20 cuttings per replication with 3 replications.

Table 4: Incidence of mulberry root rot (MRR), yield and yield loss assessment of mulberry in selected sericulture farms in Region 1 from April 2013 to December, 2013

Farm/ Location	Variety	Total area (m ²)	Yield/area (tons)	Disease severity (%)	Yield loss ¹ (tons)
Sinapangan, Bangar	cvBatac	2,246	1.677	49	3.645 a
Ortega	cvBatac	1,248	0.932	20	1.51 b
Uya-oy, Btn, L.U.	cvBatac	1,120	0.836	7	0.517 c
Narra, Btn., L.U.	cvBatac	1,691	1.262	26	1.951 b
Sto. Rosario,	cvBatac	2,131	1.591	23	1.718 b
Lacang, Sn Gabriel, L.U.	cvBatac	3,752	2.801	61	4.558 a
Baroro, Btn., L.U.	cvBatac	4,820	3.598	41	3.098 a
Pob. Sn Gabriel, L.U.	cvBatac	1,262	0.942	30	2.242 b
Naguillan, L.U.	cvBatac	1,256	0.938	20	1.498 c
Mean			1.549	32	2.38

¹Means in a column in each location with the same letter are not significantly different at 5 % level, DMRT.

Table 5: Mulberry yield and yield loss assessment as affected by mulberry root rot disease

Mulberry	Disease incidence (%)	Yield (t ha ⁻¹)	Yield loss (t ha ⁻¹)	Loss (P ha ⁻¹) ¹
MRR infected	32	7.577	2.38	1,339.97
MRR free		9.84		-

¹Mulberry leaves at P=2.00/kg.

Economic implications

Table 5 indicates the impact of the mulberry root rot *i.e.*, disease severity level of 32 % in mulberry cv. Batac can result in a leaf yield loss of 2.38 t ha⁻¹ which is equivalent to P 1,339.97.

Studies on epidemiology and species identification through molecular analyses *etc.* shall be recommended to formulate foolproof disease management strategies against this highly diverse species of pathogen attacking mulberry in the country.

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Article scientifique

CARACTERISATION ET PATHOGENIE DE *FUSARIUM* SPP. RESPONSABLE DE LA ROUILLE DE LA RACINE CHEZ LE MURIER (*MORUS* SPP.) DANS LA REGION I AUX PHILIPPINES

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RÉSUMÉ

Le mûrier (*Morus* spp.) est cultivé de manière extensive comme aliment des vers à soie (*Bombyx mori* L.). La plante est attaquée par des maladies causées par des champignons, des virus et des bactéries. Notre investigation d'une année d'Avril 2013 à Février 2014 a été conduite pour identifier et caractériser le pathogène qui cause la rouille de la racine chez le mûrier. Les différents aspects comme la symptomatologie, l'identification et la caractérisation du pathogène, l'évaluation de l'incidence de la rouille et la perte de rendement ont été couverts après étude de 23 fermes dans la région I aux Philippines. Des échantillons isolés des racines et de la rhizosphère environnante des plants de murier des 23 plantations ont révélé 115 isolats de *Fusarium* spp. Les tests de pathogénie ont montré le développement de symptômes typiques de rouille de la racine du mûrier pour les isolats des tissus infectés. La période d'incubation est de 12 à 14 jours à 24-29 °C pendant la saison des pluies et de 5-9 jours à 32-35 °C pendant l'été en serres ouvertes. Les pathogènes identifiés comme associés à la rouille du mûrier appartiennent à sept espèces de *Fusarium*, *F. oxysporum*, *F. solani*, *F. moniliforme*, *F. pseudocircicatum*, *F. phaseoli*, *F. graminearum* et *F. verticillioides*. Une sévérité moyenne de 32 % peut causer une réduction du rendement de 9,84 t/ha à 7,57 t/ha chez le cultivar Batac et ceci souligne que *Fusarium* spp. joue un rôle crucial en affectant la production de biomasse du mûrier et constitue ainsi une menace sérieuse pour sa croissance et la production de feuilles.

Mots-clés: Cultivar Batac, espèces de *Fusarium*, rouille de la racine du mûrier, pathogénie, rhizosphère, symptomatologie.



ANTIBACTERIAL FINISHING OF SILK FABRIC AS AN APPLICATION OF CHITOSAN-NEEM NANO EMULSION

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ABSTRACT

Consumer's attitude towards hygiene and active life style has created a rapidly increasing market for antimicrobial textiles which in turn has stimulated intensive research and development in the field. The present study focused on the synthesis of chitosan-neem nano complex (CNNC) for antibacterial finishing of silk fabric. The synthesis of CNNC was carried out by multiple emulsions – solvent evaporation (MESE) method. FTIR and ¹H NMR spectra were used to characterize the structure of CNNC. The nano emulsion of CNNC *i.e.*, chitosan-neem nano emulsion (CNNE) with average particle size of 85.5 nm was prepared by adding calculated amount of phosphate buffer (pH-7.4) and applied on to silk fabric by pad-dry-cure technique at different concentrations (0.5-3 %) with 70 % wet pick up during padding. The antibacterial activity and the performance properties of the treated fabrics *viz.*, tensile strength, wrinkle recovery, whiteness index, bending length and air permeability were evaluated. The finished fabric showed adequate wrinkle resistance, sufficient whiteness, slight decrease in tensile strength and more reduction in the rate of multiplication of bacteria as compared to untreated silk fabric, without hampering the comfort properties with respect to air permeability and bending length.

Key words: Antibacterial finishing, chitosan-neem nano emulsion, silk fabric.

INTRODUCTION

Textiles have long been recognized as media to support the growth of microorganisms such as bacteria and fungi. These microorganisms are found almost everywhere in the environment and can multiply quickly when basic requirements such as moisture, nutrients and temperature are convenient. Proteins in keratinous fibres and carbohydrates in cotton act as nutrients and energy sources under certain conditions. Soil, dust, solutes from sweat and some textile finishes can also be nutrient sources for microorganisms (Purwar and Joshi, 2004).

The growth of microorganisms on textiles causes unwanted effect not only to the textiles itself but also to

the wearer (Gao and Cranston, 2008). These effects include the generation of unpleasant odour, stains and discolouration of coloured fabric, a reduction in fabric mechanical strength and an increased likelihood of contamination. For these reasons, it is highly desirable that the growth of microbes on the textiles should be prohibited or minimized during their use or storage (Ghadeh *et al.*, 2012; Chen and Wei, 2014). Recently, consumer's demand for hygienic clothes and active wear has created a substantial market for antimicrobial textile products. Consequently, it is highly important to treat cellulosic and protein textiles with antimicrobial finishes, especially where the probability of microbial growth is high (Williams *et al.*, 2005; Xing *et al.*, 2007; Giri *et al.*, 2009).

Silk is the lightest, thinnest and softest natural fibre and has a large number of applications in the textile field (Xing *et al.*, 2012). Silk possesses special properties such as luminosity, high air permeability, eximious performance of absorbing moisture, water vapour transmission, thermo-insulation and compatibility to skin (Davarpanah *et al.*, 2009; Min *et al.*, 2012). So, it is widely used in areas of costume manufacture, modern industry and medical science. However, silk is a protein fibre and suffers from certain disadvantages such as obvious deficiency in antibacterial property, where all kinds of microbes can breed extremely rapidly in suitable environment and produce abnormal stimulation and even induce skin diseases. So, it is important to develop antibacterial silk fabric where aesthetics and hygiene are important aspects along with safety (Periolatto *et al.*, 2012).

A number of chemicals have been used to impart antimicrobial activity to textile goods. These chemicals include inorganic salts, organometallics, iodophores (substances that slowly releases iodine), phenols and thiophenols, onium salts, antibiotics, heterocyclics with anionic groups, nitro compounds, urea and related compounds, formaldehyde derivatives and amines (No *et al.*, 2002). Majority of such products are synthetic based and may not be environment friendly. Since the compliance to the regulations imposed by international bodies, such as EPU is essential, the textile industry continues to look for eco-friendly processes that substitute for toxic textile chemicals (Sathianarayanan *et al.*, 2010). Recently, natural materials such as chitosan, natural dyes, herbal products such as *Aloe vera*, tea tree oil, eucalyptus oil, tulsi and neem leaf extracts have been attracting the attention of research because of availability of resources, low cost, easy handling and minimum damage to the environment. In addition, they have unique properties such as non-toxicity and biological degradability (Noerati *et al.*, 2008). Nowadays, chitosan, a natural linear bio-polyaminosaccharide has opened up a new avenue in this area of research and has also been studied as a new antibacterial agent for textiles (Lim and Hudson, 2004). Protonated amino groups of glucosamine

units in chitosan may inhibit growth of microorganisms by holding negatively charged cell walls (Shin *et al.*, 2001). Extracts from different parts *viz.*, roots, flowers, leaves, seeds *etc.* of diverse species of plants also exhibit antibacterial properties. However, the major problems of chitosan as an antibacterial agent are its loss of antibacterial activity under alkaline conditions due to its loss of the cationic nature and its poor durability on textile fabrics due to lack of bonding force with fabric (Kim *et al.*, 2003). In addition, as garments are subjected to washing, the wash durability of finishes is a major issue though, chitosan and the herbal extracts show good antibacterial property after applying on silk fabric.

To overcome the above problems, chemical modification that lowers the surface energy can be used as an effective method. Despite frequent reference to textile applications, actually only a few existing reports pertain to antibacterial textiles. But in these cases, only an initial characterization of the antibacterial properties of the resulting fabric is performed without reference to changes in important textile related properties such as tensile strength, wrinkle recovery, whiteness index, comfortability *etc.* Therefore, characterization of these parameters along with comfort properties is necessary to evaluate the potential application of antibacterial textile. In this study, chitosan-neem nano complex (CNNC) was synthesized through multiple emulsion-solvent evaporation (MESE) method. The nano emulsion of CNNC *i.e.*, chitosan-neem nano emulsion (CNNE) was applied on silk fabric by conventional pad-dry-cure process and the antibacterial and physical properties of the treated fabric investigated.

MATERIALS AND METHODS

Commercially degummed, bleached and unfinished Murshidabad silk fabric (1/1 Plain, 62 ends/cm, warp 22^d reeled silk yarn, 54 picks/cm, weft 34^d reeled silk yarn, 15.98 cover factor, 56.2 g/m²) was used.

Synthesis of chitosan-neem nano complex (CNNC)

Twenty grams of neem leaf powder was mixed with

100 ml of methanol and kept at room temperature for 36 h. The supernatant was filtered twice using Whatman No. 1 filter paper and the filtrate was further used as neem extract. Chitosan solution (1%) was prepared by adding 1 g of chitosan to 1 % acetic acid (1 ml of acetic acid in 99 ml of distilled water) and stirred at room temperature for 24 h till a fine homogenous suspension was formed. The polymer solution was kept overnight at stand-still condition to remove air bubbles formed during stirring. Then, 15 ml of neem extract was added drop wise, to the prepared chitosan solution under constant stirring at room temperature. A chitosan neem complex (CNC) of blackish-brown colour was obtained which precipitated at the bottom. The precipitate was filtered and air dried. After that, CNNC was prepared using MESE technique. Chitosan solution (3 %) was added to 5 % (w/v) Tween 80 and placed in rotary shaker for 5 min. Simultaneously, 5 % Span 80 was prepared with palm oil and stirred for 10 min. Both the solutions were mixed in 9:1 ratio and stirred well for 5 min. To this, 0.01 g of tri poly phosphate (TPP) was added and kept in the rotary shaker for five minutes. The entire suspension was then incubated for 30 min in a water bath at 50 °C and then cooled. The CNNC formed was segregated from palm oil by repeated washing with petroleum ether. The CNNC was achieved with 90.37 % yield (Rajendran *et al.*, 2012).

Preparation of chitosan-neem nano emulsion (CNNE)

One tablet of phosphate buffer was deposited in a beaker and then added 50 ml of double distilled water with stirring until full dissolution. Double distilled water (50 ml) was added again with continuous stirring for 15 min. Finally, a solution of phosphate buffer of pH 7.4 was obtained. The prepared phosphate buffer solution (150 ml, pH 7.4) was poured into a suitable container followed by 30 g of CNNC. Stirring was continued for 10-15 min. A clear nano emulsion of CNNE (20 %) was formed.

Stability evaluation of CNNE

The emulsion stability was assessed by measuring the

particle size growth (Ostwald Ripening Effect) and polydispersity index (PDI) as a function of time at 5 °C. The nano emulsion prepared was filled into a bottle and flushed with nitrogen, prior to airtight closure with a plastic screw cap. Physical stability of the samples was evaluated by measuring their particle size and PDI changes at different storage period (day 0, day 30, day 60 and day 90) using dynamic light scattering (DLS) technique.

Characterization

Characterization of chitosan, neem, CNNC, silk fabric and silk fabric treated with CNNE were carried out by Fourier Transform Infrared (FT-IR) spectroscopy, Proton Nuclear Magnetic Resonance (¹H NMR) and Scanning Electron Microscope (SEM). The FT-IR spectrum of chitosan, neem, CNNC, silk fabric and silk fabric treated with CNNE were measured on Perkin-Elmer FTIR 6X with KBr discs keeping air as reference. In this analysis, a resolution of 4 cm⁻¹ was chosen and data collection was in the range of 4000 – 400 cm⁻¹. ¹H NMR spectrum for chitosan, neem and CNNC was recorded on a Bruker AV 400 (Switzerland). The surface morphology of CNC, CNNC, CNNE, silk fabric and silk fabric treated with different concentrations of CNNE were observed by using a SEM, Zeiss Model EVO 50 at 15 KV acceleration voltage with a resolution of 2.0 nm. Before placing the samples in SEM chamber, the samples were mounted onto an aluminium stud and sputter coated with gold/palladium for 180 s to prevent charging. The absorption spectrum of the reaction mixture was recorded at room temperature using UV-Vis Spectrophotometer (Model DR-324C, Time Star, India).

Fabric treatment

The silk fabric samples were padded separately with 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 % CNNE with 70 % wet pick up. Then they were dried at 95 °C for 5 min and cured at 150 °C for 3 min.

Evaluation of particle size distribution pattern

The measurement of particle size of CNNC and their

distribution pattern in CNNE were carried out using Brookhaven Instruments 90 plus, USA using dynamic light scattering technique.

Testing

Test on silk fabrics for the crease recovery angle was carried out by the Shirley Crease Recovery Tester as detailed in IS: 4681-1981 (Reaffirmed, 2004). A sample of silk, 15 mm × 40 mm dimension was cut from both warp and weft directions. The sample was carefully folded into half, kept between two glass plates and 1 kg weight was placed on top of it. After 5 min, the load was removed and the sample was placed on the fabric clamp in the tester and allowed to recover from creasing. Finally, the fabric crease recovery angle was read and recorded. The same procedure was carried out for measuring wet crease recovery angle for which each sample was immersed in water for 1 min and then placed between two pieces of blotting paper for 2 min to remove excess water (Talebpour and Holme, 2006).

Tensile strength of the fabric in both warp and weft directions were measured on an Instron Tensile Strength Tester as per the BS: 2576 (1967) method. Ten fabric samples of each treatment were extended in a direction parallel to the warp and ten, parallel to the weft so that no two samples contained the same longitudinal threads. The specimen fabric samples were cut to a size of 60 mm × 300 mm and then frayed down in the width equally at both sides to give samples which were exactly 50 mm wide. This ensured that all the threads ran the full length of the sample hence, contributing to the strength and also that the width was accurate. The rate of extension was set to 50 mm/min and the distance between the jaws (gauge length) was set to 200 mm. As per norms, any breaks that occurred within 5 mm of the jaws were rejected. The data of each treatment was averaged from the data of ten fabric samples. The measurement of tearing strength of the fabric was carried out on tearing strength tester (KARL FRANK GMBH) as per ASTM D1424-96 (Reapproved, 2004). A sample of 63 × 100 mm of the silk fabric was cut for testing.

Bending lengths of treated and untreated samples were determined according to the ASTM D1388-96 (Reapproved, 2002) protocol using a fixed angle flexometer (Paramount Stiffness Tester, Coimbatore, India). A fabric sample, 200 mm × 25 mm was first mounted on the horizontal stage under the weight of the graduated ruler specially designed for this experiment and pushed forward until it hanged downward to an angle of 41.5°. By using Reflection Spectrophotometer (Colour Eye – 2180 UV, Macbeth, USA), the fabric whiteness changes after finishing were evaluated according to ISO 105-J02:1997 Textiles. Three readings were taken for each sample and the average values were reported. Air permeability tester (Innolab India, Haryana, India) was used to measure the air permeability at 98 Pa air pressure. ASTM D 737-04 test standard was followed and an average of three readings was recorded.

The antibacterial activity was quantitatively evaluated against *Staphylococcus aureus* (ATCC 25923), a Gram-positive bacteria and *Escherichia coli* (ATCC 25922), a Gram-negative bacteria, according to ASTM E 2149-2001 test method. An incubated bacterial culture in a nutrient broth was diluted using a sterilised 0.5 mM phosphate buffer (KH₂PO₄, pH 6.8) in order to obtain a final concentration of 1.5–3.0 × 10⁵ colony forming units (CFU)/ml. This solution was used as a working bacterial dilution. The treated fabric sample (1g) was cut into small squares (5 × 5 mm) and transferred to a 250 ml Erlenmeyer flask containing 50 ml of the working bacterial dilution. All the flasks were loosely capped, placed in the incubator and shaken for 1 h at 120 rpm at 37 °C using a Wrist Action incubator shaker. After a series of dilutions using the buffer solutions, 1 ml of the diluted solution was plated in nutrient agar. The inoculated plates were incubated at 37 °C for 24 h. Then the optical density (OD) at λ_{max} (660 nm) of the solution was measured with the help of Spectrophotometer. From OD reading, the CFU/ml was calculated referring 1 OD = 2.04 × 10⁸ CFU/ml. The same treatment was carried out for untreated fabric samples. The percentage reduction of bacteria was calculated as follows:

$$\text{Reduction of bacteria (\%)} = \left[1 - 10^{-(\log_{10} \text{CFU/ml}_T - \log_{10} \text{CFU/ml}_U)} \right] \times 100$$

where, CFU/ml_U and CFU/ml_T are the colony forming units per ml of untreated and treated fabric samples, respectively. To evaluate the durability of CNNE on fabrics against several wash cycles, washing of the samples was carried out as per IS: 687-1979 by using a neutral soap (Iodet T- 0.5 %) at 40 ± 2 °C for 30 min, keeping the MLR at 1:50 followed by rinsing, washing and drying. After drying, the test samples were assessed for antibacterial activity after 10 washing cycles using the method mentioned above.

RESULTS AND DISCUSSION

FT-IR and ¹H NMR have served as the basis for structure characterization in this study. The FT-IR spectra of neem, chitosan and CNNC are shown in Figure 1. The FT-IR spectrum of neem extract is shown in Figure 1a. The spectrum of neem extract shows characteristic peaks at 3250 cm⁻¹ (hydroxyl), 1798 cm⁻¹ (ester and acetate), 1673 cm⁻¹ (olefinic linkage/ α , β -unsaturated carbonyl), 1619, 1537 and 1450 cm⁻¹ (tri-substituted double bond) and 1072 cm⁻¹ (furan) (Purwar *et al.*, 2008). In the chitosan spectrum (Figure 1b), distinctive absorption bands appear at 1651 cm⁻¹ (Amide I) and 1593 cm⁻¹ (-NH₂ bending). The absorption bands at 1156 cm⁻¹ (asymmetric stretching of the C-O-C bridge) and 1068 cm⁻¹ (skeletal vibration involving the C-O stretching) are characteristics of its saccharine structure. The aliphatic C-H stretching vibration of the polymer backbone occurred at 2923.7 cm⁻¹ (Banerjee *et al.*, 2002). The weak deformation vibration of C-CH₃ appeared at 1383.8 cm⁻¹ indicating a high degree of de-acetylation of chitosan used in this experiment (Zheng and Du, 2002). CNNC (Figure 1c) shows quite different FT-IR spectrum from Figures 1a and 1b, demonstrating the formation of nano complex between chitosan and neem. The FTIR spectrum of synthesized CNNC shows amide carbonyl stretching at 1636 cm⁻¹ and carboxylic carbonyl stretching in the range of 1710-1750 cm⁻¹. The decrease of peak at 3428.2 cm⁻¹ in Figure 1c indicates the reduction of free -NH₂ groups after formation of CNNC which is an indication of the binding of -COOMe group of azadirachtin of neem to the -NH₂ group of chitosan and formation of -NH-CO- bond (Mao *et al.*, 2004).

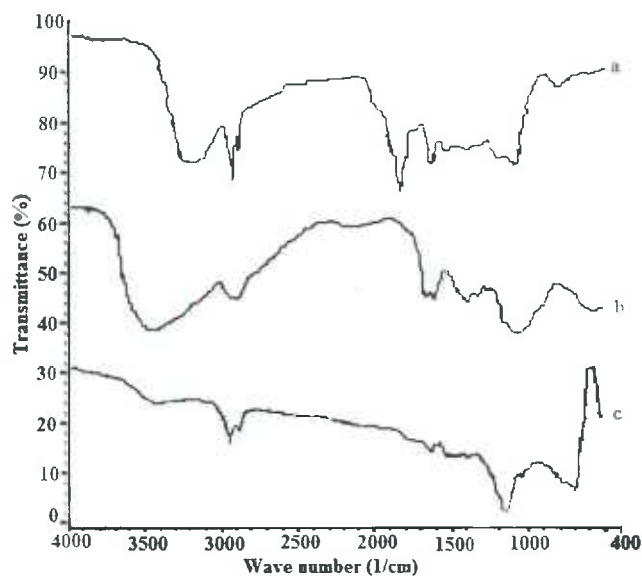


Figure 1: FT-IR spectra of a: neem, b: chitosan and c: CNNC

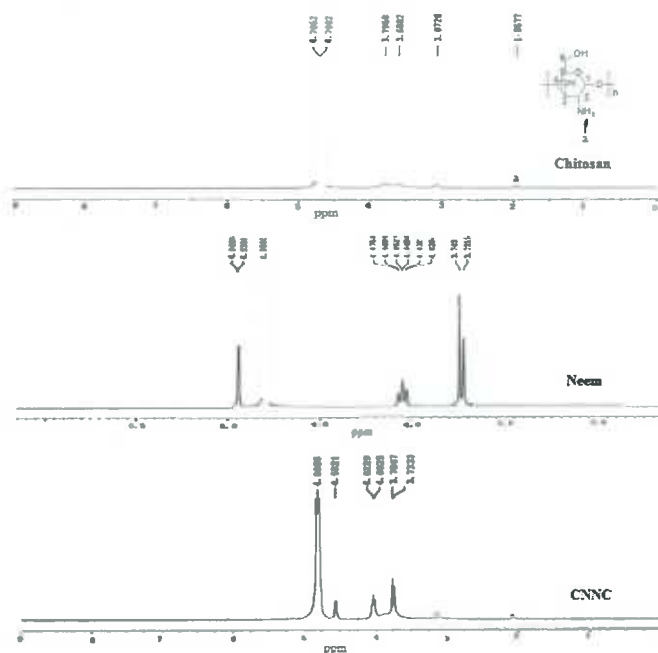


Figure 2: ¹H NMR spectra of chitosan, neem and CNNC

The ¹H NMR spectra of chitosan, neem and CNNC is shown in Figure 2. The ¹H NMR spectra of chitosan exhibited typical peaks, including the proton on the carbon bearing amino groups at 1.9577 ppm (a: -NH₂). The signals at 3.0726 ppm, 3.6082 ~ 3.7950 ppm and 4.7052 ppm are corresponding to the ring methenyl protons of chitosan backbones (H2, H3, H4, H5, H6 and H1). The chemical shifts in the ¹H NMR spectra of neem are assigned as follows: 4.94 ppm (CH-ring), 4.02-4.07 ppm (-CH) and

3.72-3.74 ppm ($-\text{CH}_2$). The ^1H NMR spectra of CNNC shows the characteristic peaks of chitosan and neem with certain chemical shifts because of the ionic interactions. The signal at 4.55 ppm is characteristic of the hydrogen at the $-\text{COOMe}$ of the neem which appeared at 4.94 ppm before the formation of CNNC through the reaction between $-\text{NH}_2$ of chitosan and $-\text{COOMe}$ of neem and formation of $-\text{NH}-\text{CO}-$ linkage between chitosan and neem.

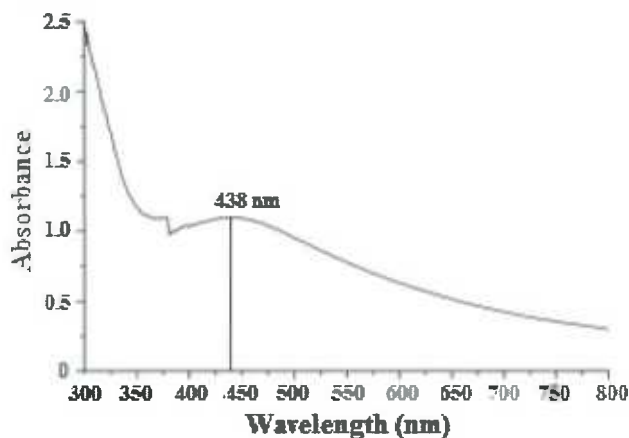


Figure 3: UV-Vis spectra of CNNE

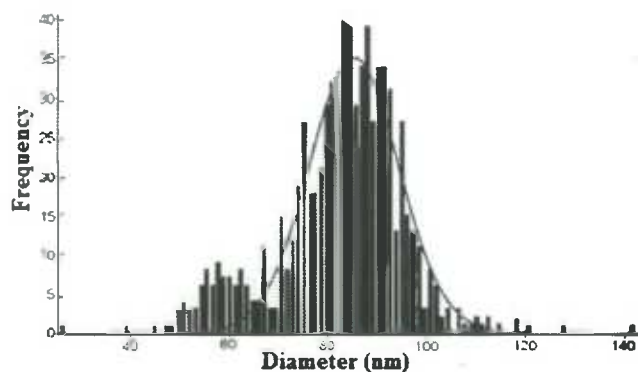


Figure 4: Histogram of particle size distribution in CNNE

The formation of CNNE from CNNC by dissolving in phosphate buffer is confirmed by the UV-Vis spectroscopy analysis (Figure 3). The change of pale yellow colour of CNNC to colourless emulsion is an indication of the formation of nano particles of CNNE within the resultant emulsion. The change in colour occurs due to the observable fact of surface plasmon excitation in the nano particles (Suman *et al.*, 2013). When the CNNC was added to the distilled water and

stirred for 10-15 min, an emulsion of pale yellow colour was obtained. After 10-15 min, the colour changes from pale yellow to a colourless one. The absorption spectra of CNNC formed in the reaction mixture was obtained by the UV-Vis analysis at the range between 300 and 800 nm, the CNNC has sharp absorbance with the highest peak at 438 nm and progressively decreased while nanometer increased (Forough and Farhadi, 2010). The aggregation of nano particles was controlled by adding tri-sodium citrate. The CNNC particles form a negative surface charge due to the adsorbed citrate ions. Each surface of CNNC particles surrounded by citrate ions, therefore repelled each other and prevented further aggregation. The histogram of nano particle size distribution is reported in Figure 4. The particles are nearly spherical and their mean diameter value is found to be 85.5 nm with a standard deviation of 9.07 nm.

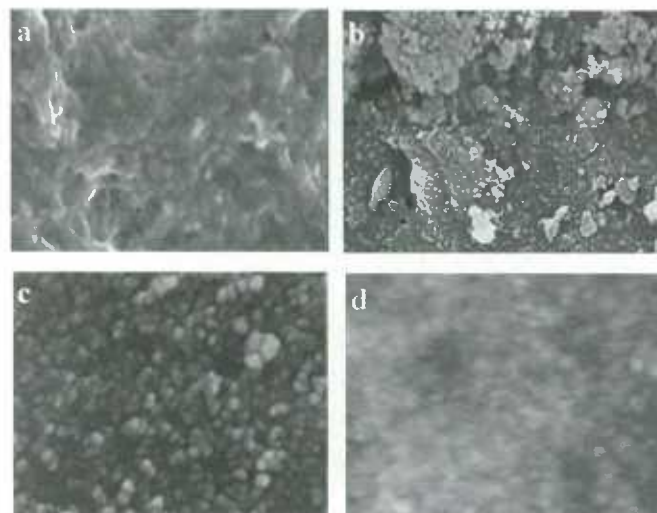


Figure 5: SEM photograph of (a) CNC (b) CNNC (c) CNNE up to 60 days of storage period and (d) CNNE after 60 days of storage period

The surface morphological features of CNC, CNNC and CNNE are depicted in Figure 5. SEM image shows that the CNC is a layered structure often called multilayered periodicity whereas the SEM image of CNNC shows an uneven morphology and roughness with higher compactness as compared to CNC. The CNNE shows almost spherical particles with average diameter of 85.5 nm which was in accordance with the dynamic light scattering analysis.

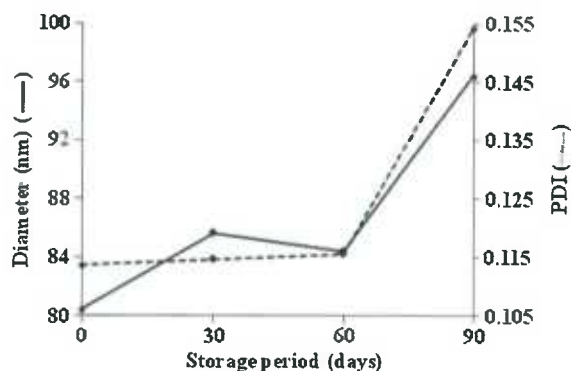


Figure 6: Effect of CNNE storage period on the growth of particle size and PDI

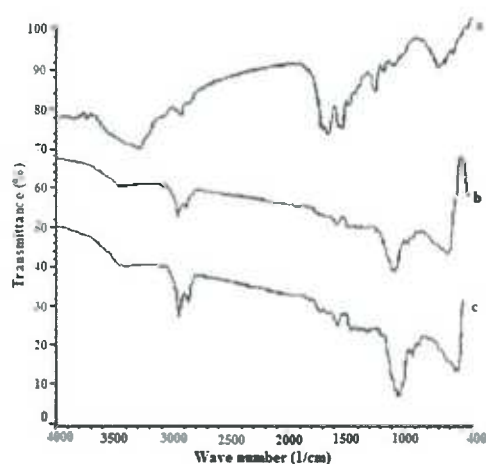


Figure 7: FT-IR spectra of a: silk fabric, b: CNNE and c: CNNE treated silk fabric

Figure 6 shows the result of nano particle size and PDI of CNNE every 30 days for a 90 days storage period. The CNNE particle size ranged from 83.4 to 84.2 nm with the PDI in the range of 0.106 to 0.116 at the end of 60 days storage period at 5 °C. Results also reveal that there are no significant differences in particle size and PDI of CNNE during the 60 days storage at 5 °C. This might be due to the fact that the nano emulsion prepared in this experiment managed to prevent oxidation effect on CNNE loaded in the disperse phase. Besides that, precautions taken during experiment such as the use of bottles with good screw cap to store emulsion and nitrogen flushing after every sampling might contribute to these results. After 60 days of storing, the particle size and PDI were increased significantly (19.4 % and 37.74 %, respectively). This may be due to the degradation of chitosan-neem after being exposed in the dispersed phase for a long period of time.

The FT-IR analysis of CNNE treated silk fabric was carried out to understand the mechanism of fixation. The untreated silk fabric (spectrum a in Figure 7) show the absorption band at 1647 cm^{-1} assigned for amide stretching and the peaks at 2925 and 3301 cm^{-1} were attributed to the C-H stretching and N-H stretching deformation. The absorption bands at 1516 cm^{-1} and 1458 cm^{-1} appeared at different intensities in the spectrum of untreated silk; these are characteristics of carboxylate anion stretching and phenolic OH bending, respectively. Carboxylate anion stretching accounts for the presence of a free carboxylic acid group at the end of the polypeptide chains and phenolic OH accounts for the presence of tyrosine and serine fractions of amino acids in the untreated silk. The spectrum of prepared CNNE (spectrum b in Figure 7) shows amide carbonyl stretching at 1636 cm^{-1} and carboxylic carbonyl stretching in the range of 1710–1750 cm^{-1} . The selective acylation of the amino groups is probably due to their superior nucleophilic character compared to the surrounding hydroxyl groups. Finishing of silk fabric with CNNE (spectrum c in Figure 7) results in the appearance of a strong absorption band in the region 1716 cm^{-1} , characteristic of ester stretching with retention/marginal intensification of band at 1562 cm^{-1} , characteristic of carbonyl anion with almost disappearance/substantial weakening of the absorption band at 1458 cm^{-1} , characteristic of phenolic OH. The trend of change in functional group pattern appears to be in line with the reaction mechanism proposed in Figure 8.

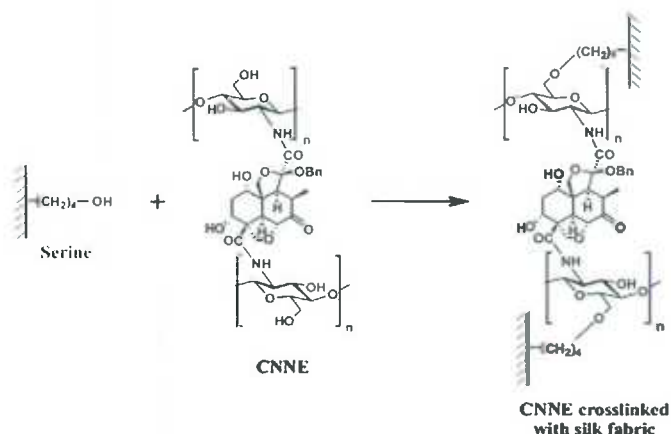


Figure 8: Mechanism of fixation of CNNE with silk fabric

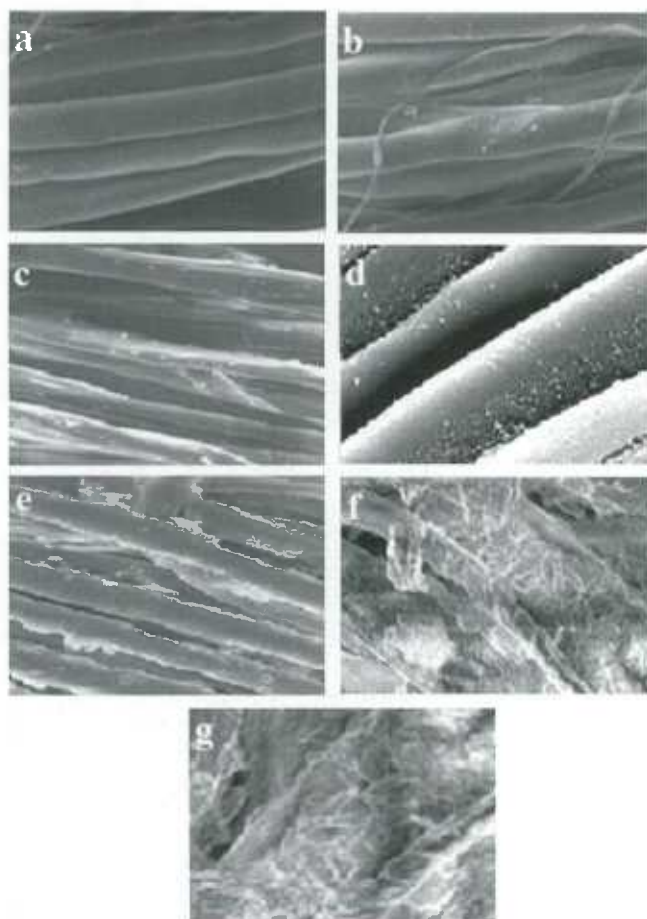


Figure 9: SEM photograph of (a) untreated silk fabric and (b-g) silk fabrics treated with different concentration (0.5-3%) of CNNE

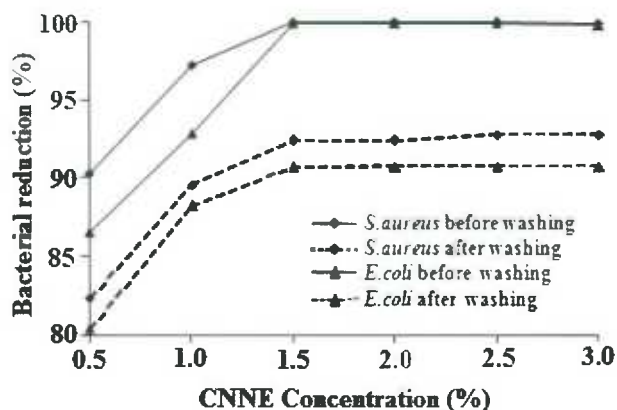


Figure 10: Effect of CNNE concentration on antibacterial activities of silk fabric before and after ten washing cycles

The SEM image as presented in Figure 9 a shows untreated silk fabric with a typically smooth surface. In

Figures 9 b-g, silk fabric samples treated with CNNE demonstrated the chitosan-neem nano particle adhesion on silk fibre and the resultant fabric surface at different concentrations of CNNE. It is clearly observed that the amount of chitosan-neem nano particles on the fabric surface and penetration into the fibre interstices increase with the increase in the concentration of CNNE from 0.5 to 1.5 % (Figures 9 b, c). In contrast, the image of silk fabric treated with 1.5 % CNNE (Figure 9 d) shows a uniform distribution of chitosan-neem nano particles on the silk fibres. This is likely due to easy penetration of chitosan-neem nano particles in the yarns and fibres or by trapping in the fibril network of the silk fibre structure with the help of the ion-dipole attraction between the CNNE and the silk fabric. At higher concentration *i.e.*, beyond 1.5 % CNNE, the deposition of nano particles on to the fabric surface is further increased which results in the agglomeration of nano particles within the fibres and on the fabric rather than penetration within the fibre interstices (Figures 9 e, f). This agglomeration ultimately forms a thin layer on to the fabric surface (Figure 9 g).

The antibacterial property (expressed by per cent reduction rate of bacteria) of the CNNE treated silk fabric before and after washing are shown in Figure 10. The activity increased as the amount of CNNE applied increased and the optimum reduction of bacteria (99.98 % for *Staphylococcus aureus* and 99.88 % for *Escherichia coli*) was obtained at 1.5 % concentration of CNNE. The antibacterial activity as proposed by a considerable amount of CNNE may coat the bacterial cell surface resulting in changes in permeability which causes death of cell by inducing leakage of the intercellular components (Shin *et al.*, 2001, No *et al.*, 2002). The antibacterial activities of the CNNE treated silk fabric was maintained reasonably (92.44 % for *Staphylococcus aureus* and 90.73 % for *Escherichia coli*) even after 10 washing cycles. The test results show that CNNE is fixed on the silk fabric by forming covalent bonds with silk fabric which are durable against repeated washing (Noerati *et al.*, 2008).

Table 1 shows the mechanical properties of untreated and CNNE treated silk fabrics. It reveals that with the increase in CNNE concentration, the DCRA and WCRA increases significantly up to 1.5 % and thereafter it remains more or less constant. This is due to the increase in the cross-linkages formed between the silk polymer molecules with CNNE. However, at higher concentration (>1.5 %), the CNNE reacts too severely with the fibre and forms a thick layer on the surface of the fabric. This reduces the resiliency of the fabric resulting in insignificant improvement in DCRA and WCRA. It is also observed that the WCRA is always lower than that of DCRA. This is due to the breaking of hydrogen bonds and some of the salt linkages between polymers with the entry of water molecules (Yang and Li, 1993). Both the tensile and tear strength of silk fabric decreases slowly up to 1.5% concentration of CNNE and then rapidly in both warp and weft directions. The CNNE reduces the friction between the fibres in a yarn and between the yarns within the fabric which results in more slippage of these entities which in turn cause reduction in load bearing capacity. This ultimately results in decrease in tensile and tear strength. The application of CNNE onto silk fabric reduces the bending length in both warp and weft directions up to 1.5 % concentration, and thereafter, it increases. At lower concentration, *i.e.*, up to 1.5 %, the droplet or the particle size of nano emulsion is so small that it can penetrate into the micro and nano structures of silk fabric very well and act as a lubricating agent between the fibres in the yarn and between the yarns of the fabric, imparting softness to the material as a whole. This

softness causes a reduction in bending length. However, at higher concentration (>1.5 %), the viscosity of the emulsion increases which ultimately results in agglomeration of nano particles. Due to this agglomeration, it is hardly possible for the nano particles to penetrate into the micro and nano structure of the substrate which ultimately leads to the deposition of the same onto the surface and formation of thick layer. This ultimately imparts stiffening to the samples. This stiffness causes a significant increase in bending length. No significant change in fabric whiteness was observed up to 1.5 % CNNE concentration. However, when the concentration goes beyond 1.5 %, the whiteness of the fabric starts reducing significantly. This may be due to increase in the severity of reaction between CNNE and fibre at higher concentration and the formation of a thick layer of CNNE on the surface of the silk fabric. However, the air permeability of the fabric remains approximately unaltered. This may be due to the porous nature of the chitosan-neem nano particle (Yang and Li, 1993).

Hence, the treatment of chitosan-neem nano emulsion (CNNE) on silk fabric at 1.5 % concentration was shown not only to impart excellent antibacterial property against Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) bacteria with good durability but also to improve wrinkle resistance with sufficient whiteness and tolerable strength loss without hampering the fabric comfort properties with respect to air permeability and bending length.

Table 1: Mechanical properties of untreated and CNNE treated silk fabrics

CNNE concentration (%)	DCRA (W+F) deg.	WCRA (W+F) deg.	Tensile strength		Tear strength		Bending length		Whiteness index	Air permeability (cm ³ /cm ² /s)
			Warp (kg-f)	Weft (kg-f)	Warp (g)	Weft (g)	Warp (cm)	Weft (cm)		
0	210	178	45	35	1046	1036	1.76	1.52	78.4	26.2
0.5	218	182	42	33	1028	978	1.58	1.47	77.2	25.9
1.0	239	192	41	31	997	962	1.43	1.40	77.1	26.0
1.5	258	212	40	29	981	929	1.40	1.38	77.0	25.9
2.0	260	213	37	26	841	814	2.66	2.17	68.6	25.6
2.5	262	212	33	24	768	758	2.71	2.31	61.3	26.0
3.0	263	212	32	20	746	739	2.75	2.33	53.7	25.6

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Article scientifique

FINITION ANTIBACTERIENNE DES TISSUS DE SOIE PAR APPLICATION D'UNE NANO-EMULSION DE MARGOUSIER - CHITOSAN

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RÉSUMÉ

L'attitude du consommateur vis à vis de l'hygiène et le mode de vie active ont créé une croissance rapide du marché pour les textiles antimicrobiens ce qui a stimulé une recherche et un développement intenses dans ce domaine. Notre étude est concentrée sur la synthèse d'un nano complexe de margousier – chitosan (CNNC) pour la finition antibactérienne des tissus de soie. La synthèse du CNNC a été menée par de multiples émulsions-évaporation de solvant (MESE). Des spectres de FTIR et de ¹H NMR ont été utilisés pour caractériser la structure du CNNC. La nano émulsion de CNNC c'est-à-dire la nano émulsion chitosan-margousier (CNNC) avec une taille moyenne de particules de 85 nm a été préparée en ajoutant des quantités calculées de tampon phosphate (pH 7,4) et appliqué sur le tissu de soie à différentes concentrations (0,5 à 3 %) par tampon avec 70 % d'humidité. L'activité antibactérienne et les propriétés des tissus traités comme la résistance à la tension, le dépliage, l'indice de blancheur, la longueur de pliage et la perméabilité à l'air ont été évaluées. Le tissu traité possède une résistance au pliage satisfaisante, une blancheur suffisante, une faible décroissance de la force de résistance et une réduction plus importante du taux de multiplication des bactéries si l'on compare au tissu non traité et ceci sans affecter les propriétés de confort en respectant la perméabilité à l'air et la longueur de pliage.

Mots-clés: Finition antibactérienne, nano émulsion de chitosan-argousier, tissu de soie.



EVALUATION OF PROMISING SILKWORM HYBRIDS FOR SUSTAINABLE COCOON PRODUCTION

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ABSTRACT

Sustainable sericulture development necessitates the development and exploitation of promising silkworm hybrids or more specifically the most adaptable ones for each location, season and climatic type. A multilocal testing programme comprising of ten silkworm hybrids (DMMMSU 209, DMMMSU 406, DMMMSU 203, DMMMSU 436, DMMMSU 408, DMMMSU 218, DMMMSU 224, DMMMSU 213, DMMMSU 346, and DMMMSU 222-check variety) was drawn with locations viz., Don Mariano Marcos Memorial State University-Sericulture Research and Development Institute (DMMMSU-SRDI), Bacnotan, La Union and Mariano Marcos State University (MMSU) Batac, Ilocos Norte, representing climatic type I; University of Rizal System (URS) Tanay, Rizal and Quirino State College (QSC) Quirino, representing climatic type III. Yield data were consolidated and analyzed. A combined analysis of variance across locations, climatic type, season and year was performed. Adaptability and stability of 10 silkworm hybrids were determined using the Bilbro-Ray procedure. DMMMSU 203, DMMMSU 406, DMMMSU 408, and DMMMSU 346 were found the most stable, producing above average yield in all seasons and sites and hence were adjudicated as adaptable to climatic types I and III while DMMMSU 346 was observed the most adapted to climatic Type III conditions in the Philippines.

Key words: Four-way cross hybrids, multilocal testing, silkworm hybrids, three-way cross.

INTRODUCTION

Sericulture is a promising agro-based industry with economic, social and ecological potentials in line with national and international programs on inclusive growth, environmental sustainability and Millennium Development Goals. However, its sustenance and development should be supported by availability of adaptable and promising silkworm hybrids which are outputs of continuous breeding efforts in Philippine government institutions like the Sericulture Research and

Development Institute of the Don Mariano Marcos Memorial State University (DMMMSU-SRDI.)

Earlier, Gapuz *et al.* (1997 a) identified DMMMSU 213, DMMMSU 203, DMMMSU 224, DMMMSU 218, DMMMSU 209 and DMMMSU 222 as the most promising hybrids. Subsequently, Bunnao *et al.* (1999) identified DMMMSU 308, DMMMSU 342 and DMMMSU 346 as the best three-way cross hybrids. Likewise, DMMMSU 408, DMMMSU 406, and DMMMSU 436 were identified by Gapuz *et al.* (1997 b) as

the most promising four-way cross hybrids.

Frankel (1958) pointed out that in meeting the demands for varieties better adapted to changing conditions, breeder is faced with the choice of breeding either for closely defined ecological conditions or for more extensive conditions which include a considerable range of environments. The latter approach requires the development of varieties possessing general adaptability; the alternative approach being breeding varieties adapted to closely defined ecological conditions.

One of the main reasons for growing genotypes in a wide range of environments is to estimate their adaptability and stability. To measure adaptability and stability of a certain genotype that is grown in a wide range of environment, Bilbro and Ray (1976) and Bonilla (1986) used regression coefficient and the coefficient of determination (R^2), respectively.

Bilbro and Ray (1976) adopted these measures in their study on environment stability of 10 cotton varieties because they believed that stability is a measure of dispersion of yields around a regression line and the slope of the regression line is a measure of adaptation that when R^2 is equal to 1, the variety is stable and when R^2 is less than 1, the variety is unstable. A variety has average adaptability when $b = 1$, below average adaptability when b is greater than 1 (adapted to favorable environments); and above average adaptability when b is less than 1 (adapted to poor environments).

Thus, to further support sericulture development and sustainability in the Philippines, this study was conducted to identify the most promising silkworm hybrid under each climatic type and season in selected sericulture potential areas in Luzon, Philippines.

MATERIALS AND METHODS

Test hybrids, testing locations and climatic types

The identified, promising silkworm hybrids were tested for their adaptability and productivity under different agro-climatic and ecological conditions as well

as in other potential sericulture areas of the Philippines. Nine silkworm hybrids viz., DMMMSU 209, DMMMSU 406, DMMMSU 203, DMMMSU 436, DMMMSU 408, DMMMSU 218, DMMMSU 224, DMMMSU 213 and DMMMSU 346 selected for the purpose consisted of five F1, one three-way cross and three double cross hybrids. They were chosen from the silkworm germplasm of DMMMSU-SRDI and subjected for multilocal testing using the commercial hybrid, DMMMSU 222 as check.

The yield performance of these promising silkworm hybrids were evaluated under different locations viz., Don Mariano Marcos Memorial State University-Sericulture Research and Development Institute (DMMMSU-SRDI), Bacnotan, La Union and Mariano Marcos State University (MMSU) Batac, Ilocos Norte, representing climatic type I; University of Rizal System (URS) Tanay, Rizal and Quirino State College (QSC) Quirino, representing climatic type III, all in Luzon, Philippines.

Data analysis

Yield data of all the ten hybrids tested were consolidated and analyzed. Data were subjected to combined analysis of variance across locations while determination of adaptability and stability was done using the Bilbro-Ray (1976) procedure. The variety means were regressed against the location means and the regression coefficient b was considered as the measure of adaptability while the coefficient of determination (R^2) was expressed as the measure of stability.

Regression coefficients approximating to 1.0 indicate average stability. When this is associated with high mean yield, hybrids have general adaptability; when associated with low mean yield, hybrids are poorly adapted to all environments. Regression value that stands above 1.0 describe hybrids with increased sensitivity to environmental change (below average stability), and greater specificity of adaptability to high-yielding environments. Regression coefficients falling below 1.0 provide a measure of greater resistance to environmental

change (above average stability), and therefore increasing specificity of adaptability to low-yielding environments.

RESULTS AND DISCUSSION

The nine silkworm hybrids and a check hybrid DM³SU 222 were used for the combined analysis of variance (ANOVA), and regression was used to measure yield stability and adaptability following Bilbro-Ray procedure.

Table 1: Combined analysis of variance (ANOVA) of 10 silkworm hybrids tested in five (5) locations

Source of variance	Degrees of freedom	Sum of squares	Mean squares	Computed F	Tabular F Level of significance	
					.05	.01
Location (L)	4	10207.3571	2551.8393			
Hybrids (H)	9	285.8694	31.7633	4.79**	1.97	2.59
H x L	36	658.6230	18.2951	2.76**	1.54	1.83
Pooled Error	100	663.0408	6.6304			

The combined analysis of variance on cocoon yield box⁻¹ of the 10 hybrids reared in five locations representing type I and type III climatic conditions showed highly significant differences among hybrids and hybrid x location effects (Table 1).

Interaction effects between hybrids and location indicate that the variability of each of the test locations contributed to the differences in cocoon yield performance. This indicates that the performance of the different hybrids on cocoon yield box⁻¹ in the different locations were not at par with each other and can be attributed to the fact that silkworms being poikilothermic, are easily affected by the prevailing rearing conditions which differs in the different growing locations (Sudhakara Rao *et al.*, 2001).

Test of mean differences using LSD showed that four hybrids significantly outyielded check hybrid DMMMSU 222, while the other hybrids produced on par

with that of the check hybrid. Results of regression analysis revealed that all the hybrids were stable with stability values ranging from 0.901 to 0.985 indicating stability of yield under any of the test locations.

Regression analysis showed that four hybrids were adaptable to favorable environment with adaptability value (b) of greater than one while five hybrids were adaptable to poor yielding environments.

Table 2: Adaptability and stability measure of 10 hybrids tested in five locations

Hybrid	Mean yield (kg/box)	Difference	Adaptability b (value)	SE coefficient	R ²	Adaptability	Stability
DM ³ SU 213	30.06	3.13**	0.942	0.142	0.936	APE	Stable
DM ³ SU 406	29.90	2.97**	0.998	0.071	0.985	APE	Stable
DM ³ SU 346	29.42	2.47**	1.157	0.092	0.981	AFE	Stable
DM ³ SU 408	29.38	2.45**	0.801	0.153	0.901	APE	Stable
DM ³ SU 203	28.65	1.71ns	0.794	0.069	0.978	APE	Stable
DM ³ SU 436	28.52	1.59 ns	1.126	0.099	0.977	AFE	Stable
DM ³ SU 224	27.44	0.51 ns	1.094	0.155	0.943	PAAE	Stable
DM ³ SU 218	27.35	0.42 ns	1.032	0.179	0.918	PAAE	Stable
DM ³ SU 222 (control)	26.93	-	0.960	0.121	0.954	APE	Stable
DM ³ SU 209	26.60	1.33 ns	0.154	1.154	0.944	PAAE	Stable

LSD 0.05 = 1.802; 1.01 = 2.388; APE - Adaptable to poor environment; AFE - Adapted to favourable environment; PAAE - Poorly adapted to all environments.

The results reveal that the different bivoltine hybrids when reared at different locations showed highly significant differences with each other in all the characters evaluated. This indicates that the productivity of the different silkworm hybrids vary when reared in different locations. These results conform to the findings of Chandrashekharaiah (1986) who reported that the degrees of expression of characters differ under various environmental conditions.

Table 3: Yield Performance of 10 silkworm hybrids tested in five (5) locations under three rearing seasons (kg/box).

Hybrid	URS	SRDI	QSC	NVSU	MMSU	Average
DM ³ SU 222 (Control)	35.42	28.58	27.13	31.76	11.77	26.93
DM ³ SU 209	40.92	31.59	19.76	28.10	10.93	26.26
DM ³ SU 406	41.40	30.23	27.36	34.26	16.26	29.90
DM ³ SU 203	38.54	29.23	27.00	30.53	17.93	28.65
DM ³ SU 436	41.74	29.57	28.13	31.80	12.03	28.65
DM ³ SU 408	37.20	30.14	31.63	31.60	16.32	29.38
DM ³ SU 218	37.55	34.38	21.80	29.93	13.07	27.35
DM ³ SU 224	39.52	30.77	20.30	33.03	13.59	27.44
DM ³ SU 213	40.01	29.05	30.50	34.80	15.93	30.06
DM ³ SU 346	42.24	32.69	23.83	34.23	14.09	29.42
Average	39.46	30.62	25.74	32.00	14.19	
Grand Mean						28.40

URS – University of Rizal System; SRDI – Sericulture Research and Development Institute; QSC – Quirino State College; NVSU – Nueva Vizcaya State University; MMSU – Mariano Marcos State University.

DM³SU 218, DM³SU 224 and DM³SU 209 had a regression coefficient approximating 1.0 ($b=1.032$, 1.099) but consistently produced below average yields. They are recognized as poor adapters to all environments. On the other hand, DM³SU 436 a double cross silkworm hybrid and DM³SU 346 a 3-way cross hybrid are typical hybrids which have expressed highly sensitive responses to changes in the environment. These hybrids yielded very little in a low-yielding environment, but responded positively as the environmental condition improved. Under the most favorable condition, they happened to be the highest yielding hybrids. These hybrids can therefore be described as being specifically adapted to high-yielding/favorable environments, and were characterized by a regression coefficient significantly greater than 1.0 ($b=1.126$ and 1.157 , respectively).

DM³SU 406, DM³SU 213, DM³SU 408 and DM³SU 203 showed average stability, with regression coefficient b of 0.942, 0.998, 0.801 and 0.794, respectively. These hybrids produced above average yields in all seasons at all sites displaying their general adaptability. The silkworm

hybrids which exhibited poor adaptability are DM³SU 224, DM³SU 218 and DM³SU 209. On the other hand, DM³SU 406 and DM³SU 346 were very sensitive to environmental changes. DM³SU 346 yielded poorly under poor environment but yielded high under the favorable conditions of type III climate of Region II.

Hence, the silkworm hybrids DM³SU 213, DM³SU 406, DM³SU 408 and DM³SU 203 are recommended for commercial cocoon production in all the different locations with Climatic Types I & Type III while DM³SU 346 could be commercially reared under good environment of Climatic type III in the Philippines.

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Article scientifique

EVALUATION D'HYBRIDES DE VER A SOIE PROMETTEURS POUR UNE PRODUCTION DURABLE DE COCONS

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RÉSUMÉ

Le développement d'une sériciculture durable nécessite le développement et l'exploitation d'hybrides prometteurs de ver à soie ou plus spécifiquement des plus adaptés pour chaque lieu, chaque saison et chaque type de climat. Un programme de tests dans de multiples localités de dix hybrides (DMMMSU 209, DMMMSU 406, DMMMSU 203, DMMMSU 436, DMMMSU 408, DMMMSU 218, DMMMSU 224, DMMMSU 213, DMMMSU 346, et la variété DMMMSU 222-check), ont été utilisés dans les localités de Don Mariano Marcos Memorial State University-Sericulture Research and Development Institute (DMMMSU-SRDI), Bacnotan, La Union et Mariano Marcos State University (MMSU) Batac, Ilocos Norte, représentant le type climatique I; l'University of Rizal System (URS) Tanay, Rizal et le Quirino State College (QSC) Quirino, représentant le type climatique III. Les données moyennes ont été consolidées et analysées. Une analyse combinée de variance parmi les localités, le type climatique, les saisons et l'année a été réalisée. L'adaptation et la stabilité des 10 hybrides de vers à soie ont été déterminées par le procédé Bilbro-Ray. DMMMSU 203, DMMMSU 406, DMMMSU 408, et DMMMSU 346 apparaissent comme les plus stables, produisant au dessus de la moyenne en toutes saisons et dans tous les sites et ont donc été répertoriés comme adaptables aux conditions climatiques de type I et III alors que DMMMSU 346 s'est montré comme le plus adapté aux conditions climatiques de type III aux Philippines.

Mots-clés: Croisements hybrides quatre voies, test multi-localisé, vers à soie hybrides F1, croisement trios voies.



TROPICAL TASAR SEED COCOON PRESERVATION DEVICE (TASAR GRAINAGE TOOL: TGT) FOR ENHANCING GRAINAGE EFFICIENCY AT FARMERS' LEVEL

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ABSTRACT

Inadequate seed support corresponding to local conditions, makes several tasar practicing tribal farmers to preserve seed cocoons for egg production on their own. Though, the seed cocoon quantity is meager, they bound to face adverse conditions, intricacies during grainage activity and threat from predators for want of amenities. The seed cocoons of tasar silkworm, *Antheraea mylitta* Drury can however be preserved at farmer's level by applying simple modus-operandi appropriate to rural background. The present study is one such attempt of processing seed cocoons for egg production, applying a handy model of Tasar Grainage Tool (TGT), which occupy less space, suits for seed cocoon and moth preservation for mating synchronization, oviposition and egg incubation for improved egg yield and hatching, besides protecting from predators. TGT functions independent of electric power, almost not require operation skill and the most importantly is cost-effective. The study revealed that TGT could maintain temperature of 25.6 to 30.2 °C and relative humidity of 72.6 to 85.7 %, irrespective of ambient climatic conditions. The improved moth emergence (13.1 %) and pairing (17.4 %), oviposition (8.5 %), fecundity (11.4 %), egg hatching (8.09 %) and shortened moth emergence span (by 7 days) against controls, besides suitability for synchronizing moth mating, indicate the impact of appliance on the rural tasar seed production activities. Hence, the TGT has application prospective for cocoon preservation, grainage and egg handling of *A. mylitta* even by unacquainted tribal rearers from rural India.

Key words: *Antheraea mylitta* Drury, grainage activity, relative humidity, tasar silkworm seed, temperature.

INTRODUCTION

Safeguarding seed cocoon and carrying out silkworm seed production in tropical tasar culture are always critical, as the impact of seed quality on crop success is paramount. Though, the tasar silkworm, *Antheraea mylitta* Drury builds cocoons out-door even under unfavorable tropical weather conditions, the cocoons used for seed production require amiable environment for their preservation to sustain the quality. Safe handling of

seed cocoons, male moths for re-use and mating synchronization, mother moths during oviposition and egg during incubation, and providing required environment are must for obtaining uniform and optimal egg hatching on a particular day (Ahsan *et al.*, 1980; Narasimhanna, 1998; Narain *et al.*, 2001; Prasad *et al.*, 2001; Reddy *et al.*, 2003, 2005, 2009 a, 2009 b, 2009 c; Reddy and Prasad, 2010). Stocks of hibernating and non-hibernating tasar seed cocoon preservation before the commencement of grainage operation is one of the important steps in

protecting the seed cocoon stocks (Koundinya, 1989; Kumar and Thangavelu, 1993; Singh *et al.*, 1993; Prasad *et al.*, 2000; Rao *et al.*, 2000; Narain *et al.*, 2001; Saheb *et al.*, 2009; Reddy *et al.*, 2009 a; Reddy and Prasad, 2010). The environment under which both the seed cocoons and male moths are protected has great influence on the quality of silkworm eggs, in terms of their fertility status and embryo development, uniformity in hatching, larval health and subsequent crop performance (Ahsan *et al.*, 1980; Singh *et al.*, 1993; Dubey *et al.*, 1994; Gaur and Upadhyay, 2001; Narain *et al.*, 2001; Prasad *et al.*, 2001; Reddy *et al.*, 2003, 2010). To attain maximum egg recovery and to avoid shortage of male moth component towards the end of grainage activity, an optimal temperature of 24 to 28 °C and relative humidity of 75 to 85 % should be provided (Koundinya, 1989; Narain *et al.*, 2001; Reddy *et al.*, 2003, 2005, 2009 c, 2010). The temperature and relative humidity conditions prevailing during grainage activity have direct influence on duration of grainage, and thus the quality and economics of silkworm seed production, egg hatching and ensuing rearing performance (Koundinya, 1989; Kumar and Thangavelu, 1993; Singh *et al.*, 1993; Prasad *et al.*, 2000; Narain *et al.*, 2001; Saheb *et al.*, 2009; Reddy *et al.*, 2009 a, 2009 c, 2010; Hussain *et al.*, 2011 a). Improper preservation of seed cocoons under fluctuating temperature and relative humidity causes pupal weakening, irregular moth emergence, weak mating and oviposition pace (Kumar and Thangavelu, 1993; Kumar *et al.*, 1995; Reddy *et al.*, 2009 c; Hussain *et al.*, 2011 b). Low humidity, particularly during moth emergence stage makes the parental moths weak and thus reduce their copulation potency and reproductive efficiency (Koundinya, 1989; Kumar and Thangavelu, 1993; Singh *et al.*, 1993; Reddy *et al.*, 2003, 2010). The role of suitable devices for proper preservation of seed cocoons, operation of tasar grainage and incubation of tasar egg for timely and healthy hatching was studied (Koundinya, 1989; Kumar and Thangavelu, 1993; Singh *et al.*, 1993; Narain *et al.*, 2001; Rath *et al.*, 2002; Reddy *et al.*, 2009 a; Reddy and Prasad, 2010). In spite of various methods and devices put into practice, none has covered all scientific steps of silkworm egg production, and the traditional methods failed to provide the necessary consistent climatic conditions for the entire period of egg handling *i.e.*, from seed cocoon production to egg hatching. The

other existing equipment in general are expensive, dependent of electric power and cannot be used effectively in rural areas for want of fabrication facility and operation ability. Hence, a need based, simple but effective device, the Tasar Grainage Tool (TGT) was fabricated and evaluated for conduct of tasar silkworm seed production efficiently. The device is found rural friendly as it is electrically independent, needs almost no skill for operation, portable and can handle tasar seed during the course of its production to hatching, in a more scientific way.

MATERIALS AND METHODS

The TGT (Figure 1) consists of three components, namely middle metal grill frame covered with wire mesh (Figure 1 a), outer gunny cloth cover (Figure 1 b) and upper plastic water container (Figure 1 c). The metal frame is made up of 10 & 8 mm size iron rods by welding with dimensions of 1.5' x 1.5' x 5' (L x B x H) having upper round shaped hoop to rest plastic water container. Four legs of metal frame are fixed at the bottom with plain round washers for stable resting in small plates filled with water (ant-wells) to avoid the ant attack. The wire mesh of one cm sieve size is fixed on all sides and upper and lower surfaces of metal frame to avoid the entry of other predators. The door is fixed to main frame with hinges on front side to open and close as per the need. A gunny cloth cover of size 1.6' x 1.6' x 5.1' (L x B x H) is fixed on outer surface of metal frame, with a slit opening on front side and small cloth strips for fixing on to metal frame. The plastic water container of 20 litres capacity with small holes evenly made at its bottom, ridge kept on upper hoop of metal frame will allow water flow on to gunny cover for maintaining required moistness of the TGT without manual application of water.

The requirement of material and fabrication cost of TGT for carrying out tasar grainage activity with 500 seed cocoons is only ₹650 (Table I) and however, the cost may vary depending on size of the device. The advantages of device are that, the required material and its fabrication facilities will be available in rural localities and can also be modified as per requirement based on the quantum and

TASAR GRAINAGE TOOL (TGT)



Figure 1 a: Metal frame with wire mesh



Figure 1 b: Gunny cloth cover



Figure 1 c: Plastic water container



Figure 1 d: Seed cocoons preservation



Figure 1: Tasar Grainage Tool (TGT) in operation



Figure 1 e: Male moths preservation



Figure 1 f: Egg incubation

location of tasar cocoons to be handled. Due to its longer self-life, easy handling, portability, accommodative choice and electrically independent operation, the estimated expenditure on TGT is meager when compared to its application value.

The device can be put into use with the sequence of dipping gunny cloth cover and fixing on metal frame

rested on water filled plates (ant-wells) kept over sand bed made on the floor of rearer's house. The plastic container kept on hoop of metal frame should be filled with water and the flow on to gunny cloth for maintaining required moisture of TGT must be ensured. Hang seed cocoons in rows from the top part of metal frame to provide uniform aeration and space in between seed cocoon rows (Figure 1 d) or keep male moths for re-use or gravid female moths for

oviposition in paper boxes (Figure 1 e) or preserve tasar eggs in plastic boxes for incubation (Figure 1 f), and can close the door of TGT for safety. Sprinkle some water initially on gunny cloth cover for uniform wetting and to accelerate the water flow from plastic container on to gunny cloth to maintain optimal humidity and temperature inside the TGT. Care to be taken that the bottom edge of gunny cloth cover not to touch ground or water filled plates kept as ant-wells to avoid predator's entry into TGT. The water level in the plastic container needs to be checked at regular intervals for maintaining required conditions, @ 1 time a day at 10.00 a. m. during seed cocoon preservation and @ 3 times a day at 6.00 a. m., 2.00 p. m. and 10.00 p. m. during grainage activity and egg incubation.

The device (TGT) was designed keeping in view of the requirement of individual tasar rearers, who usually keeps a small quantity of seed cocoons (around 500 nos.) to produce 175 to 200 DFLs for their own use, as well to have a device of handy in nature, less expensive and affordable by poor tribal rearers. The efficiency of device was studied at Central Tasar Research and Training Institute (CTRTI), Ranchi, Jharkhand & Pilot Project Centre (PPC), Goilkera, West Singhbhum district of Jharkhand for the 1st grainage season of 2010-11 (from commercial crop cocoons of 2009-10) and 2nd grainage season of 2010-11 (from seed crop cocoons of 2010-11) simultaneously; using the tropical tasar seed cocoons of commercially exploited *Daba ecorace*. The study was conducted during the period of December, 2009 to May, 2010 for the 1st grainage of 2010-11 *i.e.*, for commercial crop of 2009-10 and during July, 2010 to August, 2010 for the 2nd grainage of 2010-11 *i.e.*, for seed crop of 2010-11. Data were recorded on ambient temperature and humidity conditions prevailed then at both the places, where the device was tested and on grainage parameters for natural parental moth couplings. The impact of TGT during the course of cocoon preservation, seed production and egg handling up to egg hatching was assessed.

RESULTS AND DISCUSSION

The in-practice conventional methods of tasar seed cocoon preservation and conduct of grainage activity by farmers are, simple hanging of cocoon garlands from the

roof of rearer's dwelling house or hanging in one corner of rearer's house up to level of about 6' height from floor with a wet sand bed made on floor beneath the hanged cocoons or hanging of seed cocoon in garlands in a pagoda like hut made up of bamboo and paddy straw or nylon mesh cover underneath the tree for shade, depending upon the convenience or knowledge level of farmers, irrespective of intensity of prevailing climatic conditions of the region. However, the predators' attack is common in the above cases as the effective control measures are lacking.

Tasar silkworm seed cocoons require optimal temperature of 25-28 °C and relative humidity of 75-85 % during their preservation for sustaining pupal health and progress of metamorphosis, uniform parental moth emergence, coupling, oviposition and most importantly to lay viable eggs by mother moths (Narain *et al.*, 2001; Prasad *et al.*, 2001; Reddy *et al.*, 2003, 2009 c, 2010; Hussain *et al.*, 2011 a, 2011 b). Wide range of temperature (30 – 40 °C) and low relative humidity (35 – 65 %), which prevail during the 1st, 2nd and 3rd tasar grainage periods (June to September) and their fluctuations on different days *i.e.*, from day of spinning seed cocoon to moth emergence / oviposition and even up to hatching of tasar silkworm egg have great impact on the tasar seed production activity (Koundinya, 1989; Kumar and Thangavelu, 1993; Singh *et al.*, 1993; Prasad *et al.*, 2000; Rao *et al.*, 2000; Reddy *et al.*, 2009 a; Reddy and Prasad, 2010; Hussain *et al.*, 2011 a, 2011 b). The higher temperature and lower relative humidity that prevail during day time will adversely affect the pupal health during their preservation and reproductive vigor of moths in terms of spermatogenesis and oogenesis and thus subsequently affect the fecundity, fertility and viability (Prasad *et al.*, 2000; Rao *et al.*, 2000; Narain *et al.*, 2001; Reddy *et al.*, 2009 a; Reddy and Prasad, 2010; Hussain *et al.*, 2011 a, 2011 b). Further, the fluctuations in temperature and relative humidity will also distress the embryonic development leading to uneven and poor hatching, resulting in weak larvae and poor crops (Narain *et al.*, 2001; Prasad *et al.*, 2001; Reddy *et al.*, 2003, 2009 a, 2009 b, 2010; Hussain *et al.*, 2011 a, 2011 b).

The results of the current study on TGT has shown

Table 1: Material requirement and fabrication cost of Tasar Grainage Tool (TGT) for preserving 500 tropical tasar seed cocoons

Material	Specification	Quantity*	Cost (₹)
Meta. frame - vertical	10 mm iron rod	20 feet	80
Meta. frame - horizontal	8 mm iron rod	50 feet	125
Wire mesh	2 cm sieve size	2.5 m	100
Plastic water container	20 litres capacity	01 no.	50
Washers	Plain type	04 nos.	05
Red oxide primer	Std. make	100 ml	25
Black paint	Std. make	200 ml	50
Gunny cloth	Std. make	2.5 m	75
Meta. frame making charges	-	-	100
Gunny cloth stitching charges	-	-	40
Total cost of the Tasar Grainage Tool (₹)			650

* The quantity of the material may vary depending upon the size of TGT

reduction in moth emergence duration by 7 days and decrease in temperature by 5.3 to 6.9°C; increase in relative humidity by 33.5 to 37.3 %, improvement in moth emergence by 13.1 %, moth mating by 17.4 %, egg laying by 8.5 %, fecundity by 24 eggs / 11.4 % per DFL and egg hatching by 8.09 % (Table 2), which enlighten the impact of tool on tasar grainage activity and egg hatching. The results also indicate the efficiency of TGT in maintaining required temperature and relative humidity in comparison to conventional practices and prevailing ambient climatic conditions. The enhanced moth emergence, moth coupling over control on applying TGT indicates the role of device on synchronizing moth emergence, besides the scope of preserving male moths for coupling (re-use) during last phase of grainage operation, which is inevitable in tasar grainage activity. Also, the enhanced number in gravid female moths and improved fecundity over control batches show the impact of TGT in providing non-fluctuating climate required for egg laying activity compared to ambient conditions. Even after egg production, provision

Table 2: Comparative performance of Tasar Grainage Tool (TGT) over conventional method

Parameter	Preservation method			Improvement	CD at 5 %
	Ambient	Conventional	TGT		
Temperature (°C)	35.85±3.55	34.71±2.81	27.91±1.30	6.11	1.7
Relative humidity (%)	39.35±10.15	44.71±6.65	79.15±5.51	35.31	2.9
Cocoon preservation duration for 1st grainage (Days)	162±8.79	176±6.15	181±5.03	5	9.6
Cocoon preservation duration for 2nd grainage (Days)	32±4.85	37±3.66	41±2.75	4	1.9
Erratic emergence (%)	11.21±1.39	9.72±0.77	5.65±0.14	4.12	0.5
Emergence duration (days)	23±4.71	21±3.32	14±1.06	7	0.9
Moth emergence (%)	56.31±4.55	61.84±4.09	74.91±3.06	13.12	4.5
Sex ratio (Male : Female)	1 : 0.625	1 : 0.714	1 : 0.833	1 : 0.119	0.05
Moth mating in pairs (%)	42.9±3.75	54.1±2.31	71.5±1.94	17.40	3.3
Moth egg laying (%)	55.3±4.93	67.4±3.16	75.9±2.29	8.50	2.6
Fecundity (no. & %)	201±9.45	209±6.32	233±4.09	24 & 11.4	7.5
Egg hatching (%)	49.81±8.81	67.70±4.73	75.79±3.08	8.09	4.0

The data represent average values of 4 trials

Table 3: Cost benefit ratio of Tasar Grainage Tool (TGT)

Particulars	Conventional	TGT	Improvement
Grainage activity (days)	21	14	7 Days
Moths emerged from 500 cocoons (No.)	309	374	65 Moths
Moths mated (No.)	270	357	87 Pairs
DFLs produced (No.)	181	270	89 Dfls
Total eggs produced (No.)	37,829	62,910	25,081 Eggs
Total larvae hatched (No.)	25,610	47,679	22,069 Larvae
Cocoon : DFL ratio	2.76 : 1	1.85 : 1	0.91 Cocoons/ DFL
Net quantity of DFLs @ 209 eggs with 67.70 % of hatching	181 DFLs	337 DFLs	156 DFLs
Net expenditure @ ₹50 / day of grainage activity + TGT cost	₹1,050/-	₹700/- + ₹650/-	₹300/-
Net returns for one grainage activity with additional 156 DFLs @ ₹4 / DFL	₹724/-	₹1,348/-	(+ 624/- - 300/-) = ₹324/-
Net returns with minimum self-life of three years of TGT @ three grainage activities / year		₹324/- x 9 crops = ₹2,916/-	
Cost benefit ratio = (₹650 : ₹2,916) = 1 : 4.49			

of ideal conditions for their incubation and healthy embryogenesis is a big problem at farmer's level, which can be fulfilled using TGT, because of its ability in maintaining required abiotic conditions for egg incubation, that too independent of electricity. Thus, the TGT can provide ideal micro climate required during tasar seed cocoon preservation, silkworm egg production, and also during egg incubation. The TGT, with its easy operation, durability and multipurpose use for seed cocoon preservation, moth preservation, egg production / incubation, besides providing safety from all types of predators during entire process of tasar egg production and handling indicate its efficiency and applaud for its usage. The above results are in accordance with the earlier findings of Ahsan *et al.*, 1980; Koundinya, 1989; Singh *et al.*, 1993; Dubey *et al.*, 1994; Narain *et al.*, 2001; Prasad *et al.*, 2001; Reddy *et al.*, 2009 a, 2009 b, 2009 c, 2010 and Reddy and Prasad, 2010. Hence, with the use of TGT, the optimal abiotic factors *viz.*, temperature, relative humidity and safety required for tasar egg production and incubation can effectively be provided with less cost and at the same time, can eliminate all other disadvantages encountered with conventional methods in practice.

The cost benefit ratio (Table 3) indicates the efficacy of TGT in conducting tasar grainage activity with production of additional seed and improved egg hatching, which will fetch additional income with meager one time investment cost on gadget. Hence, by applying the cost-effective, electrically independent, simple operative, predators protective TGT; the seed cocoon / parental moth preservation, mating synchronization, oviposition perfection for better egg yield and egg incubation for higher hatching can be attained. The additional net returns of ₹324/- per one grainage activity with minimum self-life of TGT of three years and @ three grainage activities per year amounts to ₹2,916/- as net additional income, excluding the cost of TGT. Hence, the cost benefit ratio of 1:4.49 indicates TGT application as prospective for cocoon preservation, grainage and egg handling of *A. mylitta* even by the most backward tribal tasar farmers of India.

CONCLUSION

The TGT is a multipurpose, cost effective, durable and affordable implement for poor rural farmers as it is simple and electrically independent in maneuvering. The tasar seed cocoon preservation, mating synchronization of

parental moths, oviposition of gravid mother moths and even the incubation of tasar eggs, besides safeguarding them from predators can be attended utilizing TGT. Hence, the TGT has high application prospective for orderly management of tasar seed cocoons, conduct of grainage activity and handling of tasar eggs, as a rural friendly implement.

ACKNOWLEDGEMENT

The authors are thankful to Sri Subol Chandra Sit, Technical Assistant and Sri Akthar Ansari, Farm worker, Central Tasar Research and Training Institute, Central Silk Board, Ranchi, India and Sri S. B. Sekher, PPO, Pilot Project Centre, Goilkera, West Singhbhum district, Jharkhand, India for their assistance in fabrication and evaluation of the device.

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Article scientifique

EQUIPEMENT DE CONSERVATION DE COCONS POUR LE GRAINAGE DU TASAR TROPICAL (OUTIL DE GRAINAGE TASAR :TGT) AFIN D' AUGMENTER L'EFFICACITE DU GRAINAGE CHEZ LES FERMIERS

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R É S U M É

Un support inadéquate pour les œufs correspondent à des conditions locales, fait que plusieurs fermiers praticiens du tasar conservent par eux-mêmes les cocons destinés au grainage. Alors que la quantité de cocons pour le grainage est faible, ils font face à des conditions adverses, des intrusions pendant le grainage, et des menaces venant des prédateurs pour obtenir des aménagements. Les cocons du ver à soie tasar *Antheraea mylitta* Drury peuvent cependant être conservés par les fermiers en appliquant un *modus-operandi* simple adapté au statut rural. Notre étude représente une telle tentative en appliquant un modèle pratique d'outil de grainage tasar (TGT) qui occupe moins d'espace, des chambres pour la conservation des cocons et des papillons pour la synchronisation des accouplements, l'oviposition et l'incubation des œufs pour améliorer le rendement en œufs et l'éclosion en même temps que la protection vis-à-vis des prédateurs. TGT fonctionne sans électricité, ne requière presque pas d'habileté et, plus important, est peu coûteux. L'étude révèle que TGT peut maintenir la température entre 25,6 et 30,2 °C et l'humidité relative de 72,6 à 85,7 % quelles que soient les conditions ambiantes. L'émergence des papillons (13,1 %), l'accouplement (17,4 %), l'oviposition (8,5 %), la fécondité (11,4 %), l'éclosion (8,09 %) et la réduction de la durée d'émergence (de 7 jours) sont améliorés comparés aux témoins ainsi que la facilité de synchronisation des accouplements. Ceci indique l'impact de l'application sur les activités de production rurales de graines tasar. Ainsi TGT présente une application potentielle pour la conservation des cocons, le grainage et la manipulation des œufs de *A. mylitta* même par des éleveurs tribaux non avertis de l'Inde rurale.

Mots-clés: *Antheraea mylitta* Drury, activité de grainage, humidité relative, graine du ver à soie tasar, température.



Research Paper

REARING OF MULBERRY LEAF-ROLLER, *DIAPHANIA PULVERULENTALIS* (HAMPSON) (LEPIDOPTERA: PYRALIDAE) AND MASS CULTURING ITS PARASITOID, *PHANEROTOMA NOYESI* (ZETTEL) (HYMENOPTERA: BRACONIDAE) ON ARTIFICIAL DIET

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ABSTRACT

The leaf-roller, *Diaphania pulverulentalis* (Hampson) (Lepidoptera: Pyralidae) is a severe pest of mulberry, causing heavy damage to mulberry plantations, resulting in adverse effect on both quality and quantity of mulberry leaves. The pest caterpillars damage the terminal bud of mulberry plants and feed voraciously on tender leaves resulting in stunted growth and shortage of tender leaves required for chawki (young age) silkworms. *Phanerotoma noyesi* (Zettel) (Hymenoptera: Braconidae), a promising biological control agent was identified for mass release for management of mulberry leaf-roller under Integrated Pest Management programme. This parasitoid does not accept other laboratory hosts, which can be easily multiplied and it is cumbersome to mass produce on its natural host, reared on mulberry leaves. Therefore, an artificial diet was developed in our laboratory for mass multiplication of mulberry leaf-roller. The pest caterpillars were reared from 1st instar, throughout their larval life in three different densities till pupation, on artificial diet and controls were run simultaneously by feeding them on mulberry leaves. Emergence of moths from batches reared as one caterpillar/vial was very high compared to five/vial and fifty/beaker. Technique to mass produce *P. noyesi* using leaf-roller caterpillars reared on artificial diet was developed and the results are discussed.

Key words: Artificial diet, *Diaphania pulverulentalis*, *Phanerotoma noyesi*.

INTRODUCTION

Leaf-roller, *Diaphania pulverulentalis*, is a severe pest of mulberry recorded for the first time in Karnataka during 1995 (Geetha Bai *et al.*, 1997). It has spread to neighbouring states of Tamil Nadu and Andhra Pradesh also. The pest caterpillars damage the terminal bud and feed voraciously on tender leaves. Infestation begins after commencement of south-west monsoon during June and is severe during north-east monsoon and winter and then declines and tapers off by next April (Geetha Bai and Marimadaiah, 2002). An Integrated Pest Management programme comprising of components of cultural, mechanical and chemical (limited application) methods

was developed and recommended (Geetha Bai *et al.*, 1997, 2002).

Phanerotoma noyesi Zettel (Hymenoptera: Braconidae) is a commonly occurring solitary, arrhenotokous, egg-larval endo-parasitoid of mulberry leaf-roller. Biology of *P. noyesi* was studied in the laboratory (Marimadaiah and Geetha Bai, 2001). Studies on age-specific fertility life-tables of *P. noyesi* were conducted at different temperatures (Marimadaiah, 2002). Several attempts were made to mass produce this promising parasitoid using certain laboratory hosts viz., *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae), *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae), *Phthorimaea operculella* (Zeller) (Lepidoptera:

Gelechiidae), and *Sitotroga cerealella* Oliv. (Lepidoptera: Gelechiidae) which can be easily mass multiplied in the laboratory, but were not successful. At the same time, attempts to mass multiply mulberry leaf-roller caterpillars on its natural host was found to be cumbersome. Therefore, an artificial diet to mass produce mulberry leaf-roller caterpillars was developed.

Artificial diet was developed earlier for several laboratory hosts, viz., *C. partellus* (Ballal *et al.*, 1995), *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) (Nagarkatti and Satyaprakash, 1974), *Spilosoma obliqua* Walker (Lepidoptera: Arctiidae) (Narayanan, 1985), *Spodoptera litura* F. (Lepidoptera: Noctuidae) and for many other pests and a comprehensive account on this was provided by Singh and Moore (1985).

MATERIALS AND METHODS

Oviposition by leaf-roller and *P. noyesi*

Leaf-roller moths were fed on 50 % honey solution and mated moths were released for oviposition on a potted mulberry plant covered by nylon net (Figure 2). After oviposition by leaf-roller moths, the potted plant was covered by nylon net cage (1'×1'×1') and mated gravid *P. noyesi* (Figure 1) were released in the cage for parasitization for 24 h and were provided with 50 % honey solution. Eggs laid by leaf-roller moths were allowed to hatch on the mulberry plant and the caterpillars fed on tender leaves.



Figure 1: *Phanerotoma noyesi*



Figure 2: Leaf roller moths released on a potted mulberry plant for oviposition

Rearing of leaf-roller and *P. noyesi* on artificial diet

Tender mulberry leaves were harvested and dried under shade and powdered in a grinder. The ingredients used for preparation of artificial diet are, dry mulberry leaf powder (35 g), soya meal powder (35 g), corn starch (11 g), cellulose (10 g), Wesson's salt mixture (3 g), dextrose (3 g), casein (3 g), citric acid (2 g), agar (2 g), multivitaplex (2 g), linoleic acid (1 g), crotonic acid (0.4 g), sorbic acid (0.4 g), and streptomycin (0.2g). The above ingredients were mixed well, added with 300 ml of water and then cooked in pressure cooker till one whistle. This artificial diet was poured into sterilized glass vials (1"×1") for about 5 mm thickness and were closed using a cap with holes for aeration. Similarly, the artificial diet was also poured into glass beakers (1000 ml) and covered with muslin cloth. Two to three days old leaf-roller caterpillars in three different densities, viz., one/vial, five/vial or fifty/beaker were held. Similar sized glass vials and beakers were used for control batches wherein the caterpillars were fed on tender mulberry leaves offered on alternate days till pupation. Emergence of adults in treatment and control batches were recorded. These experiments were replicated five times. Similarly, leaf-roller caterpillars developed from eggs exposed to *P. noyesi* were reared and moth and parasitoid emergence were recorded.



Figure 3: Emergence of leaf roller moths and parasitoids (control)



Figure 4: Rearing of parasitized leaf roller caterpillars on artificial diet



Figure 5: Emergences of leaf roller moths and *P. noyesi* when reared on artificial diet in a cage

Cage method of rearing was also adopted wherein two to three days old, *P. noyesi* parasitized caterpillars were held on artificial diet poured on a polythene sheet in a cage (1'x1'x1'). The diet was covered by another polythene sheet till pupation stage to avoid desiccation after which it was opened (Figure 4). For control batches, parasitized caterpillars were held in a cage (1'x1'x1') and fed with tender mulberry leaves on alternate days till pupation (Figure 3). After 7 to 8 days, adult moths or parasitoids emerged (Figure 5). These adults were fed on 50 % honey solution and thus leaf-roller and *P. noyesi* were mass cultured in the laboratory. This experiment was replicated five times.

RESULTS AND DISCUSSION

Effect of larval density on moth and parasitoid emergence

When 1 or 5 leaf-roller caterpillars/vial or 50 caterpillars/beaker were reared on artificial diet or mulberry leaves, moth emergence increased with decrease in larval density (Table 1). These results indicate that moth emergence is high when caterpillars are reared singly in vials, compared to the other two batches. Similar results were obtained when parasitized leaf-roller caterpillars were reared on artificial diet (Table 2). Emergence of *P. noyesi* adults and leaf-roller moths increased with decrease in larval density. When caterpillars are reared on mulberry leaves in control batches, feeding is necessary on alternate days, which is rather cumbersome, compared to artificial diet, wherein larval food is provided only once for the entire larval period.

Mass production of *P. noyesi*

Mass production technique for *P. noyesi* parasitized leaf-roller caterpillars was developed on artificial diet in cages. Results revealed that, the total of leaf-roller and *P. noyesi* emergence was to the tune of 43.6 % (7.2 % leaf-roller moths and 36.4 % of *P. noyesi*). Similarly, in control batches, wherein fresh mulberry leaves were provided on alternate days, a total of 49.6% emergence of adults

Table1: Effect of larval density of leaf-roller (non-parasitized) on moth emergence

Treatment	No. of caterpillars /vial or beaker	Larval mortality		Pupal mortality		Moth emergence	
		No. (Mean $\pm$ SD)	%	No. (Mean $\pm$ SD)	%	No. (Mean $\pm$ SD)	%
A D	50	44.20 $\pm$ 1.30	88.40 $\pm$ 261	1.00 $\pm$ 1.00	2.00 $\pm$ 2.00	4.80 $\pm$ 0.84	9.60 $\pm$ 1.67
C	50	46.20 $\pm$ 1.92	92.40 $\pm$ 3.84	1.60 $\pm$ 1.51	3.20 $\pm$ 3.03	2.20 $\pm$ 2.16	4.40 $\pm$ 4.33
A D	5	4.04 $\pm$ 0.38	80.8 $\pm$ 7.56	0.18 $\pm$ 0.11	3.6 $\pm$ 2.19	0.78 $\pm$ 0.33	15.6 $\pm$ 6.54
C	5	4.22 $\pm$ 0.23	84.40 $\pm$ 4.56	0.04 $\pm$ 0.09	0.80 $\pm$ 1.79	0.74 $\pm$ 0.19	14.80 $\pm$ 3.89
A D	1	0.26 $\pm$ 0.05	26.00 $\pm$ 5.47	-	-	0.74 $\pm$ 0.05	74.0 $\pm$ 5.47
C	1	0.20 $\pm$ 0.10	20.00 $\pm$ 10.0	-	-	0.8 $\pm$ 0.10	80.00 $\pm$ 10.0

A D- artificial diet, C- control

Table 2: Effect of larval density on emergence of leaf-roller moths and *P. noyesi*

Treatment	No. of larvae provided	Larval mortality		Moth emergence		<i>P. noyesi</i>	
		No. (Mean $\pm$ SD)	%	No. (Mean $\pm$ SD)	%	No. (Mean $\pm$ SD)	%
A D	50	43.6 $\pm$ 1.34	87.20	2.8 $\pm$ 0.84	5.6	3.6 $\pm$ 0.89	7.20
C	50	44.2 $\pm$ 1.64	88.40	2.4 $\pm$ 0.55	4.8	3.4 $\pm$ 1.14	6.80
A D	5	4.11 $\pm$ 0.33	82.20	0.5 $\pm$ 0.52	10.00	0.4 $\pm$ 0.51	8.00
C	5	4.00 $\pm$ 0.66	80.00	0.5 $\pm$ 0.71	10.00	0.5 $\pm$ 0.53	10.00
A D	1	0.20 $\pm$ 0.42	20.00	0.3 $\pm$ 0.48	30.00	0.5 $\pm$ 0.53	50.00
C	1	0.3 $\pm$ 0.48	30.00	0.3 $\pm$ 0.48	30.00	0.4 $\pm$ 0.51	40.00

A D- artificial diet, C- control

(10.4 % leaf-roller moths and 39.2 % of *P. noyesi*) was observed (Table 3). Even though there is no significant difference in adult emergence between treated and control batches, it is cumbersome to feed the caterpillars with fresh mulberry leaves frequently. Rearing caterpillars in individual vial is cumbersome and hence cage method with ample space for larval development and aeration was found to be appropriate.

CONCLUSION

An artificial diet suitable to mass multiply mulberry leaf-roller and its promising biological control agent, *P. noyesi* was developed.

ACKNOWLEDGEMENT

The authors are grateful to the Director, Karnataka State Sericulture Research and Development Institute, Bengaluru, India for the facilities and the supporting staff for technical assistance.

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Table 3: Emergence of leaf-roller moths and *P. noyesi* reared in cage

Treatment	Replication	No. of caterpillars tested	Mortality		No. of moths emerged		No. of <i>P. noyesi</i> emerged		Total No. of adults emerged	
			No. (Mean $\pm$ SD)	%	No. (Mean $\pm$ SD)	%	No. (Mean $\pm$ SD)	%	No. (Mean $\pm$ SD)	%
A D	5	50	28.2 $\pm$ 2.16	56.4 $\pm$ 4.33	3.6 $\pm$ 3.13	7.2 $\pm$ 6.26	18.2 $\pm$ 3.83	36.4 $\pm$ 7.66	21.8 $\pm$ 2.16	43.6 $\pm$ 4.33
C	5	50	25.2 $\pm$ 1.64	50.4 $\pm$ 3.28	5.2 $\pm$ 1.09	10.4 $\pm$ 2.19	19.6 $\pm$ 0.89	39.2 $\pm$ 1.79	24.8 $\pm$ 1.64	49.6 $\pm$ 3.28
	CD		NS		NS		NS		NS	
	@ 0.05		6.334		5.325		6.119		6.783	
	@ 0.01		9.248		7.774		8.934		9.902	

A D- artificial diet, C- control

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Article scientifique

**ELEVAGE DE L'ENROULEUR DE LA FEUILLE CHEZ LE MURIER
DIAPHANIA PULVERULENTALIS HAMPSON (LEPIDOPTERA: PYRALIDAE),
ET ELEVAGE DE MASSE DE SON PARASITOÏDE *PHANEROTOMA*
NOYESI ZETTEL (HYMENOPTERA: BRACONIDAE) SUR ALIMENT ARTIFICIEL**

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RÉSUMÉ

L'enrouleur de la feuille, *Diaphania pulverulentalis* Hampson (Lepidoptera: Pyralidae) est une peste sévère du mûrier, causant de lourds dommages aux plantations, ce qui entraîne un effet négatif à la fois sur la qualité et la quantité des feuilles de mûrier. Les chenilles du parasite endommagent les bourgeons et mangent les feuilles tendres ce qui entraîne un retard de croissance et une raréfaction des feuilles tendres nécessaires pour l'élevage des jeunes vers à soie. *Phanerotoma noyesi* Zettel (Hymenoptera: Braconidae), un agent prometteur pour la lutte biologique a été identifié pour des lâchers de masse afin de contrôler l'enrouleur de la feuille chez le mûrier dans le programme de contrôle intégré des pestes. Ce parasitoïde n'accepte pas d'autres hôtes qui pourraient être aisément multipliés en laboratoire et il serait embarrassant de le produire en masse sur son hôte naturel alimenté par des feuilles de mûrier. Aussi, un aliment artificiel a-t-il été développé dans notre laboratoire pour la multiplication de masse de l'enrouleur de la feuille. Les chenilles ont été élevées à partir du premier âge, tout au long de la vie larvaire selon trois densités jusqu'à la pupaison sur un aliment artificiel, et des témoins ont été simultanément en mangeant des feuilles de mûrier. L'émergence des papillons des lots élevés à raison d'une chenille par flacon est très élevée comparée à celle des chenilles élevées à raison de quatre et cinquante par bécier. Une technique pour produire en masse *P. noyesi* en utilisant des chenilles de l'enrouleur de la feuille sur aliment artificiel a donc été développée et nous discutons les résultats.

Mots-clés: Aliment artificiel, *Diaphania pulverulentalis*, *Phanerotoma noyesi*.



Brief Note

PARASITIZATION OF SILKWORM UZI FLY, *EXORISTA BOMBYCIS* (LOUIS) BY *TRICHOMALOPSIS* SP. (HYMENOPTERA: PTEROMALIDAE) – A NEW REPORT

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ABSTRACT

The uzi fly, *Exorista bombycis* of silkworm, *Bombyx mori* has been reported to be parasitized by 20 parasitoids so far. Recently, a new species of *Trichomalopsis* Crawford (Hymenoptera: Pteromalidae) has been found parasitizing the pupae of *E. bombycis* from Karnataka, India. This is the first record of a new hyper parasitoid of the genus *Trichomalopsis*. Preliminary laboratory observations revealed that they are gregarious and complete their life cycle in 14.5 days (range: 13 to 16 days) at a room temperature of 29 ± 3 °C & RH of 57 to 78 % on uzi pupae. They prefer to parasitize only 25 % host pupae even when more pupae are available. A few important biological parameters of this new ecto pupal parasitoid are discussed in the paper.

Key words: *Bombyx mori*, *Exorista bombycis*, India, Karnataka, new species, Pteromalidae, *Trichomalopsis*.

Exorista bombycis (Louis) (Diptera: Tachinidae) commonly known as silkworm uzi fly is a major pest of the silkworm, *Bombyx mori* L., which prefers late age silkworms for oviposition and further progeny production. In the course of infestation, the host will be killed causing loss to sericulturists to the extent of 15 to 20% (Dandin *et al.*, 2000).

Of late, the strategy of biological method of insect pest suppression has gained increasing acceptance and popularity, being proved as safe, eco-friendly and specific to the target insect pest. It has been reported that hymenopterans such as *Nesolynx thymus* Girault (Eulophidae), *Trichopria* sp., (Diapriidae) and *Exoristobia philippinensis* Ashmead (Encyrtidae) are the gregarious ecto-pupal hyper-parasitoids of the mulberry silkworm uzi fly, *E. bombycis* (Pradip Kumar *et al.*, 1986).

Recently, *Trichomalopsis* sp., another gregarious pupal parasitoid of filth flies has also been observed to parasitize puparia of silkworm uzi fly (Figures 1 and 2).

This is the first record of a new species of *Trichomalopsis* on *E. bombycis* from India. However, Singh and Thangavelu (1995) have recorded *Trichomalopsis apanteloctena* (Crawford) on puparia of *Blepharipa zebina* (Walker), which attacks the pupae of tasar silkworm, *Antheraea mylitta* (Drury).

The Pest Management Laboratory of Central Sericultural Research and Training Institute, Central Silk Board, Mysuru, India as a part of exploration of new parasitoids, regularly collect uzi fly puparia from Govt. Cocoon Markets of Kollegal, Ramanagaram and Channapatna (Karnataka State) and maintain it in the laboratory in order to observe for emergence of any new parasitoid. Almost on all the occasions, there used to be emergence of *N. thymus*, which is presently the recommended bio-control agent in sericulture against uzi fly, besides *Trichopria* sp. In a similar process, during the month of March 2014, to our surprise, in addition to *N. thymus*, and *Trichopria* sp., adults of a new species of parasitoid of *Trichomalopsis* sp. (Hymenoptera:

Pteromalidae) also emerged from a few puparia of uzi fly. During identification, it was confirmed that it was not *T. apanteloctena*.

Preliminary observations made in the laboratory on *Trichomalopsis* sp. revealed that they are gregarious parasitoids and poor fliers, which complete their life cycle in 13 to 16 days at a room temperature of $29 \pm 3^\circ\text{C}$ and RH of 57 to 78 %. As per the observations of Dobesh *et al.* (1994) on house fly puparia, the parasitoid completed its life cycle in 13 days with an average fecundity of 61 (range: 29 to 130). Further, they prefer to oviposit on 2 to 3 host puparia/day even when more puparia were available. In the present study also, it was recorded that when 25 uzi fly puparia were exposed, they parasitized only three puparia though all the rest were just stung. The parasitoid is observed to have the habit of stinging the puparia and feeding on the oozing liquid. On an average, from each parasitized uzi puparium, nine parasitoids emerged (range: 8 to 12) and as per the observation of Dobesh *et al.* (1994), the mean number of emergence per house fly puparium was 6.8. Also, the longevity of the parasitoids that emerged from uzi fly puparia was 6.1 days (range: 4 to 8 days) without any food. Investigations on the biology and other aspects of the parasitoid are in progress.



Figure 1: Adult male of *Trichomalopsis* sp.



Figure 2: Adult female of *Trichomalopsis* sp.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the services of Dr. Ankita Gupta, Scientist, Insect Systematics Division, National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India for the identification of the parasitoid at genus level of *Trichomalopsis*. Acknowledgements are also due, to Dr. P. M. Sureshan, Senior Taxonomist, Zoological Survey of India, Calicut, Kerala for naming it under a new species (*uziae*) after thoroughly studying its taxonomic characters.

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Note brève

**PARASITISME DE *TRICHOMALOPSIS* SP. (HYMENOPTERA: PTEROMALIDAE)
SUR LA MOUCHE UZI DU VER A SOIE, *EXORISTA BOMBYCIS* LOUIS – UNE
NOUVELLE OBSERVATION**

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R É S U M É

On a rapporté que la mouche uzy, *Exorista bombycis* du ver à soie *Bombyx mori* est parasitée par au moins 20 parasitoïdes. Récemment, une nouvelle espèce de *Trichomalopsis* Crawford (Hymenoptera: Pteromalidae) a été repérée comme parasitant les pupes de *E. bombycis* au Karnataka en Inde. Ceci est le premier enregistrement d'un nouvel hyper-parasitoïde du genre *Trichomalopsis*. Les premières observations au laboratoire montrent qu'ils sont grégaires et ont un cycle de vie de 14,5 jours (entre 13 et 16 jours), à température ambiante de 29 ± 3 °C et 57 à 78 % d'humidité relative, sur des pupes uzy. Ils parasitent seulement 25 % des pupes même s'il y a plus de pupes disponibles. Peu de paramètres biologiques importants concernant ce nouveau parasitoïde ecto-pupal sont discutés dans cet article.

Mots-clés: *Bombyx mori*, *Exorista bombycis*, Inde, Karnataka, nouvelle espèce, Pteromalidae, *Trichomalopsis*.

NEWS CORNER

Executive Committee Meeting of ISC held at Kolkata on 12th January, 2016



The Executive Committee of International Sericultural Commission was held at Hotel Swisshotel, Kolkata, India on 12th January, 2016. Delegates from 6 countries have attended the Executive Committee. The meeting discussed and finalised few mandatory requirements on the functioning of ISC for the year 2016. During the meeting, it was decided that the 24th International Congress would be held at Bangkok during 10-14 August, 2016. The First Announcement of the Congress was released during the Meeting, which contains all details of the Congress. The Congress Organizers have also launched an exclusive website www.qsds.go.th/isccongress24th, containing the details of the Congress.

Visit of Chinese delegation to ISC



A five member delegation led by Dr. Li Long, Director, Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, Jiangsu, China visited the sericulture areas of India from 27th August to 1st September, 2015. The main purpose of the visit was to explore the possibility of collaborating with Indian seed producers to produce and supply highly productive silkworm seeds of Chinese origin to Indian sericulture farmers. Apart from meeting the ISC and CSB officials, the delegation had also met and interacted with State Sericulture Department officials of Karnataka and Private Seed Producers.

Visit of Bangladesh delegation to ISC



A 7 member delegation from Bangladesh, a Member Country visited ISC HQs on 09.12.2015. The purpose of the visit was to explore and gain first hand insight into the Indian Sericulture Industry as well as to explore ways for improving the bilateral relationship between Karnataka and Bangladesh in the field of silk industry.

Visit of Vice Governor, Guangxi Province, China to ISC



A High Power six member Delegation headed by Mr. Zhang Xiulong, Vice Governor, Guangxi Zhuang Autonomous Council, China visited the sericulture facilities in Bangalore on 14.12.2015. The delegation had also visited the office of the International Sericultural Commission and discussed on the possibility of Guangxi Province enrolling as an Associate Member.

Global Silk Production (Tonnes)

Country	2008	2009	2010	2011	2012	2013	2014
Bangladesh			40	38	42.50	43	44.5
Brazil	1177	811	770	558	614	550	560
Bulgaria	7.5	6.3	9.4	6	8.5	8.5	8
China	98620	84000	115000	104000	126000	130000	146000
Colombia	0.6	0.6	0.6	0.6	0.6	0.6	0.5
Egypt	3	3	0.3	0.7	0.7	0.7	0.82
India	18370	19690	21005	23060	23679	26480	28708
Indonesia	37	19	20	20	20	16	10
Iran	180	82	75	120	123	123	110
Japan	96	72	54	42	30	30	30
North Korea	-	-	-	300	300	300	320
South Korea	3	3	3	3	1.5	1.6	1.2
Philippines	1	1	1	1	0.89	1	1.1
Syria	0.4	0.6	0.6	0.5	0.5	0.7	0.5
Thailand	1100	665	655	655	655	680	692
Tunisia	0.08	0.04	0.12	3	3.95	4	4
Turkey	15	20	198	22	22	25	32
Uzbekistan	770.5	780	940	940	940	980	1100
Vietnam	-	-	550	500	450	475	420
Madagascar	15	16	16	16	18	18	15
Total	120396	106170	139100	129662	152846	159737	178058



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