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International Sericultural Commission (ISC)
Central Silk Board Complex, ETM Layout
Madiwala, Bengaluru - 560 068, INDIA
Tel: +91 80 26282191, 26680132, 26681663
Fax: +91 80 26681663
E-mail: sericologia@gmail.com
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IDENTIFICATION OF TWO FUNGI AND AN ANTAGONISTIC YEAST ASSOCIATED WITH THE SPOILAGE OF MULBERRY FRUIT (*MORUS ALBA* L.)

Yin Fang¹, Zi-Xu Ren¹, Cheng Chen¹, Chao Jin¹, T. R. Sivakumar¹, P. G. Ajaykrishna¹,
Jun-Qiang Jia^{1,2} and Zhong-Zheng Gui^{1,2*}

¹School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, China.

²Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China.

*E-mail: srizzgui@hotmail.com

ABSTRACT

In an attempt to prolong the shelf life of mulberry fruit, potential spoilage microorganisms were isolated and characterized from the fruit. Two fungi, *Sclerotinia sclerotiorum* and *Alternaria* sp., and an antagonistic yeast, *Sporidiobolus pararoseus*, were identified and confirmed by 18S rRNA and 5.8S rDNA sequencing. *S. pararoseus* strongly inhibited the growth of both *S. sclerotiorum* and *Alternaria* sp. Our results suggest that the yeast *S. pararoseus* might, therefore, be a potential bio-control agent for mulberry and/or other fruits.

Key words: Fungi, isolation and identification, mulberry fruit, yeast.

INTRODUCTION

Mulberry fruits are fleshy, succulent, and sweet berries, which are low in calories and contain beneficial phytonutrient compounds such as alkaloids, flavonoids, anthocyanins, polyphenols and minerals (Butt *et al.*, 2008). As part of traditional oriental medicine, this fruit has long been used in the fight against cancer, treatment of neurological diseases, inflammation and diabetes and to combat aging and bacterial infections as well (Halliwell 1992; Liu *et al.*, 2004; Ercisli and Orhan, 2007; Li *et al.*, 2009; Wang *et al.*, 2013). The mulberry fruit is at its best, with regard to flavor, texture, and nutritional value, at full maturity; however, its commercialization is limited owing to the fruit's short harvesting season, high water content (more than 70 %), fragile structure and sensitivity to storage (Ercisli and Orhan, 2007; Chen *et al.*, 2011 a). Conventional methods used for preserving other fruits and vegetables are not suitable for the thermosensitive mulberry fruit (Terefe *et al.*, 2009). Thus, the fruit is mainly consumed locally or used as an ingredient in various confectionery products such as juice, wine, jam, frozen desserts and ice cream or in traditional foodstuffs

(Ercisli, 2004; Wang *et al.*, 2013). Several technologies for the preservation of mulberry fruit have been reported, including sun drying (Doymaz, 2004) and chlorine dioxide (ClO₂) treatment (Chen *et al.*, 2011 b). However, the savory quality of mulberries can get reduced during exposure to sunlight and ClO₂ treatment and, therefore, the development of new methods to improve the storage and shelf life of mulberry fruit is necessary in order to improve its commercial sales.

One of the potential non-harmful technologies is biological control, which can lower the inoculum density of pathogens or spoilage microorganisms through biological processes (Beeyer *et al.*, 1989). Recently, biological control has been developed as an alternative to synthetic fungicide treatment. Considerable success has been achieved by using antagonistic microorganisms to control both pre- and post-harvest diseases (Janisiewicz and Korsten, 2002).

This study was aimed to identify the fungi involved in the decaying process of mulberry fruit, to confirm their pathogenicity and to investigate the possibility of using

bio-control agent(s) against them so as to prolong the shelf life of mulberry fruit.

MATERIALS AND METHODS

Mulberry fruit

Mulberry fruits (*Morus alba* L.) were harvested at full maturity from the National Mulberry Orchard of Zhenjiang, PR China. The uniformly ripe berries were selected and damaged fruits were discarded.

Isolation and identification of fungi

Isolation

Moldy mulberry fruits were cut into ca. 5 mm³ pieces, immersed in 0.85 % sterilized saline water, and cultured at 28 °C for 30 min with shaking. The cultured solution was then serially diluted to 10⁻⁴, 10⁻⁵, and 10⁻⁶ with sterilized saline water and plated onto both carboxymethyl cellulose medium (CMC) [containing CMC-Na 0.5 g/l, agar 1.8 g/l, K₂HPO₄ 0.1 %, (NH₄)₂SO₄ 2 %, NaCl 0.05 % and MgSO₄ 0.05 %; pH 7.0] and pectin medium (PM) [containing pectin 0.4 g/l, Congo red agar 2 g/l, K₂HPO₄ 0.1 %, (NH₄)₂SO₄ 2 %, NaCl 0.05 % and MgSO₄ 0.05 %; pH 7.0] agar plates. Each concentration was plated in triplicate and incubated at 28 °C for 72 h. Colonies of interest were selected for further culturing and single colony isolation on potato dextrose agar (PDA) incubated at 28 °C for 72 h.

Determination of pectinase and cellulase activities

Enzyme activity was measured using the 3, 5-dinitrosalicylic acid (DNS) method described by Chen *et al.* (2011). One unit of enzyme activity was defined as the amount of galacturonic acid (for pectinase) or glucose (for cellulase) released per hour per milliliter of enzyme solution.

Morphological characteristics

Fungi were inoculated on PDA medium and cultivated at 28 °C for 2–3 days. Colony morphology was observed

directly on the PDA plates, and characteristics such as the size, surface texture, color, form of the edge, and presence or absence of exudate were recorded. Colonies were picked from the plates by using a clean blade and placed on a glass slide. The surface morphology of the colonies was examined by light microscopy according to the methods previously described by Ma *et al.* (2014). After a further 10 days of incubation at 28 °C, fungal hyphae and spore morphology were observed under a light microscope for preliminary identification of the strain type according to the Handbook of Fungal Identification (Wei, 1979).

Identification of fungi strains by 18S rRNA sequencing

For further identification of fungal isolates, the 18S rRNA gene was amplified from genomic DNA by polymerase chain reaction (PCR). Genomic DNA was isolated using a lysozyme and protease K method. The universal primers, fP1: (5'-GTAGTCATATGCTTGTCTC-3') and rP2: (5'-TCCGCAGGTTACCTACGGA-3') were used.

PCR was performed using the following conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, followed by a final elongation cycle at 72 °C for 10 min. Amplicons of the expected size (approximately 1,800bp) were gel-purified using the DNA Gel Extraction Kit (Axygen Scientific, Silicon Valley, CA, USA) and then cloned into the pMD18-T vector (Takara, Kyoto, Japan) followed by sequencing. Sequence analysis was performed using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov>). Sequences were aligned using the ClustalX program, and a neighbor-joining phylogenetic tree was generated using the MEGA5.05 software package.

Screening and identification of yeasts

Yeast isolation

Potential yeasts were isolated from the surface of the mulberry fruit according to the methods of Assis and

Mariano (1999). Approximately, 10 g of the healthiest fruit samples were cut and resuspended by vigorous shaking in 100 ml of sterile distilled water. Serial dilutions of the yeast sample suspension were prepared in sterile distilled water. An aliquot of 0.1 ml of each dilution was plated onto yeast malt (YM) extract agar adjusted to pH 3.5 with sterile tartaric acid, and the plates were incubated at 25 °C for 72 h. All yeast colonies were examined under a light microscope, and 12 colonies were chosen based on morphological differences. The yeast isolates were repeated on YM agar to obtain pure cultures and were maintained on nutrient yeast dextrose agar (NYDA) slants. The cultures were stored at 4 °C until use.

Screening for antagonistic yeasts

A preliminary *in vitro* screening was conducted to determine whether the isolated yeasts could inhibit mycelial growth of the fungi identified. Yeasts were co-cultured with the selected fungi on PDA medium plates to test for antagonistic activity. A 5 mm mycelial disc, obtained from the edges of a 5-day-old culture of the test fungus, was inoculated onto a PDA plate (5 cm) from the yeast being tested. For control, a PDA plate was inoculated with a mycelial agar plug of the fungi alone. Each test was performed in triplicate. The plates were incubated at 28 °C under 80 % relative humidity for 6 days and were observed daily for mycelial growth. Yeast isolates that suppressed the fungal growth were considered antagonistic according to Nallya *et al.* (2012) and Huang *et al.* (2012).

Physiological and biochemical characteristics

The physiological and biochemical characteristics of the yeast antagonists were determined by detecting their ability to ferment certain sugars semi-anaerobically and to assimilate a variety of carbon compounds under aerobic conditions. Glucose, maltose, sucrose, mannose, xylose, lactose, raffinose, and starch were used as sugar and carbon sources. $(\text{NH}_4)_2\text{SO}_4$ and KNO_3 were used to detect nitrogen assimilation by yeasts. All tests were performed in triplicate.

Molecular identification of antagonistic yeasts

The molecular identification of the antagonistic yeasts was performed using an rDNA sequence comparison technique, as described by Mitchell *et al.* (1992). Primers covering the internal transcribed spacer region (ITS) were used to amplify the intervening 5.8S gene by PCR (Martorell *et al.*, 2005). The primers used were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATG-3'). Approximately, 600-bp amplicons were gel-purified using the QIAquick kit (QIAGEN, Germany). Sequencing was carried out using the Automated DNA Sequencer (3100-Avant Genetic Analyzer) and standard methods. The sequences were aligned and compared using the BLAST algorithm, and a neighbor-joining phylogenetic tree was generated using the sequences as described above.

RESULTS

Isolation of spoilage fungi

Both PM and CMC were used to screen for spoilage fungi cultured from mulberry fruits. Five monoconidial isolates (namely GA, GB, GC, GD, and GE) were isolated on the PM while only one (XZ) was isolated on the CMC. Of these isolates, GB, GC, GD, GE, and XZ were similar in colony morphology and spore structure under light microscopy and, thus, were presumed to belong to the same group of fungi. Cellulase and pectinase activities were determined for each of the isolate (Table 1). Isolates GA and GE showed the highest specific activities of CMCase (9.011 U/mg and 11.381 U/mg, respectively) and pectinase (21.971 U/mg and 20.455 U/mg, respectively). As a result, both GA and GE were selected for further morphological and molecular identification.

Morphological and molecular identification of fungi

Isolates GA and GE were inoculated onto the surface of healthy mulberry fruit and incubated at 28 °C. After 2 days, the inoculated fruit became moldy with visible hyphae that

expanded quickly over the following days. No change was observed in an un-inoculated control fruit (Figure 1). The spores were morphologically the same as the main pathogenic fungi (Figure 2).

To determine the phylogenetic relationship of these fungi with other fungi and to confirm their morphological identification, a portion of the 18S rRNA gene of GA and GE was analyzed. The sequences generated have been deposited in GenBank [GA: accession numbers KJ522780 (GA) and KJ489375 (GE)]. As shown in Figure 3, isolates GA and GE had the highest sequence homology (99 %) with *Sclerotinia sclerotiorum* and *Alternaria* sp., respectively.

Screening and identification of yeast

Twelve isolates, when grown on NYDA plates for 3 days at 25 °C, produced colonies that were oval-elongated, occurred either singly or, in clusters, were pinkish-tan colored, and with a dull and smooth surface texture (Figure 4). One isolate (Y1) was chosen for further



Figure 1: Mulberry fruit infected by spoilage fungi GA (b) and GE (c) compared with control fruit (a).

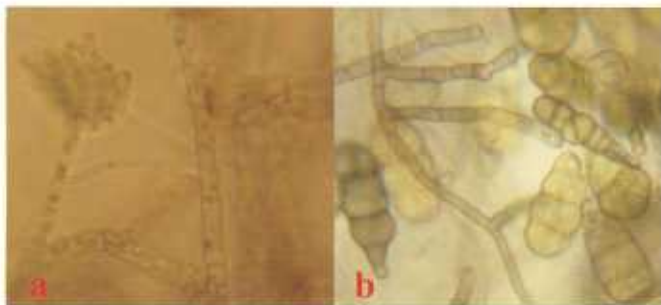


Figure 2: Morphological characteristics of fungal strain GA (a) and GE (b). Spores were observed at 40x magnification.

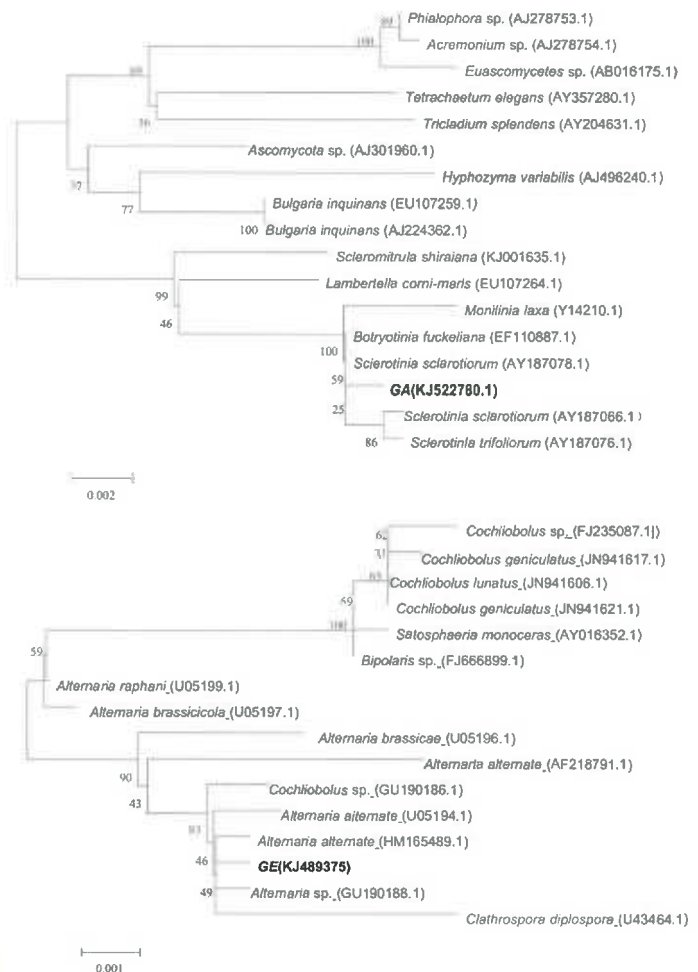


Figure 3: Phylogenetic tree based on 18S rRNA gene sequences of fungal strains GA and GE among others, constructed using the neighbor-joining method. The number of nucleotides analyzed and the corresponding GenBank accession numbers are indicated at the right of the sequence.

characterization. When viewed under a light microscope, true hyphae for Y1 were not observed. When Y1 was co-cultured with fungi GA or GE, a small zone of inhibition was observed around the Y1 inoculum (Figure 5).

Physiological and biochemical characteristics of yeast Y1

The physiological and biochemical characteristics of yeast Y1 were determined based on the color change of the substrate following fermentation of carbohydrates. The results showed that Y1 could ferment glucose, maltose, sucrose, and mannose, but not galactose, xylose, lactose,

raffinose and starch. Further, Y1 could assimilate glucose, maltose, sucrose, mannose, xylose, lactose, raffinose and starch, but not galactose (Table 2).

It was also observed that Y1 could grow in the presence of $(\text{NH}_4)_2\text{SO}_4$ but not in the presence of KNO_3 . Owing to these assimilation characteristics, Y1 was preliminarily identified as *Sporidiobolus pararoseus*.

Molecular identification of Y1

The multiple sequence alignment of the ITS region of Y1 and related yeast species is shown in Figure 6. Y1 had the highest homology (99 %) with *S. pararoseus* based on the evolutionary distance of the phylogenetic tree. The ITS sequence generated for Y1 has been submitted to GenBank under accession number KJ569246.

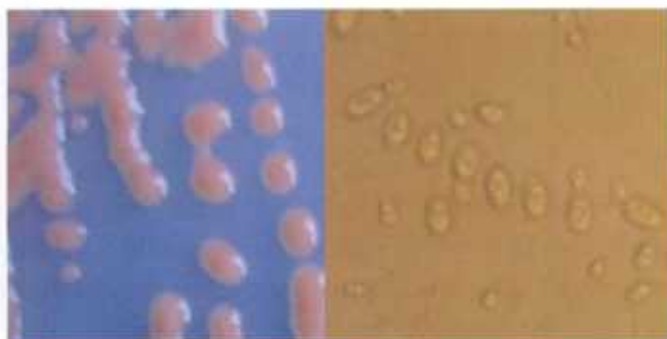


Figure 4: Morphological characteristics of yeast strain Y1. Spores were observed at 40x magnification.

Table 1: Specific activities of CMCase and pectinase from the isolated fungus

Isolate	CMCase specific activity ($\text{U} \cdot \text{mg}^{-1}$)	Pectinase specific activity ($\text{U} \cdot \text{mg}^{-1}$)
GA	9.011	21.971
GB	6.753	12.283
GC	5.208	12.578
GD	8.775	12.515
GE	11.381	20.455
XZ	5.288	6.242

Table 2: Fermentation and assimilation of carbohydrates by yeast Y1

Type	Carbohydrate fermentation	Carbon source assimilation
Glucose	+	+
Maltose	+	+
Sucrose	+	+
Mannose	+	+
Galactose	-	-
Xylose	+	+
Lactose	+	+
Raffinose	+	+
Starch	+	+



Figure 5. Morphological characteristics of co-culture of strain Y1 with GA (a) and GE (b).

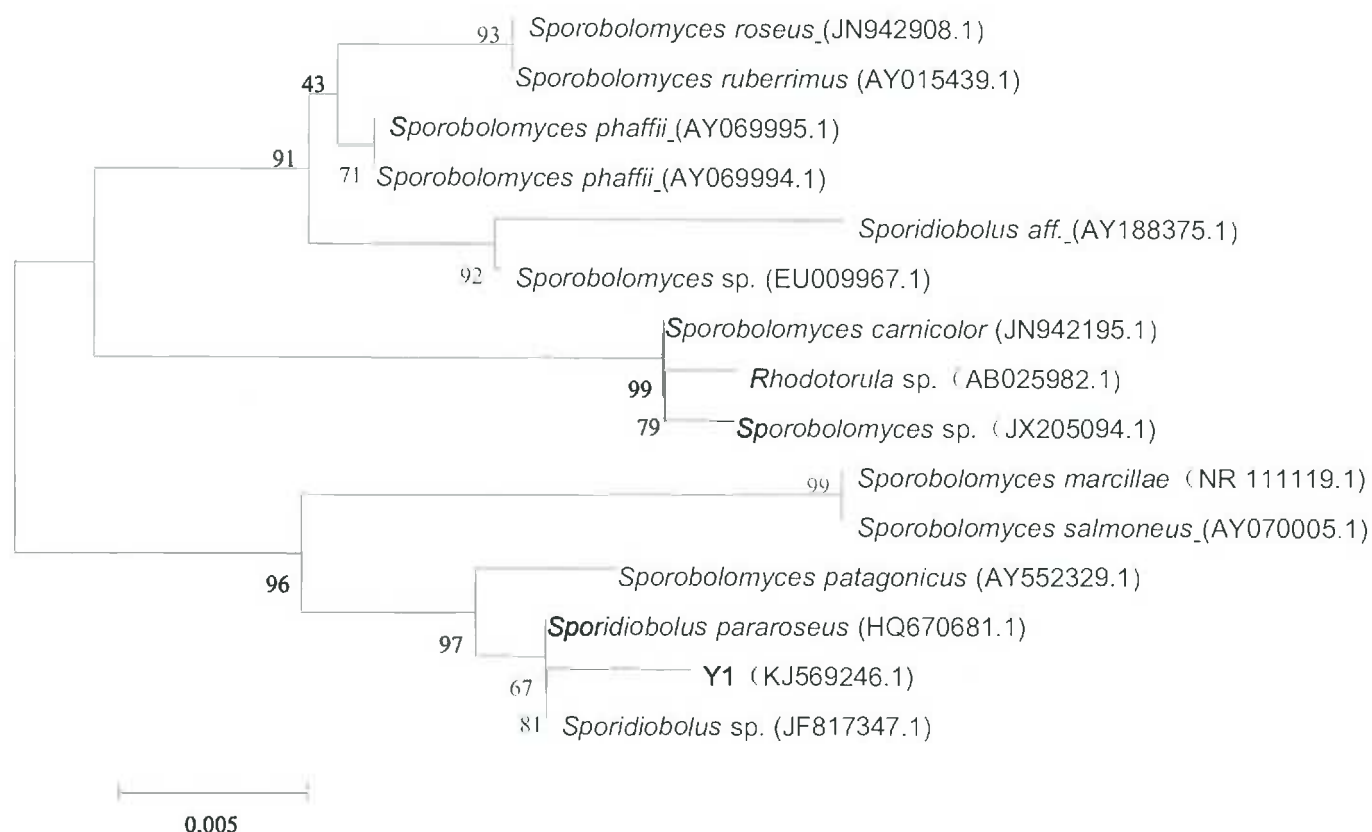


Figure 6. Phylogenetic tree based on 5.8S rDNA gene sequences of strain Y1 and other homologous strains. The number of nucleotides analyzed and the corresponding GenBank accession numbers are indicated at the right of the sequence.

DISCUSSION

Mulberry cultivation has traditionally focused on promoting the production of leaves to feed the silkworm (*Bombyx mori* L.) in countries where sericulture is practiced, such as China, India, Thailand and Brazil. In these countries, there is little or no market for the mulberry fruit, primarily owing to its perishability and ease of spoilage (Chen *et al.*, 2011).

Many spoilage fungi have been reported for different fruits, e.g., apple, cherry, pear, peach, grape, strawberry, and citrus fruit. These fungi include *Alternaria alternata*, *Botrytis cinerea*, *Geotrichum candidum*, *Glomerella cingulata*, *Monilinia fructicola*, *Mucor piriformis*, and *Rhizopus stolonifer* (Vinas *et al.*, 1998; Schena *et al.*, 2003; Lahlali *et al.*, 2004; Qin *et al.*, 2004; Torres *et al.*, 2006; Zhang *et al.*, 2007; Zhao *et al.*, 2007; Morales *et al.*,

2008; Chandrasekaran *et al.*, 2016). All of these fungi can produce polygalacturonase, which results in the decay of the fruit. In this study, cellulase and pectinase activities were used to screen for potential spoilage fungi. This method has been previously used by Al-Hindi *et al.* (2011) to successfully identify spoilage fungi in other fruits. In the present work, two fungi, *Sclerotinia sclerotiorum* and *Alternaria sp.*, were isolated and identified by both morphological and 18S rRNA sequencing.

S. sclerotiorum is globally known as a fungal pathogen that damages fruits and vegetables. This damage can be primarily attributed to the secretion of cellulase, pectinase, and other hydrolytic enzymes that soften the fruit or vegetable and lead to rotting (Collmer and Keen, 1996; Li *et al.*, 2004). *S. sclerotiorum* also produces oxalic acid, which is a major pathogenic factor that not only directly harms plants, but can also bind calcium on the surface of

the plant, leading to wilting (Godoy *et al.*, 1990; Maxwell and Lumsden, 1990). *Alternaria* sp. is another important group of fungi responsible for fruit and vegetable diseases. It adapts well to its environment and has a wide host range (Li *et al.*, 1993). A previous study has shown that *Alternaria* sp. can produce more than 70 different toxic metabolites known as alternaria toxins, which can cause acute and chronic poisoning in humans and animals (Li, 2001). Spores of *Alternaria* sp. are also important allergens causing allergic respiratory disease (Lopez *et al.*, 1989) and skin allergies (Chapman, 1999).

Recently, a number of different microorganisms including bacteria, filamentous fungi, and yeast have been shown to protect fruits and vegetables against pre-harvest and post-harvest pathogens (Qing and Shiping, 2000; Alakali, 2010). In particular, yeasts have been extensively studied because of a number of characteristics that make them useful for bio-control purposes, such as low nutritional requirements, ability to colonize dry surfaces for long periods, and ease of production in large quantities (Chanchaichaovivat *et al.*, 2007). In addition, unlike mycelial fungi and bacteria, yeasts generally do not produce allergenic spores, mycotoxins, or antibiotic metabolites (Droby and Chalutz, 1994). Additionally, yeasts make good bio-control candidates because they are non-hazardous (Arras and Arru, 1997; Arras *et al.*, 1999) and provide an extra source of vitamins, minerals, and essential amino acids to the consumer (Hussein *et al.*, 1996).

In the present study, the red yeast, *S. pararoseus*, was isolated from mulberry fruit for the first time. *S. pararoseus* species is a ballistosporous yeast belonging to phylum *Basidiomycota*. Reports reveal that *S. pararoseus* has antagonistic activity that could be used to control various fruit diseases. Previous studies showed that *S. pararoseus* could inhibit the growth of *Botrytis cinerea* in grapes (Raspor *et al.*, 2010) and *Monilinia fructicola* in nectarines (Janisiewicz *et al.*, 2010). Moreover, Machado and Bettiol (2010) demonstrated that *S. pararoseus* was able to suppress sporulation of *B. cinerea* on the leaf discs of lilies. In addition, the *in vitro* tests carried out in this

study indicate that this yeast, strongly inhibits the two fungi *S. sclerotiorum* (GA) and *Alternaria* sp. (GE) and may therefore be a potential bio-control agent against other pathogenic fungi found in other fruits. Further studies are required to understand better the antagonistic mechanisms of *S. pararoseus* and its potential use as a bio-control agent for fruits.

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Article scientifique

IDENTIFICATION DE DEUX CHAMPIGNONS ET D'UNE LEVURE ASSOCIES A LA DETERIORATION DU FRUIT DU MURIER (*MORUS ALBA* L.)

Yin Fang¹, Zi-Xu Ren¹, Cheng Chen¹, Chao Jin¹, T. R. Sivakumar¹, P. G. Ajaykrishna¹,
Jun-Qiang Jia^{1,2} and Zhong-Zheng Gui^{1,2*}

¹School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, China.

²Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China.

*E-mail: srizzgui@hotmail.com

RÉSUMÉ

Dans le but de prolonger la conservation du fruit du mûrier, des microorganismes potentiellement responsable de sa détérioration ont été isolés à partir du fruit et caractérisés. Deux champignons, *Sclerotinia sclerotiorum* et *Alternaria* sp., et une levure antagoniste, *Sporidiobolus pararoseus*, ont été identifiés et confirmés par le séquençage de l'ARNr 18S et de l'ADNr 5,8S. *S. pararoseus* inhibe fortement la croissance de *S. sclerotiorum* et de *Alternaria* sp. Nos résultats suggèrent que la levure *S. pararoseus* peut, ainsi, être un agent potentiel de bio-contrôle pour le fruit du mûrier et d'autres fruits.

Mots-clés: Champignons, isolement et identification, fruit du mûrier, levure.



IDENTIFICATION OF SNP MARKERS LINKED TO GENES CONTROLLING POST-COCOON TRAITS IN THE MULBERRY SILKWORM, *BOMBYX MORI*

S. Sreekumar^{1*}, S. K. Ashwath² and Keiko Kadono-Okuda³

¹Central Sericultural Germplasm Resources Centre, Central Silk Board, P. B. No. 44, Thally Road, Hosur 635109, Krishnagiri Dist., Tamil Nadu, India.

²Central Sericultural Research and Training Institute, Srirampura, Mysuru 570008, Karnataka, India.

³Insec.-Plant Interaction Research Unit, National Institute of Agrobiological Sciences, 1-2, Owashi, Tsukuba 305-8634, Japan.

*E-mail: sreecsrti@yahoo.co.in

ABSTRACT

For the identification of DNA markers closely linked to post-cocoon traits in the mulberry silkworm, bulked segregant analysis (BSA) was carried out using the low yielding polyvoltine race, Pure Mysore and the high yielding bivoltine race, CSR2 as parents which showed contrasting features for the selected traits. The data on filament length and denier were recorded in the parents, F1 as well as the high and low bulks of F2 progeny. Two hundred and forty SNP primers representing all the 28 linkage groups (LG) in silkworm were screened with genomic DNA of parents, PM, CSR2 and their F1 progeny. Forty-eight primers showed distinct polymorphism between the parents showing a clear size difference in the amplified products and the co-dominant expression of these polymorphic bands were observed in the F1 where two bands were detected. Screening of these 48 informative primers among the parents, F1 and bulks of F2 progeny revealed that SNP primer no. 09082 of LG 9 and SNP primer no. 12059 of LG 12 are closely linked to the QTL controlling the traits of filament length and denier, respectively. The results of the present study has shown the prospects of using SNP markers in silkworm breeding programme for improving cocoon traits in the low yielding polyvoltine breeds of India leading to the maximisation of silk yield and quantity.

Key words: *Bombyx mori*, bulked segregant analysis, denier, filament length, QTL, SNP.

INTRODUCTION

The indigenous polyvoltine silkworm strains being reared and multiplied in the tropical climate of India for the past two hundred years are characterized by shorter larval duration; lower body, cocoon and shell weight, with lower silk content; shorter filament, thin denier and poor silk quality. But their survival potential is high. On the other hand, the bivoltine strains are of temperate origin being reared in countries such as China and Japan, and are endowed with longer larval duration with higher body, cocoon and shell weight with high raw silk recovery, longer filament, thick denier and better silk quality. New breeding strategies adopted during the last few years in India have resulted in evolving a number of productive silkworm strains through extraction from Japanese hybrids (Basavaraja *et al.*, 1995; Datta *et al.*, 2000 a; Datta *et al.*, 2000 b). However, though of high silk content, these

breeds were susceptible under the high germload and low input-cum-low management situation of Indian tropical climate and hence are being popularized in favourable seasons only with progressive farmers who can provide the optimal rearing environment. Even today, the bulk of the silk in India is produced by a polyvoltine × bivoltine cross strain and is not of the required International standard. As a result, India imports large quantities of silk from China. Hence the polyvoltine strains need to be improved by incorporating genes carrying the desired traits so that they produce high quality silk. Molecular markers associated with quantitative trait loci (QTL) are reported for many crops and many important traits. After linkage between QTL and a molecular marker is determined, the QTL can be transferred to a different genetic background by marker assisted selection. For identification of DNA markers closely linked to the selected character, one of the strategies adopted to single

out from a large number of markers, the few that are tightly linked to the target gene is by way of bulked segregant analysis (BSA). This technique has been successfully used to identify DNA markers linked to downy mildew resistance gene in lettuce (Micheltore *et al.*, 1991), leaf rust resistance gene in barley (Pouslen *et al.*, 1995), malaria and nematode resistance genes in mosquitos (Severson *et al.*, 1999), powdery mildew resistance gene in wheat (Chantret *et al.*, 2000), sex determination in aphids (Gadau *et al.*, 2001), vaccine response in chicks (Kaiser *et al.*, 2002), QTL for grain quality in rice (Govindaraj *et al.*, 2005), sex expression in shrimps (Khamnamtong *et al.*, 2006), genetic mapping in zebrafish (Bradley *et al.*, 2007), clubroot resistance genes in oilseed rape (Werner *et al.*, 2008), red wing patterning in Heliconius butterflies ((Baxter *et al.*, 2008), for isolation of sex-linked SCAR markers in yellow catfish (Wang *et al.*, 2009), and powdery mildew resistance gene in soybean (Kang and Mian, 2010). Recently we have detected one SNP marker linked to cocoon traits through the strategy of bulked segregant analysis (Sreekumar *et al.*, 2011).

The present investigation was carried out with the objective of detecting DNA markers closely linked to post cocoon-traits viz., filament length and denier (filament size) in mulberry silkworm, *Bombyx mori*. To achieve this goal, parents showing contrasting features for the selected traits were used and SNPs were screened in the parents, F₁ and F₂ bulks by employing bulked segregant analysis.

MATERIALS AND METHODS

Silkworm breeds used and raising of F₁ and F₂ progeny

Silkworm breeds viz., Pure Mysore (PM) and CSR2 which show large differences for the post-cocoon traits were selected for the present study. The parents were reared under the standard rearing conditions and the F₁ generation of PM x CSR2, and F₂ by inbreeding of F₁ progeny, were raised, which were reared simultaneously.

Estimation of post-cocoon parameters

After completion of the larval period, the mature larvae were shifted to plastic mountages for cocooning. On the sixth day after mounting, the cocoons of parents, F₁

and F₂ progeny were harvested. Generally, cocoon filament is reeled by cooking the cocoons in hot water of 90-95°C for 4-5 minutes to soften the cocoon and unwind the filament smoothly. But in the present study, as DNA has to be collected from the live pupa inside the cocoon, cold reeling procedure was followed by dipping the cocoon in 0.8 % NaOH solution for 3-5 min. Reeling was carried out using the Eppovette (single cocoon reeling machine) having the wrap reel of 1.125 m periphery swift with an indicator. The filament was attached to the reel and rotated manually and the total filament length was recorded for individual cocoons of parents and progeny. After completion of reeling, the live pupae were dipped in 5 % Acetic acid solution to neutralise alkalinity, thoroughly washed in water and preserved for DNA extraction. The reeled silk filament was dried in hot air oven for 2 h and silk weight was recorded using analytical balance.

The denier (filament size) denotes weight in grams of 9000 m of raw silk which was calculated using the following formula:

$$\text{Denier} = \frac{\text{Weight of the raw silk reeled in grams}}{\text{Length of the raw silk reeled in metres}} \times 9000$$

DNA extraction

Genomic DNA was extracted from the parents, F₁ and F₂ progeny using the live pupae obtained after cold reeling employing the standard phenol-chloroform method. The ethanol precipitated DNA was resuspended in TE (5 mM Tris-HCl, pH 8.0, 0.5 mM EDTA). Purified DNA was checked in 0.8 % agarose gel containing ethidium bromide. Quantification of DNA was done using Ultraspec-1100 pro UV-spectrophotometer (Amersham BioSciences, Hongkong). All the samples of DNA were diluted to a concentration of 20 ng/μl in TE.

Primers and PCR

A total of 240 SNP primers (forward and reverse) pertaining to the 28 linkage groups (Yamamoto *et*

al., 2006; 2008) generously supplied by Insect Genome Research Group, National Institute of Agrobiological Sciences, Tsukuba, Japan, were used to amplify the DNA of parents and F_1 progeny. PCR reaction consisted of 20 ng of template DNA, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Fermentas Inc, Maryland, USA), 1 μ M of SNP primer, and 0.45 U of *Taq* DNA polymerase (Fermentas Inc, Maryland, USA) in 10 μ l volume. Thirty-five cycles of PCR were performed on a PTC 200 Thermal Cycler (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 visualized under UV light.

RESULTS

Detection of polymorphism between parents and F_1 progeny

The genomic DNA of parents, PM, CSR2 and their F_1 progeny were amplified with 240 SNP primers of 28 linkage groups in silkworm (Table 1). Out of these, a total of 48 primers yielded distinct polymorphism between the parents showing clear size differences in the amplified products between the parents and the co-dominant expression of these polymorphic bands were observed in the F_1 where two bands were detected. The remaining 192 primers showed monomorphic pattern as shown by identical banding profiles in the parents and F_1 s. The highest polymorphism of 80 % was found in the LG 9, followed by LG 16 (75 %) and LG 12 (57.1 %). In the case of LGs 6, 7, 15, 17, 19, 20, 21, 23, 24, 25 and 28, no polymorphism was noticed. The overall polymorphism was found to be 20 %. The details of primers showing distinct polymorphism between the parents are presented in Table 2.

Post-cocoon parameter data in parents, F_1 and F_2 progeny

In the mulberry silkworm, filament length and denier are the two key post-cocoon traits which ultimately

Table 1: DNA polymorphism detected using parents and F_1 progeny

Linkage group	Primers screened (No.)	Primers showing monomorphism (No.)	Primers showing polymorphism (No.)	% of polymorphism
1	4	3	1	25.0
2	6	4	2	33.3
3	19	15	4	21.1
4	12	10	2	16.7
5	8	6	2	25.0
6	6	6	0	0.0
7	12	12	0	0.0
8	6	5	1	16.7
9	5	1	4	80.0
10	11	8	3	27.3
11	9	4	5	55.6
12	14	6	8	57.1
13	18	16	2	11.1
14	9	8	1	11.1
15	11	11	0	0.0
16	8	2	6	75.0
17	6	6	0	0.0
18	9	8	1	11.1
19	7	7	0	0.0
20	4	4	0	0.0
21	9	9	0	0.0
22	16	12	4	25.0
23	4	4	0	0.0
24	6	6	0	0.0
25	3	3	0	0.0
26	12	11	1	8.3
27	3	2	1	33.3
28	3	3	0	0.0
Total	240	192	48	20.0

determine the yield and quality of silk. Hence, in the present investigation, these two traits were selected for the analysis. The mean and standard deviation of the filament length and denier are listed in Table 3. PM and CSR2 differed for filament length by almost three folds and the

Table 2: Details of SNP primers showing polymorphism between the parents

Sl. No.	Primer ID	Linkage group	Sequence	
			Forward	Reverse
1	01065	1	ACTGACGAGCTCCCTCTGAA	TTCTGTACGACAATACCGTTCG
2	02034	2	TTTTTATTATATACGTCCCC	CATAAGTTTGCGTGTAAAGC
3	02051		AGAAAATACTGTTACTCGCTAGG	CAAACCTAAAAATGCAAATGG
4	03070		TTCGAACCGTITGTTGAAGTT	TGAACATGATTTTGACCCCC
5	03071	3	GGAAATTTACCAATGACATGAA	TCGGCTAAGGTTTCAACCAA
6	03079		TCGTAATCGTCACTTTTAACCTC	GCACGTCTGGGTGTAGTTGA
7	03085		CGCAGCAAAATTAGGGTTGT	GCACTGCTCTTGCTAGGGTC
8	04110	4	CTGTGCTGACTATCCGGTCA	CAACTATCAGTAAATGAAACCGACA
9	04124*		CTCTCGATGCTTTCAGGACC	AAATTCGGTAGCCAGAGCCT
10	05053	5	CATGGAAGGAAGAAAATACCCA	TGTTGTGTCAACGCCAATTC
11	05106		GCAAACCTCGAAATCCGATA	GTTATGTAACCCGTGTGGGC
12	08047	8	CATGATAGGAGAGTTATAGCGTCCG	CCTCCCTTCAAGCCGAAAC
13	09075	9	TGGAATGTTTAAATGCACGC	GTTTGTGTACAGAGCGCGGTT
14	09080		AACCGCAATTTAACGTCAC	ATGTTTCCCGAATCTGGTTG
15	09082*		G GTTG CATAACTAAGCTGCG	TTGGCGTGATTCTTAATACCG
16	09083	10	TGGCCAAATTGGGAATTAC	CATCATCTTCATCATCTCATCA
17	10098		CGAATACGCATCGTGTCAA	TGCAACGGTCTTAAATGAGG
18	10105		TCGCGATGTGAGTAATGGTG	CATGACATATTCGCAAAACG
19	10112	11	CTGATCATAGCTCTGTAATTATTATGC	TAGATTCAAGAAACGGGCCAC
20	11049		ACACGTGTCCAGTGTTCAT	TCGATATCGGAGGTTCGAGT
21	11075		TGGCACCTTAAAGTTACTGG	CAAACATGTGTTCAAAGTCG
22	11092	12	TAAACAGTTCGTTTGCCGAA	AGTAAACGCATATTGCCCCC
23	11105		TGTCTTCTCTGGAGGGTTG	TCCATTATCACTTCATTACAAATCA
24	11108		TGGGATAAGGTGGAGAAAAAGA	GTTCCACGGGGAGCAACTAT
25	12047	13	AGTGTCTTGTGTCATTCTGG	CCAGTTAAGCATTATAGCC
26	12059*		GCTGTGCTGTATTAGTGCCG	GCAAGCAACAATGTTCCAAA
27	12073		CGGTGGAAAAATTAACCTATGGG	CGACTAGTAGTTTCTTTAGTTTCGC
28	12077	14	CAATGAAGAAGTTTGAAGGGCT	TTCTCGTAAATATTATCATCAAAACA
29	12079		AAACTAAGCTTTTGGGTCCGA	GCGCCATTCTAAGTGACCAT
30	12080		AAATTGCGAGCTTGACACG	AAGCGGAGGCTCTGGAAT
31	12081	15	TGGCTTCATTCTAGAAGACATTTT	TGAAGTTTCAATGCAACCAA
32	12082		TCGACGGCTTACTTAGTGAGTG	ATACCTCTCAGTGGGCAACG
33	13129		CCAAACATTTCTTTGGTCCG	GGTCTCAAGTATAGTTACAACGGATG
34	13138	16	TTACGTAGCGCGTCAAAATC	TTAAGATTGAAGATGGAAGATAGAGA
35	14050		TGTAGAGTAACCGACTATGATGATGA	TTTCAACTAGCAGTTCGCTATCTT
36	16006		GTGGTCTTCTCCAGACAA	CGATCATGCCACTCTGTAAA
37	16015	17	GACTCTGGTACCTGGATGTGG	TCGTAGACGACTTAAAGTTCCG
38	16035		CGTTACTAAAGCCTCTCCG	AACGAGATCATACGCCAAAAA
39	16057		AACGAGATCATACGCCAAAAA	GCTTGAGACGGGTGAAGAAT
40	16058	18	CTCCGTCTACAACGCTTTT	TCGAGTACGCATATCCTAATTATACG
41	16059		CAAGGATAAAGAAGAAGAACTCACC	TGTCAITCCAGCATCTTGGT
42	18086		GACCCACTGAGAAGATCCCA	CGTTTCATCAATTCACAGAACA
43	22054	22	AAACACAACCTTGGCCTCCAC	AAAGGGAGTCTGGCTTAGA
44	22075		CCAGAGATCATTTTGTCAACTT	GGGCCATTCTCTACTGAAA
45	22090		CCGTAGCGTAACAAACGACA	CGATATAGGCTTCACAGGTGTTT
46	22094	26	GACAGCTGCCCCGAGCTATTA	CAGTTGCTAGGCAAAGAGCA
47	26048		TCCAACCCCACTAAATTGTC	GAATCTTAGGTTTCAAATTAGGCA
48	27044		TTTAGGCACTACAAGCACCG	CACAGAAACGCCAAGCATT

* Primers showing linkage with cocoon (Sreekumar *et al.*, 2011) and post-cocoon traits.

Table 3: Mean values with standard deviations and 't' value for post-cocoon trait data in the parents, F₁ and F₂ progeny

Parent / Progeny	Filament length (m)	t value	Denier	t value
Pure Mysore (P1)	331±59	12.2***	2.07±0.37	4.01***
CSR2 (P2)	905±137		2.77±0.41	
PM x CSR2 F ₁	717±111		2.56±0.14	
PM x CSR2 F ₂ (Mean)	653±140		2.37±0.37	
PM x CSR2 F ₂ (High bulk)	744±130		2.63±0.23	
PM x CSR2 F ₂ (Low bulk)	561±78	3.8***	2.11±0.29	4.44***

Note: t value is the result of student's 't' test for filament length and denier between means of parents, F₂ bulks and BC bulks

*** denotes significance at 0.001 %.

denier is significantly low in PM (2.07) when compared to CSR2 (2.77). In the case of the F₁, the values of these two traits are much higher than the mid parent value, indicating the dominance of CSR2 in expression of the above traits. The mean values of the F₂ progeny for both filament length and denier was found to be intermediate between the two parents possibly due to wide segregation of the parental genes for low and high values. Based on the values of filament length and denier in the F₂ progeny, high and low bulks of top ten and bottom ten cocoon samples were selected in the F₂ progeny. The high and low bulks also showed significant differences between them. The statistical analysis using the Student's 't' test also confirmed highly significant differences between the parents as well as F₂ bulks.

Identification of DNA marker linked to post-cocoon traits through Bulk Segregant Analysis

The genomic DNA of parents, PM, CSR2, their F₁ progeny, F₂ high and low bulks for filament length as well as denier were amplified with the 48 informative SNP primer pairs which showed distinct polymorphism between the parents. It was observed that except primer No.09082 of Linkage group (LG) 9 and primer No. 12059 of LG12, all the remaining 46 primers showed polymorphism between the parents and co-dominant expression in the F₁ as revealed by 2 bands inherited from both the parents. However, both the high and low bulks of F₂ showed identical pattern with amplified products of same size found in either PM or CSR2, indicating absence of linkage with either filament length or denier traits.

In the case of SNP primer No. 09082 of LG 9, the low yielding parent PM showed the amplified product of 0.4 kb while the high yielding parent CSR2 had a heavier PCR product of 0.8 kb (Figure 1a). In the F₁, co-dominant expression of two bands of 0.4 kb and 0.8 kb was observed. In the high filament length bulk of F₂, 0.8 kb product found in CSR2 was present, while in the low filament length bulk, 0.4 kb band specific to PM was amplified. Further, the 0.8 kb product specific to CSR2 was found in all the high bulk individuals, while the 0.4 kb product observed in PM was present in all the low bulk individuals (Figure 1b). These results clearly showed that the DNA regions amplified by the primer No. 09082 of LG 9 is closely

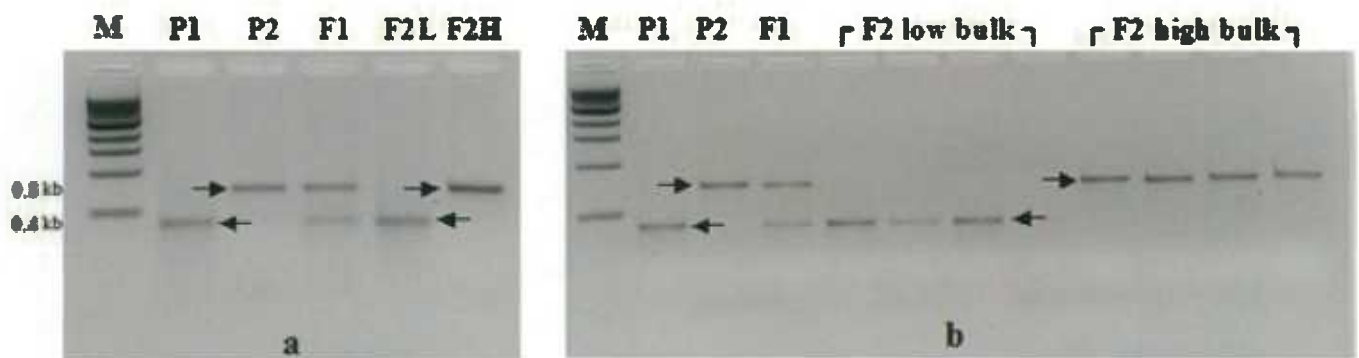


Figure 1: Amplification profiles of SNP primer No.09082 of 9th linkage group in parents, F₁ and F₂ low and high filament length bulks: Arrows indicate polymorphic SNP products of 0.4 kb in PM, low yielding and 0.8 kb in CSR2, high yielding parent. F₁ shows co-dominant expression of both products revealed by two bands. F₂ low filament length bulk shows the 0.4 kb product of low yielding parent PM, while F₂ high filament length bulk has the 0.8 kb band of high yielding parent CSR2 (a). Individual screening of high and low bulks also shows the same pattern in all samples (b).

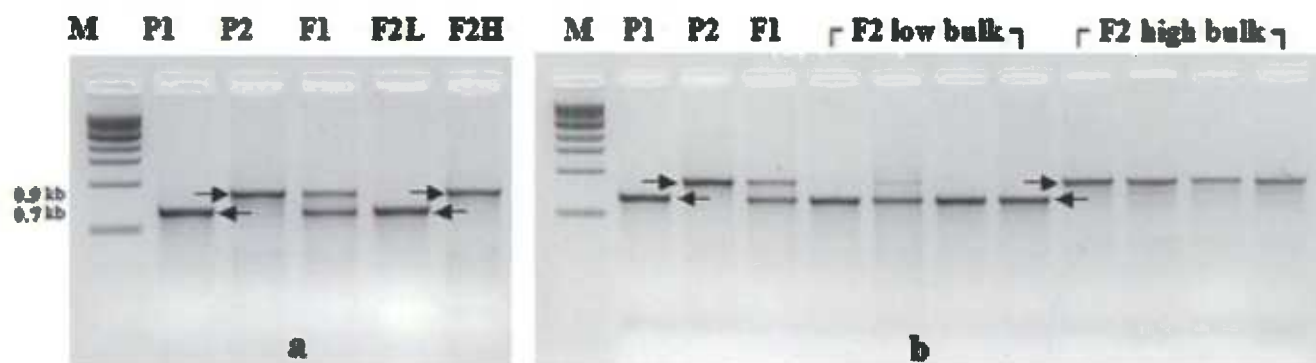


Figure 2: Amplification profiles of SNP primer No.12059 of 12th linkage group in parents, F₁ and F₂ low and high denier bulks: Arrows indicate polymorphic SNP products of 0.7 kb in PM, low yielding and 0.9 kb in CSR2, high yielding parent. F₁ shows co-dominant expression of both products revealed by two bands. F₂ low denier bulk shows the 0.7 kb product of low yielding parent PM, while F₂ high denier bulk has the 0.9 kb band of high yielding parent CSR2 (a). Individual screening of high and low bulks also shows the same pattern in all samples (b).

linked to quantitative trait loci (QTL) controlling the filament length character.

Similarly, for SNP primer No.12059 of LG 12, the low yielding parent PM showed the amplified product of 0.7 kb while the high yielding parent CSR2 had a heavier PCR product of 0.9 kb (Figure 2 a). In the F₁, co-dominant expression of two bands of 0.7 kb and 0.9 kb was observed. In the high denier bulk of F₂, 0.9 kb product found in CSR2 was present, while in the low denier bulk, 0.7 kb band specific to PM was amplified. The individual profiles in the high and low bulks also confirmed these findings (Figure 2 b). These results clearly indicated that the PCR products amplified by the primer No. 12059 of LG 12 is closely linked to quantitative trait loci (QTL) associated with the denier trait.

DISCUSSION

To identify DNA marker closely linked to any gene or genomic region, two strategies have proven effective in locating from a large number of markers, the few that reside near a targeted locus. One is by using 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. Any DNA polymorphism between the DP and NIL is likely to be linked to the target gene (Young and Tanksley, 1989). Using this strategy, high activity digestive amylase genes from the Indian polyvoltine breeds used as DPs have been introgressed into the genetic background of high yielding bivoltine breeds of temperate origin with “null” type of amylase and NILs were developed with DP type of amylase (Ashwath *et al.*, 2001). Using these NILs, RAPD markers linked to the high activity digestive amylase genes in silkworm have been identified (Ashwath *et al.*, 2010).

The other strategy, which is more generally applicable is referred to as bulked segregant analysis (BSA), which relies on the use of segregating populations of F₂. The strategy of BSA involves comparing two pooled DNA samples of individuals from a segregating F₂ population originating from a single cross. Initially, F₁s will be raised between the parents showing contrasting phenotypes for the target trait (*eg.* high and low or resistant and susceptible) and F₂ progeny will be generated out of this. Using the selected informative DNA markers which show distinct polymorphism between the parents, the DNA profile of the parents, F₁ progeny and F₂ bulks segregating for the target trait (high and low or resistant and susceptible) will be compared. The DNA markers that are polymorphic between the bulks are likely to be linked to the target gene.

BSA has several advantages over the use of near-isogenic lines to identify markers in specific regions of the genome. Genetic walking will be possible by multiple rounds of BSA; each new pair of bulks will differ at a locus identified in the previous round of analysis. This approach will have widespread application both in those species where inbreeding is possible and in those that are obligatorily outbreeding (Michelmore *et al.*, 1991). Further, the simplicity and low cost of BSA have led to its use for more complex traits, including traits whose genetic control is unknown. This method is often restricted to segregating generations which are simplest and cheapest to produce, such as backcross (BC) and F_2 generations. For dominant marker systems, the F_2 is considered as the best generation to conduct BSA (Mackay and Caligari, 2000).

We used SNP primers supplied by Silkworm Genome Research Group Tsukuba, Japan for the detection of polymorphism between the two breeds *viz.*, PM and CSR2 both of which are very popular in the field and have contrasting features related to silk production properties. Initially, we amplified the template DNAs of parents and F_1 progeny with 240 SNP primers belonging to all the 28 linkage groups in silkworm. Out of these 240 primers, 48 showed distinct polymorphism between the parents which was confirmed by the co-dominant expression of both the polymorphic products in the F_1 . The overall polymorphism was found to be 20 %. This low level of polymorphism could be attributed to the fact that silkworm strains used in the present study are different from that of the original SNP studies by Yamamoto *et al.* (2006), who used their own standard strains, p50T and C108T which are used routinely for molecular linkage map construction. Further, in the present study, only deletions or insertions could be detected which would result in different sized amplified products but nucleotide substitutions are unlikely to affect DNA fragment migration in agarose gels which may escape detection even if they are polymorphic. But the original studies used sequence based methods which would also detect nucleotide substitutions.

A linkage map of 534 SNPs based on BAC end

sequences in the silkworm *B. mori* using the male backcross population of standard strains, p50T and C108T has been constructed (Yamamoto *et al.*, 2006) and this map was subsequently extended to 1755 SNPs (Yamamoto *et al.*, 2008). However, out of the 240 SNP primers used in the present study, only 58 SNP markers were located in the linkage map and out of these, only 11 showed polymorphism between the parents.

Lu *et al.* (2004) identified eleven QTLs using a backcrossed population derived from a cross between C100 and Dazao and with AFLP technique, they mapped two QTL for whole cocoon weight on LGs 6 and 19; three QTL for cocoon shell weight on LG 3, 14, and 19 and three QTL for cocoon shell percentage on LGs 2, 14 and 19. Recently, an integrated map of SSR markers was constructed by Zhan *et al.* (2009) who have identified six QTL involved in cocoon weight, cocoon shell weight, cocoon shell percentage and pupal weight. A composite interval mapping of QTLs employing AFLP markers was carried out (Lie *et al.*, 2010) using the backcross of Dazao and C100 strains and 19 putative QTL associated with cocoon weight, cocoon shell weight, pupa weight and cocoon shell percentage were located. Previously, we found that the SNP primer No. 40124 of LG4 showed its linkage to both cocoon weight and shell weight traits (Sreekumar *et al.*, 2011). It is a well established fact that both cocoon weight and cocoon shell weight are strongly positively correlated (Chatterjee *et al.*, 1992; Zhan *et al.*, 2009) and the genetic relatedness of these traits is corroborated by earlier findings which showed the possibility of a QTL in the LG 4, which controls both these traits. Pleiotropic role of one ISSR marker significantly associated with many biomass traits such as larval weight, cocoon weight, shell weight, shell percentage and floss content has also been reported (Pradeep *et al.*, 2007). All these studies have indicated the possibility of presence of QTL for cocoon weight and cocoon shell weight in the LGs 1, 3, 4, 6, 10, 11, 12, 14, 15, 19, 21, 22 and 23.

Though many QTL have been identified for cocoon traits using DNA markers, so far, no reports are available on the QTL for post-cocoon traits. In the present investigation, the 48 informative SNP primers which showed distinct polymorphism between the parents were

used for amplification of the genomic DNA of parents, F₁ and two bulks of high and low post-cocoon traits and out of the 48 primers, two primers, namely No. 09082 pertaining to LG 9 and No.12059 of LG 12 showed clear polymorphism between the F₂ bulks of high and low post-cocoon traits indicating the linkage of the DNA segments amplified by these primers of LG 9 and LG12 with the genes controlling filament length and denier traits, respectively. Thus, the present study is the first report on the DNA markers associated with post-cocoon traits *viz.*, filament length and denier.

CONCLUSION

In this study, efforts have been made to identify DNA markers linked to post-cocoon traits, which play a vital role in determining the quality and quantity of silk. Bulk segregant analysis carried out employing SNP primers among the parents showing large differences for post-cocoon traits, their F₁ and bulks of F₂ progeny clearly showed the linkage of DNA segments amplified by SNP primers of 9th and 12th linkage groups with the QTLs governing filament length and denier traits. The results of the present study have shown the prospects of using SNP marker assisted selection in the silkworm breeding programmes for improving post-cocoon traits in the low yielding polyvoltine breeds of India leading to maximisation of silk yield and quality.

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Article scientifique

IDENTIFICATION DE MARQUEURS SNP LIES AUX GENES CONTROLANT LES CARACTERES POST-COCON CHEZ LE VER A SOIE DU MURIER, *BOMBYX MORI*

S. Sreekumar^{1*}, S. K. Ashwath² and Keiko Kadono-Okuda³

¹Central Sericultural Germplasm Resources Centre, Central Silk Board, P. B. No. 44, Thally Road, Hosur 635109, Krishnagiri Dist., Tamil Nadu, India.

²Central Sericultural Research and Training Institute, Srirampura, Mysuru 570008, Karnataka, India.

³Insect-Plant Interaction Research Unit, National Institute of Agrobiological Sciences, 1-2, Owashi, Tsukuba 305-8634, Japan.

*E-mail: sreecsrti@yahoo.co.in

R É S U M É

Pour l'identification de marqueurs ADN étroitement liés aux caractères post-cocon chez le ver à soie, une analyse de ségrégation en masse (BSA) a été conduite en utilisant la race polyvoltine à faible rendement, Pure Mysore, et la race bivoltine à haut rendement, CSR2, comme parents car ils montrent des faits contrastés pour les caractères sélectionnés. Les données relatives à la longueur du filament et au titre ont été enregistrées chez les parents, la F1 et également pour les groupes élevé et bas de la F2. Deux cent quarante amorces SNP représentant tous les 28 groupes de liaison (LG) ont été criblées avec l'ADN génomique des parents et de leur progéniture F1. Quarante huit amorces montrèrent un polymorphisme distinct entre les parents ce qui indique une différence de taille claire dans les produits amplifiés et l'expression co-dominante de ces bandes polymorphiques a été observée en F1 où deux bandes sont détectées. Le passage au crible de ces 48 amorces parmi les parents, la F1 et les groupes de la F2 révèle que l'amorce SNP n° 09082 de LG 9 et l'amorce n° 12059 de LG 12 sont étroitement liées au QTL contrôlant la longueur et le titre du filament respectivement. Nos résultats montrent la possibilité d'utiliser les marqueurs SNP dans les programmes de lignage pour améliorer les caractères du cocon chez les lignées polyvoltines à faible rendement de l'Inde afin de maximiser la quantité de soie.

Mots-clés: *Bombyx mori*, analyse ségrégation en masse, titre, longueur du filament, QTL, SNP.



ESTERASE α , β - THE BIOCHEMICAL MARKERS FOR QUANTITATIVE AND QUALITATIVE TRAITS OF SILKWORM, *BOMBYX MORI* L.

A. K. Verma*, G. K. Chattopadhyay, N. B. Kar, A. K. Saha and N. Suresh Kumar

Silkworm Breeding, Genetics and Molecular Biology Laboratory, Central Sericultural Research and Training Institute, Murshidabad, Berhampore 742101, West Bengal, India.

*E-mail: dr.anilkumarverma@gmail.com

ABSTRACT

M.Con.1, M.Con.4 and M.Con.5 are multivoltine (V_3) congenic breeds developed through introgression of high cocoon shell weight (SCW) character from bivoltine syngenic line into high survival multivoltine syngenic line. Similarly, high survival character from multivoltine was introgressed in bivoltine to develop bivoltine congenic breeds viz., B.Con.1, B.Con.4 and B.Con.5. Syngenic lines developed based on isozyme possessor have different anodic native protein molecular masses for amylase, esterase, acid phosphatase and malate dehydrogenase in haemolymph at pH 7.0 and 8.5. Only esterase isozyme pattern depicted that 232 and 223 kDa native proteins, the possessor of β -specific esterase from donor multivoltine breeds introgressed to respective congenic bivoltine breeds B.Con.1, B.Con.4 and B. Con.5 at respective pH 7.0 and 8.5. These breeds possess survival rate equal to that of the donor even during highly variable and unfavourable climate during April to September. It indicates that thermo-tolerant β -specific esterase is one of the markers associated with high survival. Similarly, 185 and 180 kDa native protein molecular masses, the possessor of α -specific esterase from donor bivoltine breeds introgressed to multivoltine congenic breeds M.Con.1, M.Con.4 and M.Con.5 at respective pH 7.0 and 8.5 causing gradual transgression of cocoon shell weight, was considered as biochemical marker for high shell weight. Transgression of target trait took place gradually after successive development of recurrent back cross lines RBL1, RBL2 and RBL3 for both the traits. The other two native protein masses, 176 & 141 kDa at pH 7.0 and 164 & 137 kDa at pH 8.5- the possessor of non specific α β -esterase are common in all multivoltine or bivoltine silkworm strains. Isozyme possessor native proteins of esterase in haemolymph of different breeds are discussed.

Key words: Bivoltine (V_2), congenic breed, esterase, introgression, multivoltine (V_3), recurrent back cross lines, syngenic line.

INTRODUCTION

Hybrid plants and animals can withstand unfavourable climatic situation therefore facing less chance of crop failure. Many workers have reported the occurrence of heterotic genetic interaction in hybrids of a number of plants (Singh and Singh, 1971; Cregan and Busch, 1978; Borghi *et al.*, 1988) and livestock (Stonaker, 1963). It was Harada (1949, 1961) and Yokoyama (1974) who initiated hybrid culture in sericulture at commercial level in Japan and thereafter, the very visible impact of it has

resuscitated farmers to go for hybrid culture in the commercial fields of Japan sericulture. Gradually, the use of hybrids has become a traditional practice at commercial level in tropical countries with a conception that the breeds used in hybridization are pure lines. But the key problem of tropical silkworm breeds or strains is that they have highly heterogenic gene pool resulting in higher quantitative and qualitative variations in hybrids leading to deficiency in the desired result. During the last decade, a few improved multivoltine and bivoltine breeds were developed by different Institutes and from time to time,

different hybrids were raised among the developed breeds to utilize hybrid vigour / heterosis at commercial level (Dutta, 1984; Subba Rao *et al.*, 1990; Bhargava, 1993; Rao *et al.*, 1998) but the results couldn't make much impact in the eastern and north-eastern parts of the country where the new hybrids did not get popularized except for N x NB4D2. In plant system, use of inbred lines for developing congenic lines was reported previously. Moentono (1988) utilized the inbred line for high combining ability for grain yield and root lodging resistance. Torigoe *et al.* (1987) studied the difference between inbred and hybrids in terms of morphological and physiological characters of maize. Tanksley *et al.* (1981), Tanksley and Rick (1980) and Moentono (1988) used inbred/ near isogenic/ congenic lines for agricultural crop improvement on tomato, maize and corn. Blondon *et al.* (1994) stated that congenic line is suitable for biochemical tools when searching for genetic marker/ molecular marker linked to the introgressed locus.

Zubay (1987) introduced BH2 gene from B strain mice to A strain mice through development of near isogenic line for studying histocompatibility and serological test for presence of H2 antigen, thus suggesting a possibility to develop a new variety/ strain for fleshy and disease resistant animal. No report was available for developing congenic breed (Con.B / Near Isogenic line (NIL) using any multigenic trait as a target character on beneficial insect. Thereafter, emphasis has been on to develop isozyme based multivoltine (V_3) and bivoltine (V_2) syngenic lines (Chattopadhyay *et al.*, 2001 a, b, c) with an interest to introgress the target trait (dealt with multiple genes) to develop congenic breeds of bivoltine for horizontal tolerance as rearing of bivoltine breed has become a major impediment due to poor survival during adverse seasons in tropical sericulture country like India. Similarly, multivoltine breeds of tropical race, in general have poor quality silk but withstand adverse climatic situation and are less susceptible to diseases. In this context, multivoltine congenic breeds offer high scope for high cocoon shell weight to produce good quality silk without rearing of bivoltine as P1 and can be used directly at commercial level that can provide better performance

than normal multi x bi hybrid. Use of congenic hybrids was not reported elsewhere but presently from our laboratory, it was recorded that hybrid of congenic breeds or congenic x syngenic line provides heterobeltiotic genetic interaction on cocoon shell weight and total yield along with improvement in other economic characters (Verma *et al.*, 2003, 2005). During the course of development of syngenic lines, esterase isozyme pattern depicted that one thermo-stable ($80 \pm 1^\circ\text{C}$) β -esterase band naturally present in haemolymph of multivoltine breed CB5 (Chattopadhyay *et al.*, 2001c) along with Nistari and M6DPC resulting in horizontal resistance. It is also been observed that high shell weight bivoltine breeds JPN and D6 are the carrier of one common protein in haemolymph, the possessor for α -esterase for high shell weight with good quality silk (Chattopadhyay *et al.*, 2013). Developed syngenic lines of multivoltine and bivoltine and their respective congenic breeds are utilised to find out native protein molecular mass possessor of specific enzyme/ enzymes for target traits *i.e.*, high survival and high shell weight after introgression. Among the isozymes *i.e.*, amylase, esterase, acid phosphatase, alkaline phosphatase and malate dehydrogenase studied, only esterase is observed to have relationship with the above target characters and hence been discussed here.

MATERIALS AND METHODS

Silkworm breeds

Three tropical multivoltine breeds of silkworm *viz.*, CB5, M6DPC and Nistari with horizontal tolerance (high survival) and two temperate breeds *viz.*, JPN and D6 with high cocoon shell weight and quality silk procured from Germplasm stocks of Central Sericultural Research and Training Institute, Berhampore, West Bengal were considered for this study. Silkworm rearing was conducted in different seasons following the standard rearing method. Three multivoltine and two bivoltine lines were derived from the selected silkworm breeds by continuous selection, based on phenotypical traits such as larval marking, cocoon shape, cocoon colour, egg character as well as physiological parameters *viz.*, scotophase duration

in different stages of development *i.e.*, II, III and IV instars and maturation period for spinning. Further, digestive juice and haemolymph from these derived lines were collected to analyze the banding patterns in respect of esterases, amylase, acid phosphatase, alkaline phosphatase and malate dehydrogenase.

Collection of digestive juice and haemolymph

From each derived line, digestive juice and haemolymph were collected from 20-25 individuals on the 5th day of 5th instar. Digestive juice was collected in chilled microcentrifuge tubes pre-coated with 1% EDTA (chelating agent) to arrest the enzyme activities. From the same individual larvae, haemolymph (~ 200-300 μ l) was also collected by puncturing the prolegs, in chilled microcentrifuge tube pre-coated with 0.1 % phenylthiourea to prevent melanisation. The injured prolegs were sealed immediately with liquid paraffin to stop further bleeding in order to keep the larvae alive for further screening and rearing. The collected haemolymph samples were centrifuged at 2511 g and supernatant was stored at -20°C (Chattopadhyay *et al.*, 2001 b, c).

Polyacrylamide Gel Electrophoresis (PAGE): Ten μ l each of digestive juice and haemolymph were electrophoresed at pH 7.0 and 8.5 separately, under non-denatured conditions on a 7.7 % polyacrylamide gel. The gel was run at constant voltage until the tracing dye reached the bottom of the gel. Following electrophoresis, gels were washed in 0.5 M boric acid (pH 4.1) for 30 min followed by two changes of ice-cold distilled water. Gels were stained for α - and β -esterase activities separately by the procedure of Simms (1965) by using α - or β -naphthylacetate as substrates for α - or β -esterase, respectively. Controls were similarly incubated but in the absence of α - and β -naphthylacetate. Gels were documented in the System GDS-7600-UVP. The relative mobility (R_f value) was calculated for each polypeptide utilizing the formula: $R_f = \text{Distance of protein migration} / \text{distance of dye migration}$. The heat stable esterase band was detected by incubating at 80 $\pm$ 1°C for 10 minutes prior to the addition of α - or β -naphthylacetate as substrate. All

the chemicals used were procured from EMARK India Ltd. Mumbai.

Syngenic line development: Three multivoltine breeds, CB5, M6DPC and Nistari and two bivoltine breeds, JPN and D6 were considered for syngenic line development. From each breed, worms with similar phenotypical characters *viz.*, larval marking, cocoon colour and cocoon shape and similar physiological characters *viz.*, moulting duration, different stages (II, III and IV) duration and spinning duration, were taken. Digestive juice and haemolymph were collected as mentioned above from selected worms of each breed. Anodic and cathodic amylase, esterase, acid phosphatase and malate dehydrogenase isozyme patterns at pH 7.0 and 8.5 were documented for each larva. From each breed, individual larvae showing similar/ homogenous banding pattern for different isozyme similar to that of parents were selected for successive generations. These lines were allowed for sib-mating for 15 generations to develop syngenic lines (Figure 1). In the present study, only esterase isozyme pattern of syngenic lines *viz.*, CB5Lm5, M6DPE, Nistari, JPNp and D6p are highlighted.

Congenic breed development: After fixation as syngenic lines, one each of multivoltine among three lines and bivoltine from two lines were considered separately as receptor and donor parent, respectively for each crossing to develop high cocoon shell weight multivoltine congenic breed and its reciprocal for horizontal resistant (high survival/ tolerant) bivoltine congenic breeds. For M.Con.1, CB5Lm5 as a recipient and JPNp as donor, for M.Con.4, M6DPE as a recipient and D6p as a donor and for B.Con.1 and B.Con.4, their reciprocal crosses, respectively were used. For M.Con.5, Nistari plain (p) as recipient and D6p as donor and for developing B.Con.5, its reciprocal was used. In F₁, only those cocoons having high cocoon shell weight and other phenotypic characters as that of receptor parent were selected. Thereafter, consecutive recurrent back crossing was conducted for 10 to 15 generations in a similar fashion and finally sib mating was done between male and female moths emerged from larvae having maximum homogeneity to receptor parent

with higher cocoon shell weight closer to donor parent resulting in RBL1 (Figure 2). As the target character did not appear in RBL1, second crossing between donor and RBL1 was done following the procedure as for RBL1. Though some improvement was observed for this cross (RBL2), the target was not reached to the level of that of donor. Therefore, RBL2 was again considered as a recipient and crossed with donor to develop RBL3 that showed further improvement but still failed in complete achievement of the target trait like donor. Hence, RBL3 was crossed again with donor to raise RBL4 adopting the same method which showed the introgression of the target trait of donor and was considered as Congenic Breed (Con.B). In some cases, fifth or sixth time crossing with donor parent is needed for successful introgression of desired trait controlled by multiple genes (Figure 2).

To develop high survival bivoltine congenic breed, survival percentage was calculated on the basis of total

no. of moths emerged but not on the number of good cocoons harvested as there may be further mortality before emergence during unfavourable seasons. DFLs produced during unfavourable season were cold stored to be used in the next unfavourable season after three successive favourable seasons (Chattopadhyay *et al.*, 2013).

RESULTS

Four apparent native proteins of molecular mass of 232 kDa as possessor of β specific esterase, 185 kDa as α specific esterase and the other two of 176 and 141 kDa as α - β non specific esterase were identified at pH 7.0 (Figure 3). The 232 kDa β -specific esterase protein which is exclusively present in multivoltine lines (Figure 3, Lane 1) was introgressed in developed bivoltine congenic breeds viz., B.Con.1, B.Con.4 and B.Con.5 resulting in horizontal tolerance (high survival) from respective donor (Figure 3, Lane 7, 8 and 10). On the other hand, 185 kDa native protein, the possessor of α -specific esterase is

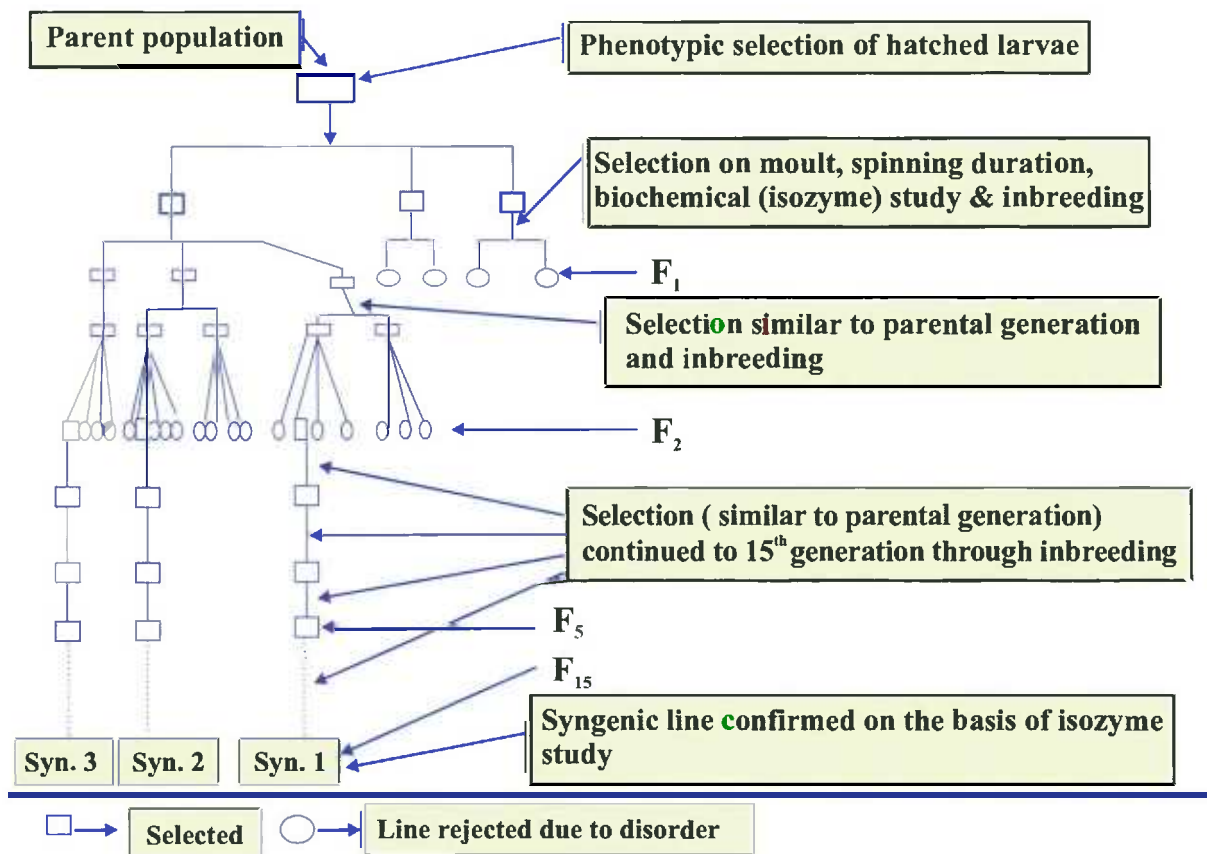


Figure 1: Scheme for development of syngenic lines in silkworm, *Bombyx mori* L.

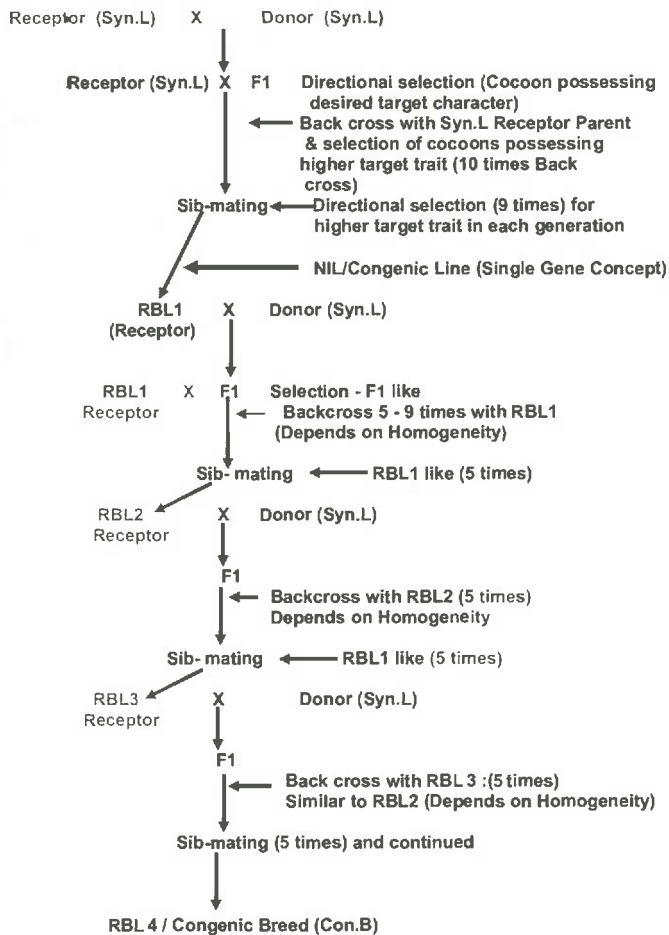


Figure 2: Scheme for development of congenic breeds dealing with multiple genes

For a character controlled by a single gene, only the steps up to RBL1 is required, but for multigenic trait, up to RBL4 or more may be necessary for complete introgression

exclusively present in donor bivoltine syngenic lines JPN β and D6 β (Figure 3, Lane 6 and 9) corresponding to their recipient and introgressed in developed multivoltine congenic breeds viz., M.Con.1, M.Con.4 and M.Con.5 (Figure 3, Lane 3, 4 and 5) possessing high shell weight. The other two proteins as non specific α - β esterase are common in all the lines.

At pH 8.5, five apparent native proteins of molecular mass of 223, 180, 164, 154 and 137 kDa are present as esterase possessors (Figure 4) wherein 223 kDa as β -specific esterase is present in multivoltine breeds (Figure 4, Lane 1 and 6) and B.Con.1, B.Con.4 and B.Con.5 (Figure 4, Lane 7, 8 and 10). The native protein

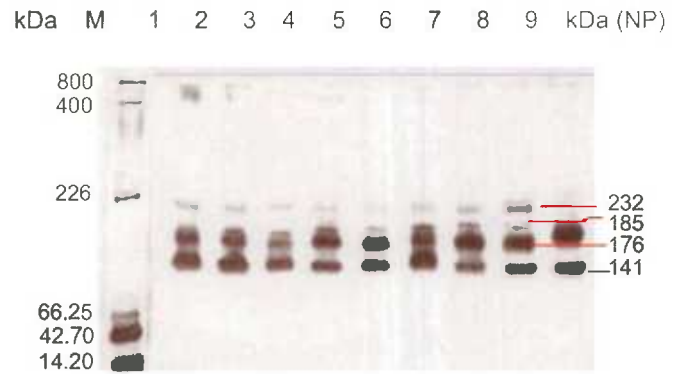


Figure 3: Vertical 7.7 PAGE of esterase isozyme pattern in haemolymph at pH 7.0: M - Marker, Lane 1 - CB5-Lm5, M6DPE/ Nistari plain (p), Lane 2 - M.Con.1, Lane 3 - M.Con.5, Lane 4 - M.Con.4, Lane 5 - JPN β , Lane 6 - B.Con.1, Lane 7 - B.Con.4, Lane 8 - B.Con.5, Lane 9 - D6 β

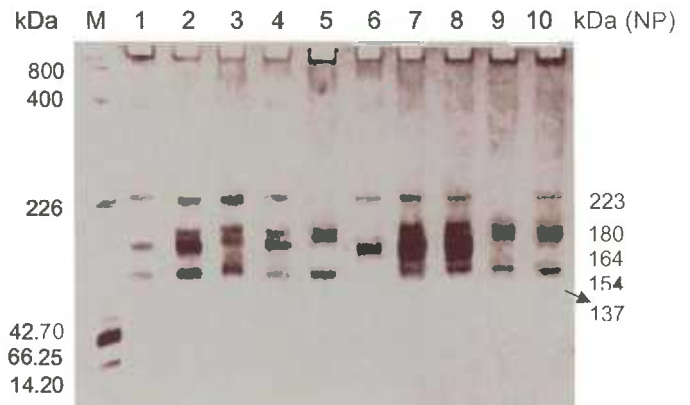


Figure 4: Vertical 7.7 PAGE of esterase isozyme pattern in haemolymph at pH 8.5: M - Marker, Lane 1 - N+p/CB5-Lm5/M6DPE (no pattern difference), Lane 2 - M.Con.1, Lane 3 - M.Con.4, Lane 4 - M.Con.5, Lane 5 - JPN β , Lane 6 - Np, Lane 7 - B.Con.1, Lane 8 - B.Con.4, Lane 9 - D6 β , Lane 10 - B.Con.5

of 180 kDa α -specific esterase possessor is exclusively present in bivoltine and congenic breeds M.Con.1, M.Con.4 and M.Con.5 (Figure 4, Lane 2, 3 and 4). The other two native proteins of viz., 164 and 137 kDa non specific α - β esterase are common in all the lines except Nistari plain (Figure 4, Lane 6). Presence of one more non-specific α - β esterase of 154 kDa was documented in developed bivoltine congenic breeds viz., B.Con.1, B.Con.4 and B.Con.5 (Figure 4, Lane 7 and 8).

The average survival in developed bivoltine congenic breeds viz., B.Con.1, B.Con.2, B.Con.3 and B.Con.4

Table 1: Performance of multivoltine congenic breeds during favourable seasons (November - February)

Breed	Yield/10000 larvae		Cocoon weight (g)	Shell weight (g)	Shell %	Filament length (m)	Denier
	No.	Weight (kg)					
CB5Lm5 (receptor)	9016	9.61	1.152	0.175	15.23	433	2.65
M.Con.1	8948	13.116	1.400	0.261	18.64	735	2.81
JPNp (donor)	8533	9.681	1.447	0.272	18.86	863	2.72
M6DPE (receptor)	8935	9.44	1.422	0.202	14.21	680	2.19
M.Con.4	8908	13.115	1.523	0.260	17.07	685	2.77
Nistari plain(p)	9125	6.80	1.135	0.125	11.01	331	1.65
M.Con.5	9115	12.537	1.469	0.255	17.34	765	2.77
D6p (donor)	8633	12.34	1.585	0.275	17.35	850	2.52
CD at 5 %	440.99	1.232	0.113	0.022	0.752	23.70	0.158

Table 2: Performance of bivoltine congenic breeds during unfavourable seasons (April to September)

Breed	Survival %	Yield /10000 larvae (kg)	Cocoon weight (g)	Shell weight (g)	Shell %	Non-breakable filament length (m)	Denier
CB5Lm5 (donor)	69.71	7.12	1.106	0.153	13.83	385	2.70
B Con.1	59.23	7.16	1.262	0.239	18.93	702	2.62
B Con. 2	56.18	6.27	1.258	0.232	18.44	696	2.54
B Con. 3	61.28	7.52	1.272	0.212	16.7	675	2.68
JPN (receptor)	No survival						
D6p (receptor)	No survival						
B Con. 4	65.48	9.93	1.310	0.257	19.61	705	2.67
M6DPCLmEl(donor)	68.33	7.15	1.121	0.148	13.20	392	2.15
CD at 5%	8.56	1.33	0.133	0.021	0.785	21.82	0.145

during unfavourable seasons were 59, 56, 61 and 65 %, respectively in comparison to very less or no survival in receptor bivoltine breeds JPNp and D6p (Table 2). Agrahayani (November) crop is the main commercial crop of Eastern India, reared in congenial environment. The rearing of preceding P1 crop is done during September-October under the prevailing extremes of temperature (40-45 °C) and humidity (90-98 %), resulting in very poor survival / failure in the rearing of pure bivoltine parents. On the contrary, bivoltine congenic lines due to multivoltine blood can be reared under these conditions with moderate success in the field. The shell weight in developed multivoltine congenic breeds viz., M.Con.1, M.Con.4 and M.Con.5 during

favourable season were 0.251, 0.260 and 0.255 g, respectively in comparison to 0.175, 0.202 and 0.113 g in recipient multivoltine breeds CB5Lm5, M6DPE and Nistari, respectively (Table 1).

DISCUSSION

Syngenic multivoltine and bivoltine lines were developed initially to attain homogeneity first phenotypically, physiologically and thereafter, based on different isozyme possessor native protein pattern similarity in specific enzymes viz., Amy, Est, Acph, and Mdh and continuous sib mating done under such selection pressure. It is vivid from a series of studies conducted on isozyme possessor native protein pattern in

haemolymph of RBLs and finally on Congenic breeds, that only esterase isozyme pattern provided very discrete isozyme pattern behaviour between recipient and donor parents by the presence of specific band in high cocoon shell weight bivoltine and high survival multivoltine breeds. The present study demonstrates that β -esterase isozyme possessor apparent native proteins of 232 and 223 kDa at pH 7.0 and 8.5, respectively are exclusively present in haemolymph of multivoltine lines and all developed bivoltine congenic breeds but absent in bivoltine syngenic lines. This suggests that the presence of 232 and 223 kDa native proteins, the possessor of β -esterase in haemolymph, a thermo-tolerant $80 \pm 1^\circ\text{C}$ enzyme at respective pH is considered as one of the biochemical markers associated with higher survival supporting the rearing performance data. Further, it has also been documented that native proteins, 185 kDa and 180 kDa, the possessor of α -specific esterase at respective pH, present only in bivoltine lines with high shell weight were introgressed in high survival multivoltine breeds to develop high cocoon shell weight multivoltine congenic breeds M.Con.1, M.Con.4 and M.Con.5. Rearing data also confirmed that the apparent native protein associated with the improvement of cocoon shell weight (0.261, 0.260 and 0.255 g) of multivoltine congenic breeds over receptor (0.175 and 0.202 and 0.112 g) as another biochemical marker for high cocoon shell weight.

Consequent to highly heterogenic gene pool in the tropical silkworm, it becomes difficult to trace the level of introgression (Young *et al.*, 1988) of gene/genes in any evolved breed. So the importance of syngenic lines is explained here that helped to understand clearly the isozyme pattern difference between recipient and donor. Starin and Zeven, 1981; Tanksley *et al.*, 1981 and Zeven *et al.*, 1983 stated that Congenic breed / Near Isogenic line is the most suitable material to be examined for Biochemical or Genetic marker/ Molecular marker linked to introgressed locus. Development of syngenic line provided homogeneity in esterase isozyme pattern among multivoltine and also in bivoltine under selection pressure and is the most important breeding strategy to understand

and identify biochemical marker. Our finding corroborates with this view and suggests that the Congenic breeds are more useful for quality improvement and stress tolerance in tropical country like India with special reference to Eastern India. The identified markers are in use in further breeding programme involving high quality Japanese and Chinese races to suit tropical climate.

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LES α , β - ESTERASES, MARQUEURS BIOCHIMIQUES POUR LES CARACTERES QUANTITATIFS ET QUALITATIFS CHEZ LE VER A SOIE, *BOMBYX MORI* L.

A. K. Verma^{*}, G. K. Chattopadhyay, N. B. Kar, A. K. Saha and N. Suresh Kumar

Silkworm Breeding, Genetics and Molecular Biology Laboratory, Central Sericultural Research and Training Institute, Murshidabad, Berhampore 742101, West Bengal, India.

^{*}E-mail: dr.anilkumarverma@gmail.com

R É S U M É

M.Con.1, M.Con.4 et M.Con.5 sont des lignées polyvoltines (V_3) congéniques développées par introgression du caractère du poids de coque soyeuse élevé (SCW) d'une lignée syngénique bivoltine dans une lignée syngénique polyvoltine à survie élevée. De même, le caractère survie élevée d'un polyvoltin est introgressé dans un bivoltin pour développer des lignées congéniques bivoltines telles que B.Con.1, B.Con.4 et B.Con.5. Les lignées syngéniques développées basées sur la présence d'un isozyme ont des masses moléculaires de protéines anodiques natives différentes pour l'amylase, l'estérase, la phosphatase acide et la malate déshydrogénase dans l'hémolymph à pH 7,0 et 8,5. Seul le profil des isozymes de l'estérase donne des protéines natives à 232 et 223 kDa, le possesseur de la β -estérase spécifique des lignées polyvoltines donneuses étant introgressé dans les lignées bivoltines congéniques respectives B.Con.1, B.Con.4 et B.Con.5 aux pH 7,0 et 8,5. Ces lignées possèdent un taux de survie égal à celui du donneur même pendant un climat très variable et défavorable de avril à septembre. Ceci indique que la β -estérase spécifique est l'un des marqueurs associés au taux de survie élevé. De même, les protéines natives de 185 et 180 kDa, le possesseur de l' α -estérase spécifique des lignées bivoltines donneuses est introgressé vers les lignées congéniques M.Con.1, M.Con.4 et M.Con.5 aux pH respectifs de 7,0 et 8,5 ce qui induit une transgression graduelle du poids de la coque soyeuse considérée comme marqueur biochimique pour le poids de coque élevé. La transgression du caractère cible prend place progressivement après des croisements de retour récurrents des lignées RBL1, RBL2 et RBL3 pour les deux caractères. Les deux autres masses de protéines natives, 176 & 141 kDa au pH 7,0 et 164 & 137 kDa au pH 8,5 le possesseur de l' α β -estérase est commun chez toutes les souches polyvoltines ou bivoltines.

Mots-clés: Bivoltine (V_2), lignée congénique, estérase, introgression, polyvoltine (V_3), croisements de retour récurrents, lignée syngénique.



Research Paper

EFFECT OF SUBMERGED CULTURE PARAMETERS ON GROWTH AND ANTIMICROBIAL ACTIVITY OF *PAENIBACILLUS LAUTUS* RRT AY-2, AGAINST SOME PATHOGENS OF *ANTHRAEA ASSAMENSIS* HELFER

D. K. Gogoi¹, S. P. Sandilya¹, P. M. Bhuyan¹, P. Dutta¹, K. Neog¹, L. S. K. Singh² and T. C. Bora²

¹Department of Biotechnology, Central Muga Eri Research and Training Institute, Central Silk Board,
Jorhat 785700, Assam, India.

²Department of Biotechnology, North East Institute of Science and Technology, Council of Scientific and Industrial Research,
Jorhat 785006, Assam, India.

E-mail: gkdeep@rediffmail.com

ABSTRACT

Antheraea assamensis Helfer, which is endemic to North-eastern India and produces golden yellow silk, is prone to various entomo-pathogenic microorganisms due to outdoor nature of rearing. A bacterial strain isolated from a hot-spring of India and identified as *Paenibacillus lautus* RRT AY-2 was found to possess antimicrobial potentialities against some of the muga silkworm pathogens. The *in-vitro* culture parameters of the bacterium were optimized for growth and maximum production of the antimicrobial agent. Maximum growth as well as production of the antimicrobial agent was recorded at 35 °C with pH 7.5 in liquid media supplement with glucose and beef extract as C and N sources, respectively. *P. lautus* RRT AY-2 reached its maximum growth on 48 h of incubation, while it recorded the maximum antimicrobial activity at 54 h and specific rate of product formation was maximum in between 18 and 24h. Amendment of glutamic acid, aspartic acid and leucine in combination with glucose enhanced the growth as well as biosynthesis of the bioactive molecule. Glucose concentration of 1.2 % and NaCl at 2 % was found to be optimum for production of the antimicrobial agent. The solvent extracted crude antimicrobial compound was partially purified by Thin Layer Chromatography. The TLC purified active compound with UV λ-max 260 nm in ethyl acetate has got the lowest minimum inhibitory concentration (MIC) against *Bacillus sp.*, whereas the highest MIC was recorded against *Pseudomonas aeruginosa*. This bacterium may be utilized on muga silkworm to develop colonization resistance against pathogenic microbes as an alternative strategy for disease management.

Key words: Antimicrobial agent, hot-spring, MIC, *Paenibacillus lautus* RRTAY-2.

INTRODUCTION

Eastern Himalaya including entire North East India belongs to the Indo-Burma mega-biodiversity hot-spot (Myers *et al.*, 2000), which constitute plenty of hot-springs (Saha *et al.*, 2005; Bora *et al.*, 2006). The affluence in thermophilic microbial diversity of the region is reaffirmed by identification of new gammaproteobacterium, *Aquimonas voraii* sp. (Saha *et al.*, 2005) and *Flavobacterium indicum* sp. nov. (Saha and

Chakrabarti, 2006) from a warm-spring of Nambor Wild Life Sanctuary, Assam, India. Though a number of hot-springs have been reported from Indian sub-continent (Chatterjee and Guha, 1968; Majumdar *et al.*, 2000), very few efforts have been made to explore the potentialities of prevailing microbial wealth (Ghosh *et al.*, 2003).

Muga silkworm, *Antheraea assamensis*, endemic to North-eastern regions of India produces the unique glistening golden yellow silk. The whole of sericulture

industry revolves around this insect in the North-eastern India as it offers livelihood security to over 0.75 million people. Being poikilothermic in nature, muga silkworms are highly prone to diseases such as flacherie (Guha *et al.*, 1999), microsporidiosis and occasionally virosis (Jolly *et al.*, 1986). The major pathogenic microbes that affect the muga silkworm are *Pseudomonas aeruginosa* strain AC3 (Choudhury *et al.*, 2002), *Bacillus thuringiensis* (Das *et al.*, 2010), *Fusarium moniliforme*, *Beauveria bassiana*, *Penicillium digitatum* and *Aspergillus* sp. (Das *et al.*, 2007). Virulent strains of *Pseudomonas aeruginosa* strains causing flacherie in muga silkworms, *A. assamensis* (Guha *et al.*, 1999; Choudhury *et al.*, 2002) were reported to be resistant to different antibiotics and plant extracts (Choudhury *et al.*, 2002). Under tropical climate, out of every 5-6 crops per annum, sericulturists experience 2-3 crop failures due to disease outbreak. Bacterial flacherie is the most threatening disease of muga silkworm with percentage disease prevalence (PDP) up to 70 %, followed by grasserie caused by virus (2 to 47 %) and muscardine (10-47 %) caused by fungus (Choudhury *et al.*, 2002).

Members of the bacterial genus *Paenibacillus* produce antibacterial compounds such as polymyxin, octopytin and baciphelacin (Slepecky and Hemphill, 1991) and an antifungal compound (Chung *et al.*, 2000). Spore forming bacterial species of *Paenibacillus polymyxa* are able to produce antibiotic compounds, for example, polymixin group (Vogler and Studer, 1966), gatavaline (Nakajima *et al.*, 1972), LI-F complex (Seldin *et al.*, 1999), fusaricidin A (Kajimura and Kaneda, 1996), gavaserin and saltavalin (Pichard *et al.*, 1995). Recently, a bacteriocin like compound named polyxin was reported from *P. polymyxa* strain isolated from regional fermented sausages (Piuri *et al.*, 1998).

Culture parameters viz., temperature, pH, incubation period (Digrak and Eluk, 2001; Vahidi *et al.* 2004) and chemical factors viz., carbon, nitrogen supplement (Adinarayana *et al.*, 2001), and amino acid amendment in

combination with carbon source (Noaman *et al.*, 2004) and mineral salt (Basak and Mazumdar, 1975; Sailer *et al.*, 1993) play a major role in the production of antimicrobial agent by microorganisms.

Considering the increasing demand for bio-control and management strategies against the dominant infectious diseases of *A. assamensis*, we took up an endeavour, to characterize the antimicrobial property of thermophilic bacteria isolated from a hot-spring located at Golaghat district of Assam. This study provides an account of the efforts made to investigate and optimize the effects of various culture parameters on growth, bioactivity and metabolite biosynthesis by *Paenibacillus lautus* RRT AY-2 against some muga silkworm pathogens.

MATERIALS AND METHODS

Isolation of bacteria

The bacterial strain was isolated by standard serial dilution method (Collins *et al.*, 1989), from sediment sample of Borpung, a natural warm water-spring situated in Assam, India. The prevailing temperature and pH of the warm-spring water with sediment recorded during the collection were 48 °C and 8.5, respectively. The strain was further maintained in nutrient agar (Hi-Media, Mumbai) culture medium (Peptone 0.5 %, Beef extract 0.15 %, Yeast extract 0.15 %, NaCl 0.5 %, Agar 1.5 %; pH 7.4).

Characterization and identification of the bacterium

The bacterial isolate was subjected to characterization both morphologically and biochemically according to the methods described by Holt *et al.* (1994). The identification was carried out on the basis of microscopic observation (ZEISS, Axioskop 40), morphology, biochemical characters and 16S rDNA homology. The genomic DNA of bacterial isolate RRT AY-2 was prepared as described in Marmur's protocol (Johnson, 1994) and rDNA sequencing was carried out through the courtesy of IGIB, New Delhi, India. The phylogenetic similarity search was performed by using NCBI Genbank databases blast service.

Test organisms and *in-vitro* antibiosis

Five microbial test pathogens of muga silkworm, comprising of two bacteria - *Pseudomonas aeruginosa* and *Bacillus* sp. and three fungi - *Fusarium moniliforme*, *Beauveria bassiana* and *Aspergillus* sp. were considered for *in-vitro* antibiosis of the isolate RRT AY-2. The test pathogens were isolated from the diseased silkworm larvae collected from farmers' field. The surface sterilized dead larvae were dissected to collect the contents from surface area, foregut, midgut and hindgut. Isolation was done as per standard serial dilution technology on Nutrient agar, BH Infusion agar and RB Chloramphenicol agar (Hi-media Ltd., Mumbai).

Antimicrobial assay of the bacterial culture filtrate and extract was carried out by agar well diffusion method (Schillinger and Lucke, 1989) and standard disc diffusion method (Acar, 1980). Mueller hinton agar (MHA) and PDA (Hi-media Ltd.) media were used for bioassay against bacterial and fungal test pathogens, respectively.

Estimation of growth and metabolite production

The bacterial growth was determined according to the dry weights of cell in mg/50 ml. Production of active metabolite was estimated by calibrating a standard curve from concentrations of the pure active compound versus optical density at UV λ -max (SPECORD 210) as described earlier by Gogoi *et al.*, 2008.

Optimization of media

Optimization of culture conditions and media supplements on growth and bioactive metabolite production was carried out where sterilized sediment water was considered as basal medium. The parameters viz., incubation period, temperature, pH, carbon and nitrogen supplement, effect of glucose concentration, salinity and amino acid amendment were considered for optimization.

Incubation period, temperature and pH: The bacterial

isolate RRT AY-2 was inoculated into the basal medium supplemented with glucose and beef extract and then incubated under shaking condition (150 rpm) up to 15 days at 32 °C.

In order to assess the effect of temperature on growth and antimicrobial agent production, the bacterium was cultured under various temperature regimes ranging from 15 to 60 °C. In addition, to determine the effect of initial media pH, the bacterial isolate RRT AY-2 was cultured under different pH levels, ranging from 3 to 11 at 35 °C for 10 days. The growth and the bioactive metabolite production in comparison to control were determined for every experiment by comparing cell dry weight and UV λ -max of the culture filtrate.

Carbon and Nitrogen supplement: Various carbon sources such as glucose, lactose, glycerol, fructose, maltose, galactose, starch, mannitol, sucrose, ribose and xylose and nitrogen sources viz., yeast extract, beef extract, peptone, $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , NH_4Cl and NH_4PO_4 were supplemented individually into the basal medium at a concentration of 1 %. The bacterial isolate RRT AY-2 was inoculated to the carbon and nitrogen amended basal media and incubated at 35 °C for 6 days at 150 rpm in gyratory shaker (Kuhner ISF-1-W). The respective biomass *vis-à-vis* metabolite production was recorded.

Effect of glucose concentration: The effect of different concentrations of glucose (0-4 %) on growth and biosynthesis of active metabolite was studied and maximum antibiotic production was recorded.

Salinity: The effect of salinity on growth and bioactive metabolite production was determined by culturing the bacterium in various NaCl concentrations ranging from 1-10 %, into the basal media amended with 1 % glucose. The biomass accumulation and bioactive metabolite production for each NaCl concentration were estimated as stated earlier.

Amino acid amendment: A number of amino acids were amended at a concentration of 0.1 mg/ml in combination

with glucose. The effect on growth and bioactive metabolite production was estimated under required incubation conditions.

Specific rate of product formation (qp): The specific rate of active metabolite production (qp) was calculated according to the following equation:

$$qp = 1/X (dP/dt)$$

Where, X is the biomass concentration ($\mu\text{g/ml}$), P is antimicrobial agent concentration and t is time. The derivative dP/dt was calculated according to the method proposed by Le Duy and Zajic (1973) and using software for graphical analysis Origin PRO-7.5.

Extraction of bioactive compound

The bacterial culture was centrifuged at $7796 \times g$ (Sigma 3K30) for 10 minutes and the supernatant was extracted by mechanical shaking with equal volume of ethyl acetate, thrice. Afterwards, the solvent fraction was treated with Na_2SO_4 (Rankem) and removed at reduced pressure using rotary evaporator (Buchi R-114). The residue was weighed and then redissolved in ethyl acetate for later use.

Purification

Purification was carried out by thin layer chromatography (TLC) device on silica gel (Merck Ltd.) using petroleum ether-ethyl acetate (Rankem) gradient (10:1 $\rightarrow$ 1:10) as running solvent system. TLC purified fractions were recovered and bioassayed to find out the pure active fraction.

MIC and MFC

MIC and MFC were determined according to Boruwa *et al.* (2004) by inoculating equal size of test organisms to 5 ml each of nutrient broth (NB) and potato dextrose broth (PDB) in different tubes. Serial dilutions of the active compound were added simultaneously and MICs of the

bacterial test pathogens were determined after 48 h of incubation by removing 10 μl of the contents from each tube and spreading them onto NA plates. After 24 h of incubation at 35°C , growth of the colony was observed. MFCs were determined in molten M2 agar base (Hi-media Ltd.) by following the same procedure after 48 h of incubation at 28°C .

Statistics

Statistical analyses were carried out by calculation of the means and standard deviations of the data. Duncan's multiple range test (DMRT) was done to compare the sample means at significance level of $p < 0.001$ (Gomez and Gomez, 1984).

RESULTS

The strain RRT AY-2 isolated from warm spring was a gram positive, rod shaped, endospore forming, motile bacterium; optimum temperature and pH for growth was 35°C and 7.5, respectively. The physiological and biochemical characteristics of the isolate determined are provided in Table 1. The 16S rRNA gene partial sequence has got GenBank accession no. DQ299946 and it was compared to that of different strains obtained from the NCBI database using the neighborjoining method (Thompson *et al.*, 1994). The results provided in Table 2 show that RRT AY-2 has the highest similarity (99 %) with *Paenibacillus lautus* AB073188 and *Paenibacillus lautus* AJ491842. Hence, the bacterial strain was further named as *Paenibacillus lautus* RRT AY-2. Comparison against 16S rRNA gene sequences held in GenBank indicates that the organism is related phylogenetically to members of the family 'Paenibacillaceae'.

Extraction, purification and assay of bioactive compound

The active culture filtrate was solvent extracted and four major fractions with different R_f values from preparative TLC plate were considered for antibiotic bioassay. The fractions recovered from TLC plates were

Table 1: Morphological, physiological and biochemical characteristics of *P. lautus* RRT AY-2

Characteristic	Result	Characteristic	Result
Morphological		Biochemical	
Shape	Rod	Catalase	+
Gram stain	+	Urease	+
Pigment	-	Oxidase	+
Endospore formation	+	Citrate utilization	+
Motility	+	Nitrate reduction	+
Physiological		Starch hydrolysis	+
Aerobic growth	+	Indole production	-
Anaerobic growth	+	Gelatin liquefaction	+
Optimum temperature	35°C	D-glucose utilization	+
Growth temperature range	10-50°C	D-Mannitol utilization	+
Optimum pH	7.5	Sucrose utilization	+
Growth pH range	5-9	Glycerol utilization	+
		NaCl tolerance	<6 %

Table 2: Identity of *P. lautus* RRT AY-2 compared with partial strains in the Gen-Bank database

Organism	Strain	Accession No.	% Identity
<i>Paenibacillus lautus</i>	JCM 9073	AB073188	99
"	C2	AJ491842	99
"	NRRL B-379	D85394	99
"	NRRL B-377	D85609	99
"	CCM 3892	D78472	98
"	S138	AY509231	98
"	NRS-666T	D78473	98
"	RS601	DQ789323	98
"	CP183ca	AJ871418	98
"	CP183ca	AJ871461	98
"	RS301	DQ789320	98
"	Blautus-JR	Af478100	98

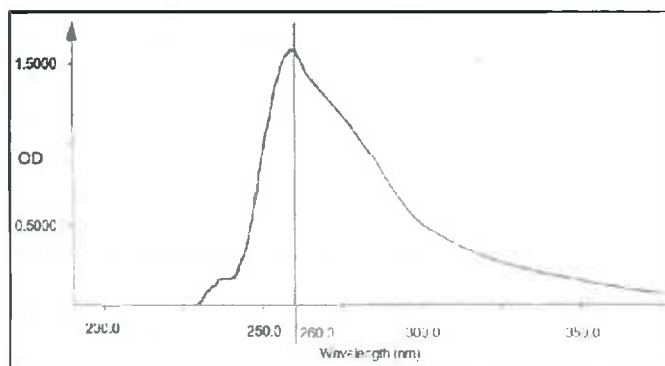
solvent eluted, dried *in-vacuo* and dissolved in Dimethyl Sulfoxide (E Merck Ltd.) for bioevaluation by agar well diffusion method. Fraction with R_f value 0.542 in ethyl acetate and petroleum ether (1:4) as running solvent exhibited inhibition zones against almost all the test pathogens (Table 3). The purified compound was soluble in methanol, ethyl acetate, chloroform and DMSO

whereas, sparingly soluble in water and petroleum ether. The TLC purified active compound showed UV λ -max in ethyl acetate at 260 nm (Figure 1).

The bioactive compound exhibited the lowest MIC of 45 μ g/ml against *Bacillus* sp., followed by *F. moniliforme* (55 μ g/ml) and *B. bassiana* (60 μ g/ml) whereas the highest

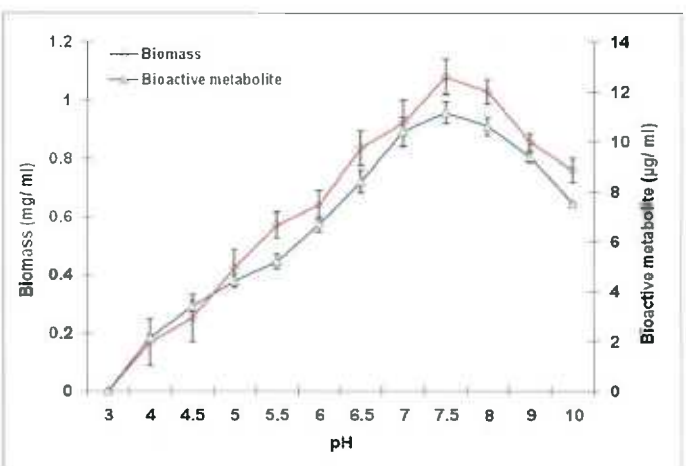
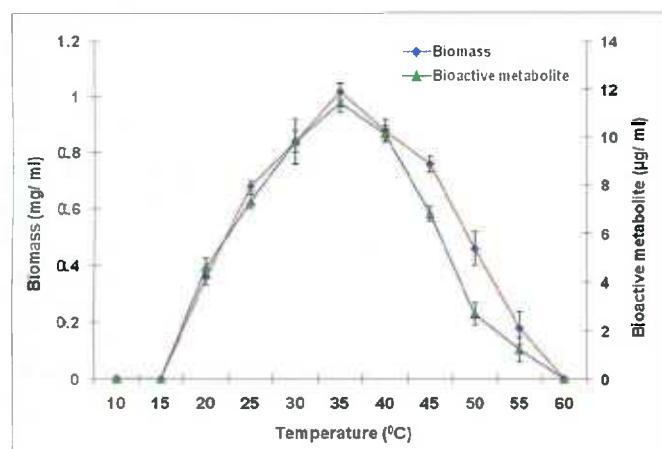
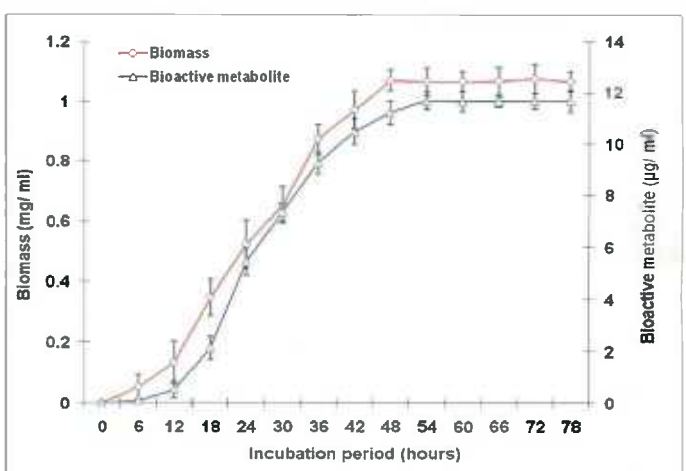
Table 3: *In vitro* antibiosis of the four TLC purified fractions against the test organisms with respective R_f values

Test organism	R_f values (Inhibition zone diameter in mm)			
	0.32	0.45	0.52	0.83
<i>Bacillus</i> sp.	-	-	22 (± 2)	-
<i>Pseudomonas aeruginosa</i>	-	-	18 (± 2)	-
<i>Fusarium moniliforme</i>	-	17 (± 1)	20 (± 2)	-
<i>Beauveria bassiana</i>	-	-	21 (± 2)	-
<i>Aspergillus</i> sp.	-	-	20 (± 2)	-

Concentration of the fractions = 100 $\mu\text{g/ml}$ **Figure 1: UV λ -max of the bioactive compound isolated from *P. laetus* RRT AY-2****Table 4: Minimal inhibitory concentrations (MIC) of the TLC purified secondary metabolite isolated from *P. laetus* RRT AY-2**

Test organism	MIC ($\mu\text{g/ml}$)
<i>Bacillus</i> sp.	45 (± 2)
<i>Pseudomonas aeruginosa</i>	65 (± 2)
<i>Fusarium moniliforme</i>	55 (± 2)
<i>Beauveria bassiana</i>	60 (± 2)
<i>Aspergillus</i> sp.	65 (± 2)

Data are mean of observations on three independent experiments.

**Figure 3: Influence of pH on growth and biosynthesis of secondary metabolite by *P. laetus* RRT AY-2****Figure 2: Influence of temperature on growth and biosynthesis of secondary metabolite by *P. laetus* RRT AY-2****Figure 4: Influence of incubation period on growth and biosynthesis of secondary metabolite by *P. laetus* RRT AY-2**

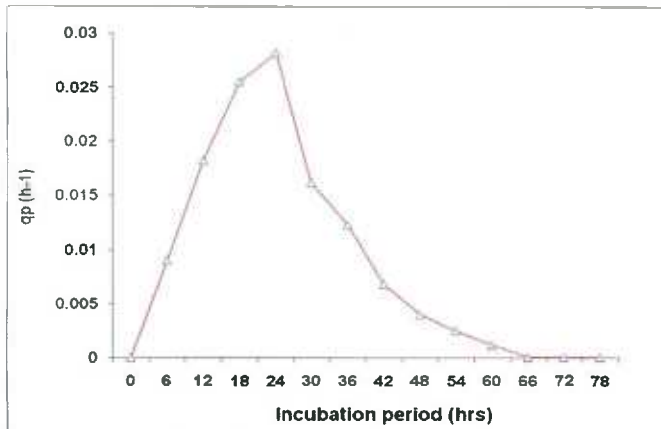


Figure 5: Specific rate of bioactive metabolite production (qp) by *P. lautus* RRT AY-2

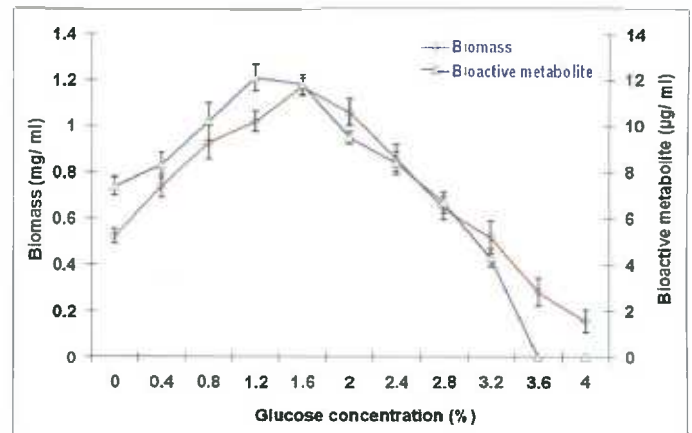


Figure 8: Influence of glucose concentration on growth and biosynthesis of secondary metabolite by *P. lautus* RRT AY-2

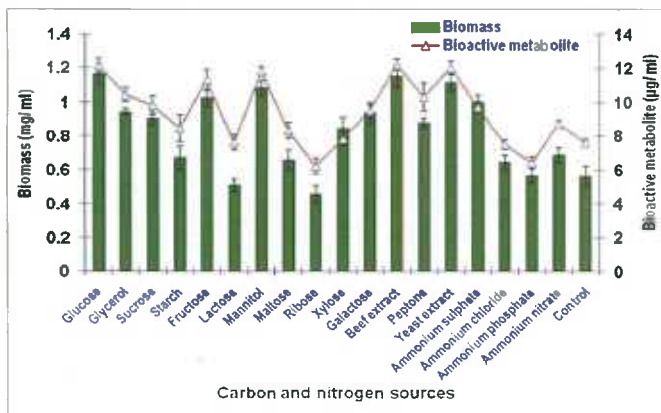


Figure 6: Influence of carbon and nitrogen sources on growth and biosynthesis of secondary metabolite by *P. lautus* RRT AY-2

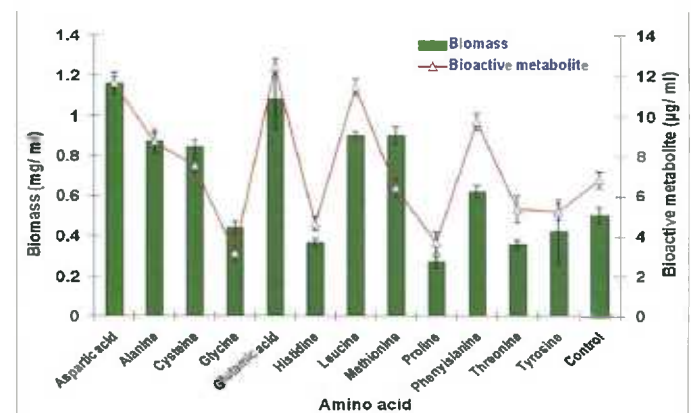


Figure 9: Influence of amino acids amendment with glucose on growth and biosynthesis of secondary metabolite by *P. lautus* RRT AY-2

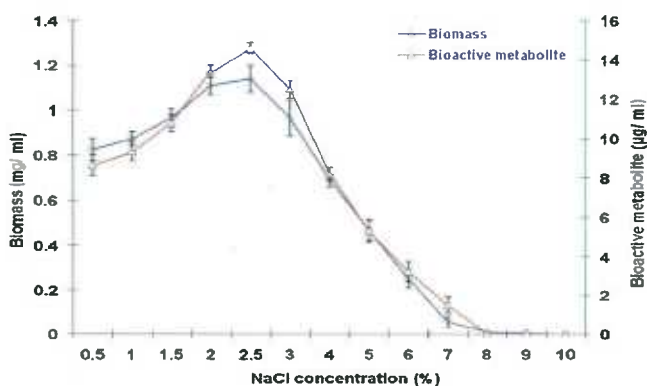


Figure 7: Influence of NaCl concentration on growth and biosynthesis of secondary metabolite by *P. lautus* RRT AY-2

MIC was recorded against *P. aeruginosa* and *Aspergillus* sp. (65 µg/ml) (Table 4).

Influence of culture parameter

The bacterial strain *P. lautus* RRT AY-2 produced the highest biomass (5.1 mg / 50 ml) as well as antimicrobial metabolite (11.4 µg / ml) at 35 °C on submerged culture (Figure 2). The optimum growth of the bacterium was observed at 35 °C. The strain could not grow at a temperature below 15 °C and growth as well as metabolite biosynthesis was almost ceased at above 55 °C. The pH 7.5 is optimum for maximum growth (5.4 mg/50 ml) and production of antimicrobial agent (11.16 µg/ ml) by *P. lautus* RRT AY-2 (Figure 2). Growth of the bacterium was

arrested at pH below 4 and above 10. Hence the temperature and pH regime of 35 °C and 7.5, respectively were considered as the standard for other experiments.

Incubation period up to 48 hours was found enough for maximum growth (5.35 mg / 50 ml) of *P. lautus* RRT AY-2 and the highest amount of bioactive metabolite (11.68 µg / ml) was estimated on 54 h culture filtrate (Figure 4). The bacterial dry cell weight and metabolite biosynthesis remained almost constant after 48 and 54 h of incubation period, respectively. Highly significant specific rate of bioactive metabolite biosynthesis (qp) was recorded on 18 to 24 h (0.169 6h⁻¹) and it was followed by 12 to 18 h (0.153 6h⁻¹) culture (Figure 5). The bacterium, after 54 h incubation period was set for optimum growth and metabolite synthesis in further experiments.

Impact of carbon, nitrogen source, glucose concentration and salinity

The bacterial strain *P. lautus* RRT AY-2 was cultured on basal medium supplemented with various carbon and nitrogen sources, and results exhibited significant effect on growth as well as secondary metabolite production (Figure 6). Highly significant growth (5.8 mg / 50 ml) as well as biosynthesis of antimicrobial agent (12.14 µg / ml) was detected in glucose supplemented medium. In addition, fructose (11.27 µg / ml) and mannitol (11.8 µg / ml) containing media also promoted the bioactive metabolite production in comparison to the control.

As for growth of *P. lautus* RRT AY-2, beef extract was optimized as the best (5.76 mg / 50 ml) nitrogen supplement, while biosynthesis of antimicrobial agent was recorded almost same on beef extract (12.2 µg / ml) and yeast extract (12 µg / ml) amended media (Figure 6). In addition, peptone (10.3 µg/ml), ammonium sulphate (9.7 µg/ml) and ammonium nitrate (8.7 µg/ml) supplement also provided higher antimicrobial agent than in control basal medium.

Concerning the concentration of glucose in liquid

medium, 1.6 % (w/v) glucose provided the highest growth (5.86 mg / 50 ml), whereas, higher production of antimicrobial agent was recorded at glucose concentrations of 1.2 % and 1.6 %. No antibiotic production was detected for the bacterium grown at glucose concentration above 3.6 % and a slower rate of growth was observed in a medium with glucose concentration below 1.2 % (Figure 7).

Similarly, 2.5 % NaCl concentration in liquid medium produced the highest biomass (5.72 mg / 50 ml), whereas, 2 % NaCl was found to be optimum for biosynthesis of antimicrobial agent (11.84 µg / ml) by *P. lautus* RRT AY-2 (Figure 8). Consequently, the concentration ranging from 1 to 4 % NaCl was recommended for growth and secondary metabolite synthesis by the bacterium, which was detected to get decreased above 5 % NaCl concentration.

Amino acid amendment

Amendment of a few amino acids in combination with glucose promoted the growth as well as biosynthesis of antimicrobial agents by *P. lautus* RRT AY-2. Among the amino acids screened, aspartic acid in combination with glucose provided the highest growth (5.8 mg / 50 ml), while glutamic acid enhanced the biosynthesis of antimicrobial agent (12.4 µg / ml) by the bacterial strain. Besides, phenylalanine (9.68 µg / ml), alanine (8.7 µg / ml), leucine (8.6 µg / ml) and cysteine (7.5 µg / ml) amendment with glucose also provided higher quantity of antimicrobial agent in comparison to the control basal medium (Figure 9).

DISCUSSION

Several *Bacillus* and related genera including *Paenibacillus* sp. have been tested as potential antimicrobial agents as they produce a range of antibiotics and form resistant spores (Weid *et al.*, 2003). Antimicrobial agent produced by *P. lautus* strain RRT AY-2 demonstrated antibacterial and antifungal activity against entomo-pathogenic microorganisms, suggesting that the strains or its metabolites could be used as an alternative or

as supplement to manage infectious diseases of muga silkworm. To determine novel bioactive secondary metabolites, many modern techniques such as high-throughput screening of different biological sources have been employed (Grabley and Thiericke, 1999; Maier *et al.*, 1999). As an alternative, effective screening process can be achieved through systematic manipulation of culture parameters for potential organisms. As far as impact of culture conditions are concerned, there is confusion between achieving maximal growth rates and maximal antibiotic yields because conditions that allow higher growth could be unfavourable to antibiotic production (Audhya and Russell, 1974; Chisti and Moo-Young, 1993). The growth and yield of bioactive compounds by potential microorganisms can sometimes be promoted by optimizing physical and chemical factors (Calvo *et al.*, 2002; Llorens *et al.*, 2004).

The bacterium, *P. lautus* RRT AY-2 isolated from warm-spring sediment sample produces antimicrobial agent with broad spectrum *in-vitro* activity against some microbial pathogens of muga silkworm. The results revealed that the active metabolite extracted from the bacterium is more effective against gram positive bacteria and fungi. Gram negative bacterial pathogens were less sensitive to the bioactive compound.

In this study, the incubation temperature of 35 °C, was found to be optimum for growth as well as biosynthesis of bioactive metabolite. A decrease in growth and bioactive metabolite production was observed when the incubation temperature was higher or lower than the original native temperature. Higher temperature had some adverse effect on the metabolic activities of the microorganisms and it has been reported by different workers that the metabolic activities of the microorganisms become slow at lower temperature (Adinarayana *et al.*, 2001).

The initial pH value of the culture medium can also have a significant effect on the production of secondary metabolite (Yakoby *et al.*, 2000; Singh, 2002). We have recorded optimum pH level at 7.5 for growth and bioactive metabolite production, whereas, acidic pH was

not found suitable for antibiotic production in this study. According to Buchanan and Ayres (1975), the initial pH of medium is not a determinant factor for antibiotic production, but Keller *et al.* (1997) showed the reduction in secondary metabolite production when the pH was increased. The pH is related to permeability characteristics of the cell wall and membrane, hence it affect either ion uptake to or its loss from the nutrient medium (Hansen, 1968).

Regarding the incubation period, the highest production of the antimicrobial compound was recorded at 48 h of incubation. The trophophase (the growth phase) of *P. lautus* RRT AY-2 lasted for 48 h, but the idiophase (production phase) reached its peak at 54 h of incubation and remained almost constant after 60 h. Griffiths and Saker (2003) reported excretion of bioactive metabolites by *C. raciborskii* to occur as cultures moved into the post-exponential phase of growth, while Egorov (1985) postulated that maximum antimicrobial activity was attained after reaching the maximum value of the biomass.

Most of microbial culture media contain carbon and nitrogen sources and their supplement which strongly influence the growth as well as metabolite production by microorganisms. Regarding carbon source, simple sugars such as glucose, fructose, sucrose as sole carbon source enhanced growth as well secondary metabolite production rather than more-complex carbon compounds *viz.*, starch, galactose, xylose, mannitol *etc.* (Calvo *et al.*, 2002). The simple carbohydrates through catabolism process, provide intermediates leading to primary as well as secondary metabolites in addition to CO₂, water and energy (Turner, 1971). After depletion of the favoured carbon source, the subsequent carbon source is used for metabolite biosynthesis, rather it was also reported that readily utilized carbon sources yield less antibiotics than do slow utilizing one (Demain, 1986).

While considering the effect of different nitrogen sources on growth and metabolite biosynthesis, beef extract and yeast extract were found to be the best. On the other hand, amendment of some amino acids in

combination with glucose enhanced growth as well as secondary metabolite production, while most of them found to possess negative effect. Out of 11 amino acids screened, aspartic acid, glutamic acid, leucine and phenyl alanine have shown remarkable influence on enhancement of bioactive metabolite production by *P. lautus* RRT AY-2. Amino acids that form pyruvate were found the best for growth and antimicrobial agent production and they may have some role by sharing their carbon ring or both carbon and nitrogen skeleton into the primary or secondary metabolism processes (Noaman *et al.*, 2004).

Glucose concentration at 1.2 % in the culture media provided the conditions for maximum production of antibiotics, whereas, 1.6 % of glucose concentration led to maximum growth of *P. lautus* RRT AY-2. High concentration of glucose, phosphate or ammonium is generally regarded as repressor of secondary metabolisms (Zhang *et al.*, 1996) and maximum cell growth rates can inhibit secondary metabolite production (Gallo and Katz, 1972). The carbon/nitrogen ratio can also affect the type and yield of metabolite synthesis (Bruckner and Blechschmidt 1991; Candau *et al.*, 1992). Maximum antibiotic production by *P. lautus* RRT AY-2 was obtained at 2 % NaCl concentration in culture media, while an increase in salt concentration reduced the growth and secondary metabolite yield.

In conclusion, the *in-vitro* antimicrobial potentiality of *P. lautus* RRT AY-2 is affirmed by its convincing MIC against some virulent muga silkworm pathogens. The optimized culture conditions *i.e.*, 35 °C, pH 7.5, 48 h incubation, glucose as carbon and beef extract as nitrogen source, 1.2 % glucose and 2 % NaCl concentration and amendment of glutamic acid in glucose facilitate growth as well as bioactive metabolite biosynthesis. Results suggested that the best conditions for biosynthesis of antimicrobial agent was slightly different from the best conditions for growth of the bacteria, however these findings will facilitate to further complete chemical characterization of the bioactive molecule. Study on the functional role of *P. lautus* RRT AY-2 in lepidopteran

insect may lead to develop a beneficial microbial consortium to enhance silkworm health and productivity. One of the greatest benefits conferred by antagonistic bacteria on insect is colonization resistance to invading pathogens. As muga silkworms are unique bioresources, exploratory studies on beneficial microflora would offer opportunity for their better utilization in silkworm host defense, especially against infectious diseases.

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Article scientifique

EFFET DES PARAMETRES DE CULTURE IMMERGEE SUR LA CROISSANCE ET L'ACTIVITE ANTIMICROBIENNE DE *PAENIBACILLUS LAUTUS* RRT AY-2, CONTRE CERTAINS PATHOGENES DE *ANTHERAEA ASSAMENSIS* HELFER

D. K. Gogoi^{1*}, S. P. Sandilya¹, P. M. Bhuyan¹, P. Dutta¹, K. Neog¹, L. S. K. Singh² and T. C. Bora²

¹Department of Biotechnology, Central Muga Eri Research and Training Institute, Central Silk Board, Jorhat 785700, Assam, India.

²Department of Biotechnology, North East Institute of Science and Technology, Council of Scientific and Industrial Research, Jorhat 785006, Assam, India.

*E-mail: gkdeep@rediffmail.com

RÉSUMÉ

Antheraea assamensis Helfer, qui est endémique du nord-est de l'Inde et produit une soie jaune d'or, est confronté à des microorganismes entomopathogènes variés en raison de son élevage en plein air. On a trouvé qu'une souche bactérienne isolée d'une source indienne et identifiée comme *Paenibacillus lautus* RRT AY-2 avait des potentialités antimicrobiennes contre certains pathogènes du ver à soie muga. Les paramètres de culture de la bactérie ont été optimisés pour la croissance et la production maximale d'agent antimicrobien. La croissance maximale ainsi que la production d'agent antimicrobien ont été enregistrées à 37°C et pH 7,5 dans un milieu liquide supplémenté en glucose et en extrait bovin comme source de C et de N. *P. lautus* RRT AY-2 atteint sa croissance maximale après 48h d'incubation, alors qu'il présente son activité antimicrobienne maximale à 54h et le taux de production est maximum entre 18 et 24h. L'ajout d'acide glutamique, d'acide aspartique et de leucine en combinaison avec le glucose augmente la croissance ainsi que la biosynthèse de la molécule bioactive. Une concentration de glucose de 1,2% et de NaCl de 2% est optimale pour la production de l'agent antimicrobien. Le composé antimicrobien brut extrait par le solvant a été partiellement purifié par chromatographie en couche mince (TLC). Le composé ainsi purifié qui présente une absorption maximale dans l'UV à la longueur d'onde de 260nm dans l'acétate d'éthyle donne la plus faible concentration inhibitoire (MIC) contre *Bacillus sp.*, alors que la plus forte MIC a été enregistrée contre *Pseudomonas aeruginosa*. Cette bactérie peut être utilisée sur le ver à soie muga afin de développer une résistance contre les microbes pathogènes.

Mots-clés: Agent antimicrobien, source, Concentration Inhibitoire Maximale, *Paenibacillus lautus* RRT AY-2.



EVALUATION OF ERI SILKWORM (*SAMIA RICINI* DONOVAN) GENOTYPES USING GGE BILOT TECHNIQUES

K. Velayudhan^{1*} and S. Masilamani²

¹Eri Silkworm Seed Production Centre, Central Silk Board, Hosur 635109, Tamil Nadu, India.

²Research Extension Centre, Central Silk Board, Krishnagiri 635115, Tamil Nadu, India.

*E-mail: velayudhancsb@gmail.com

ABSTRACT

Five genotypes of eri silkworm (*Samia ricini* Donovan) viz., Borduar, Dhanubhanga, Khanapara, Nongphoh and Titabar were studied with an objective to find out the productive and adaptable genotype for Tamil Nadu climatic conditions. The selected five genotypes were reared in all seasons for a period of two years (2012-14). The data were analysed using GGE biplot analysis (Yan, 2001) which is a recent biometrical application and widely used in developed countries for multi-environment trials (METs). The results generated in graphical forms represented three important aspects viz., i) which-won-where pattern, ii) inter-relationship among environments and iii) mean performances and stability of genotypes. Biplot analysis revealed that the genotype, Dhanubhanga had high stability for high survival and Borduar, Nongphoh and Titabar possessed superior economic characters of cocoon. Therefore, using these genotypes as parents, appropriate breeding techniques can be drawn to evolve a productive hybrid of eri silkworm with high survival and better cocoon traits in order to sustain and popularise ericulture in South India, especially Tamil Nadu.

Key words: Biplot analysis, eri silkworm, genotype-environment interaction, GGE biplot, *Samia ricini* (Donovan).

INTRODUCTION

Among the non-mulberry silkworm species, the eri silkworm (*Samia ricini* Donovan) stands exclusive that it has been completely domesticated and reared indoor (Joshi, 1992). It is a multi-voltine insect completing six to seven generations in a year. The word "Eri" is derived from the Sanskrit term "Erranda", which refers to the Castor plant, *Ricinus communis* L., the primary host plant. The bi-products from eri-culture also find uses ranging from fertilizers in rural areas to pharmaceutical industries which could be tapped to increase income of the farmers manifold in the long term (Altman *et al.*, 2003; Kim *et al.*, 2003; Reddy, 2008; Sutherland *et al.*, 2009).

The stability of cocoon yield and other economic traits in eri silkworm over a range of environments is important but, to attain it, is a challenge to breeders. The main

reason for this being the widely varied performance of the hybrids under different environmental conditions. Eri silkworm, is known to exhibit a high degree of genotype-environment interaction. A stable hybrid performing consistently well under varied environmental conditions, is therefore, the need of the industry. Perusal of literature showed that very few studies have so far been taken up in mulberry silkworms with reference to their stability under varied environments (Thiagarajan *et al.*, 1994). However, no such studies have been undertaken to assess stability of eri silkworm genotypes reared in diverse environmental conditions of Tamil Nadu.

If all the genotypes respond similarly to all the environments tested, their relative performance in other environments may be predicted with some confidence. In a genotype x environment (g x e) interaction, the relative performance of genotype changes from environment to

environment. Though rearing of silkworm is conducted under controlled laboratory conditions, the silk cocoon yield and yield contributing parameters are greatly affected in different seasons (Ueda *et al.*, 1969; Gabriel and Rapusas, 1976; Thiagarajan *et al.*, 1993 a, 1993 b). Recently, ericulture has been introduced in southern states of India using castor and tapioca as food plants. Therefore, it is necessary to evaluate the eri silkworm of different eco or land races in an attempt to identify the most stable races with respect to larval and cocoon characters of economic importance in different rearing seasons under Tamil Nadu climatic conditions.

The earlier stability models (Eberhart and Russell, 1966; Perkins and Jinks, 1968; Freeman and Perkins, 1971) involve regression analysis for single trait at a time and stability measures are expressed as numerical output, and therefore interpretations for multiple traits or component traits are found to be less effective and inaccurate. The GGE biplot methodology is by far, the best approach for visual analysis of large two-way and multivariate datasets, particularly from the field of breeding and genetics research, which has not been attempted in silkworm till date. The cocoon yield in an environment is a mixed effect of genotype main effect (G), environment main effect (E) and genotype x environment interaction (GE) effect (Thiagarajan *et al.*, 1994). Since only G and GE are relevant for the purpose of genotype evaluation, the term GGE was coined by Yan *et al.*, 2000.

Silkworm genotypes must be evaluated on the basis of multi-environment trials (MET) to ensure that the selected breeds have acceptable performance in variable environments within the target region and to meet the many facets of the demand from the producers, processors and the consumers. Silkworm breeders aim at selecting breed that perform well in a wide range of diverse environments. To identify such well buffered genotype, detailed study on economic traits and breed's response to a change in environments is essential. Highly productive silkworm genotypes should be identified through multi-environment trials (METs) and on the basis of multiple

traits. Perusal of literature indicated that in silkworm (mulberry, tasar, eri and muga) as on today, no study has been reported using GGE biplot analysis as detailed by Yan (2001). Therefore, for the first time, an attempt has been made to measure the stability of selected eri silkworm genotypes using the biplot technique which is a recent application and widely used biometrical model in developed countries.

MATERIALS AND METHODS

The study was conducted in the rearing house of the Eri P2-Basic Seed Farm (A Unit of Eri Research and Training Institute, Central Silk Board, Ministry of Textiles, Govt. of India), Hosur. Hosur (Krishnagiri District, Tamil Nadu, India) is situated at an elevation of 942 meters from mean sea level with latitude 12° 45' 0"N and longitude 77° 51' 0"E. The minimum temperature is 14 °C in December-January, and attains a maximum of 36 °C during April. The relative humidity ranges from 37 % during March to 90 % during October. The annual rainfall is 800 to 1000 mm. The experimental materials selected for the present study comprised of 5 genotypes *viz.*, Borduar, Dhanubhanga, Khanapara, Nongphoh and Titabar which were procured from the germplasm collection of Central Muga, Eri Research and Training Institute, Ladoigarh, Assam. Details of the selected genotypes are presented in Table 1. The selected five genotypes were reared in all seasons for a period of two years (2012-14).

Experiment was conducted from hatching of the eri silkworm eggs till harvesting of cocoons. Sum of 1000 worms of each genotype in five replicates of 200 each were maintained separately in wooden trays (60 cm x

Table 1: Characteristics of experimental genotypes of eri silkworm

Acc. No.	Genotype	Larval body colour	Cocoon colour
SR-001	Borduar	Plain & Zebra on yellow and blue	White
SR-006	Dhanubhanga	Plain yellow and blue	White
SR-003	Khanapara	Plain yellow and blue	White
SR-004	Nongphoh	Plain yellow and blue	White
SR-002	Titabar	Plain & Zebra on yellow and blue	White

90 cm). The tender leaves of castor (*Ricinus communis*) were fed four times a day until the larvae reached III instar stage and semi tender leaves and mature leaves were fed when they were IV and V instar stage, respectively. Required temperature and relative humidity for different instars during rearing were maintained in the experimental chamber. Cocoon harvesting was carried out on sixth day after spinning. The rearing performance of each genotype was assessed for the parameters viz. , fecundity (number of eggs laid by a single female moth), hatching (%), larval weight (g), larval duration (days), effective rate of rearing (ERR by number), cocoon weight (g), cocoon shell weight (g) and cocoon shell percentage. The data were analysed for G x E interaction using GGE biplot analysis (Yan, 2001) which is a windows application that fully automates biplot analysis.

GGE biplot analysis is a windows application that fully automates biplot analysis and results generated in graphical forms represented the following three important aspects:

- i) The which-won-where pattern
- ii) Inter-relationship among environments
- iii) Stability of genotypes

There are two major tasks for MET data analysis. The first is to determine whether the target region is homogenous or should be divided into different mega-environments; the second is to select superior genotype for a given mega environment based on the multiple traits. The biplot technique provides a powerful solution to this problem. The concept of biplot was developed by Gabriel (1971) to graphically display a rank-two matrix, which is a matrix resulting from multiplying a matrix with two columns by a matrix with two rows. The significance of this concept is that if a two-way data set can be sufficiently approximated by a rank-two matrix, then it can be graphically displayed and investigated.

RESULTS AND DISCUSSION

Multiple environment trials (METs) are usually quite large, and it is difficult to interpret the general pattern of

the data without some kind of graphical presentation.

I) The which-won-where pattern

For fecundity, genotypes viz., Nongphoh and Dhanubhanga were found to be consistent for all the seasons studied among which the latter one was found to be vertex genotype and the remaining were inconsistent (Figure 1). An interesting feature of this view of GGE biplot is that it displays the vertex cultivar that had the highest yield in different environments. These vertex genotypes are the most responsive genotypes since they had the longest distance from the origin. For hatching per cent, genotype Borduar was displayed as the vertex genotype for winter and spring seasons and genotype Titabar was designated the vertex genotype for summer, rainy-I (south-west monsoon during June to September) and rainy-II (north-east monsoon during October to December) seasons (Figure 2). Similarly, for cocoon shell weight, genotype Titabar was the vertex genotype of spring season and Borduar for the remaining seasons (Figure 3). The polygon view of a biplot provides the best way for visualizing the interaction patterns between entries and testers. Since the vertex entries have the largest distances from the origin, they are the most responsive to the change of testers relative to other entries within respective groups. Entries located near the biplot origin are less responsive to the change of testers.

ii) Inter-relationship among environments

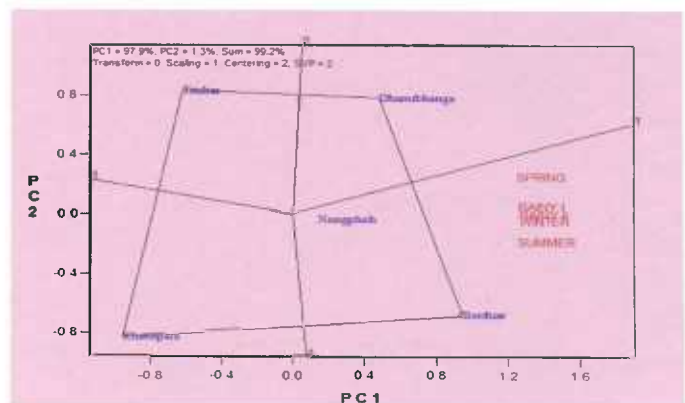


Figure 1: Fecundity (No.) – Which genotype wins where and the best for which environment

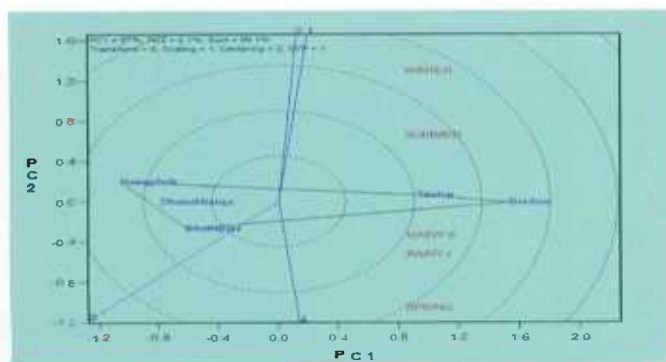


Figure 2: Hatching (%) – Which genotype wins where and the best for which environment

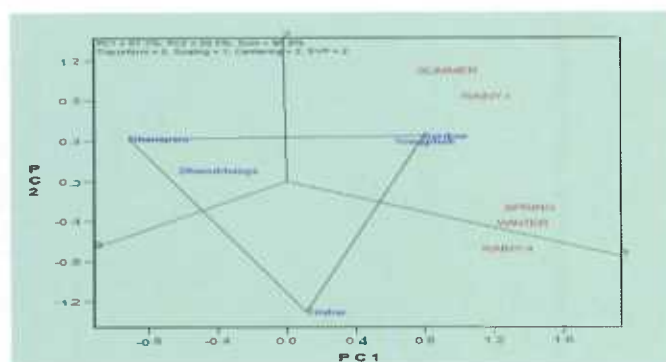


Figure 3: Cocoon shell weight (g) – Which wins where and the best for which environment

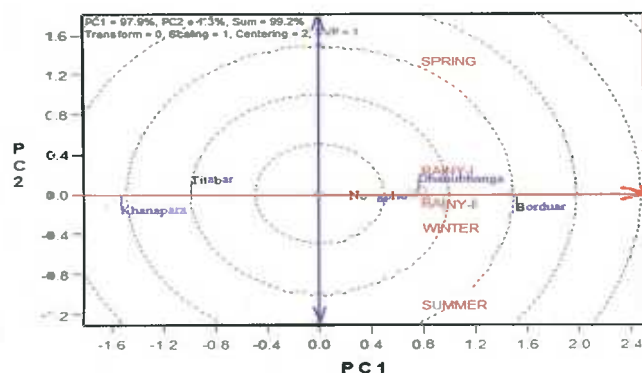


Figure 4: Fecundity (No.) – Biplot showing relationship among environments

GGEbiplot is used to display interrelations among various breeding objectives (traits) based on genetic, environmental or phenotypic values. This helps the breeder to achieve a system understanding of his animal

or plant, to identify appropriate breeding strategies, to identify superior breed (as packages of traits), superior parents (good in a particular trait) and appropriate traits for indirect selection for a target trait. The GGE biplot showing inter-relationship among environments for fecundity is presented in Figure 4. Rainy-I, rainy-II and winter seasons were seen as closely related with respect to fecundity of the genotypes and placed close to ideal circle in the biplot. Though the fecundity in summer season was found to be on the higher side, it showed less consistency among the genotypes irrespective of the environmental effect. Among the 5 breeds studied, none of them exhibited stable performance with respect to the fecundity in summer season. However, spring season was depicted as the most suitable for high fecundity for the all the 5 genotypes studied besides showing high consistency and therefore, spring season can be exploited for grainage purpose to produce more number of eggs.

For single cocoon weight (Figure 5), rainy-I and summer were found to be more closely related and showed highly stable performance than the remaining seasons in terms of mean values. From the biplot, it is visualized that there are two distinct groups of environments formed. One group consisted of the rainy I and summer seasons with linear scaling in the range of 90 to 100 and the second group comprised the remaining seasons within the linear scaling range of 0 to 20. Though values of cocoon weight were observed to be higher than their population mean value in group II, there was less consistency or stability among the genotypes. In biplot, linear scaling is a fair indicator of stability, lower the values, lower the stability and *vice versa*. Similar relationship among the environments was recorded in the case of cocoon shell weight also (Figure 6).

For cocoon shell percentage (Figure 7), winter season stood apart with lower values than their population mean besides showing less consistency in their performance. The eri genotype viz., Titabar showed average stability with values close to the population mean value of cocoon shell percentage and Borduar had high shell percentage with above average stability in all the environments except

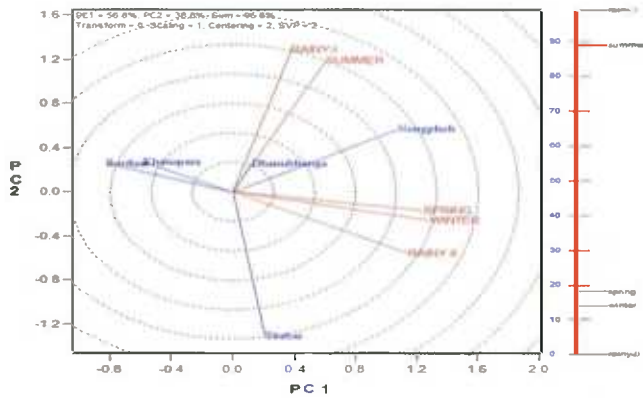


Figure 5: Cocoon weight (g) – Genotype vs. Environment relationship

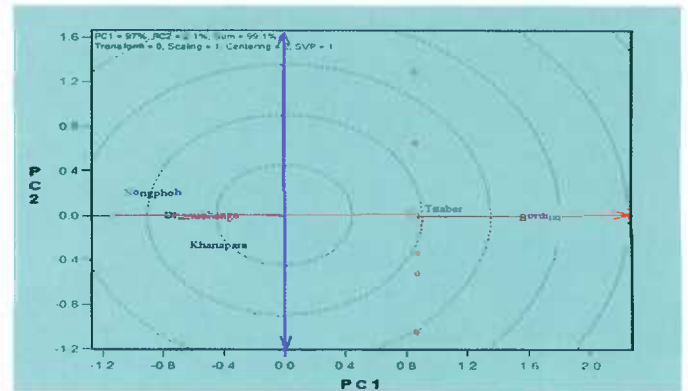


Figure 8: Hatching (%) – Ranking of genotypes based on both mean and stability

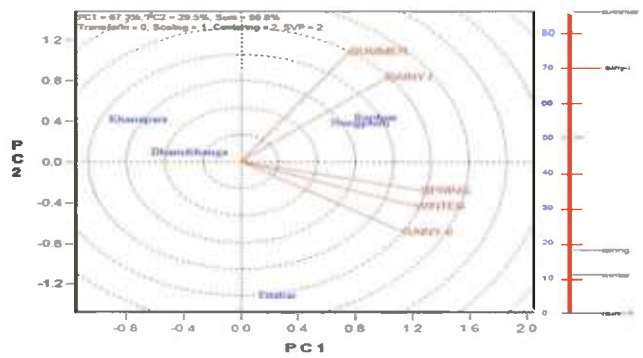


Figure 6: Cocoon shell weight (g) – Biplot showing linear scaling of environments

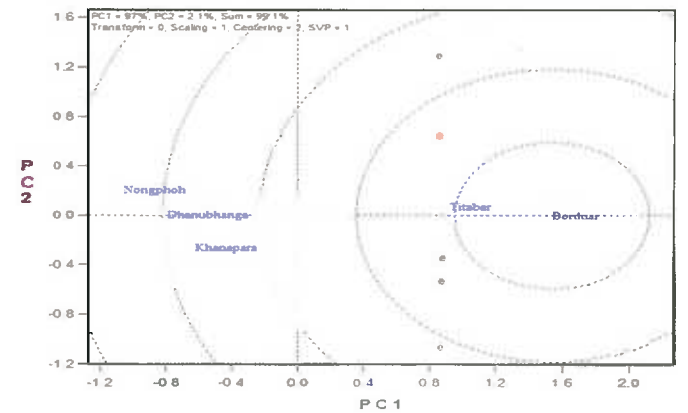


Figure 9: Hatching (%) – Comparison against genotype Borduar

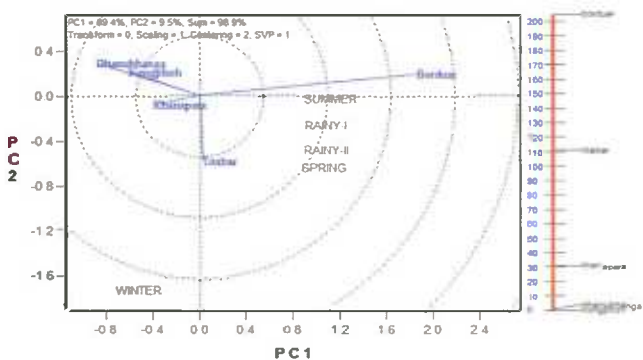


Figure 7: Cocoon shell percentage – Biplot showing linear scaling of genotypes

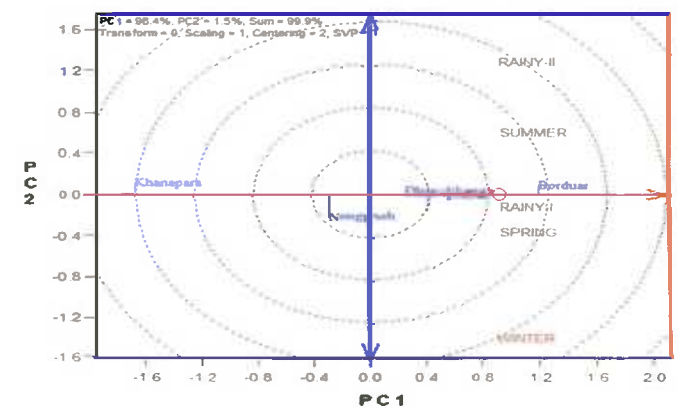


Figure 10: Larval weight (g) – Ranking of genotypes based on mean and stability

winter season.

iii) Stability performance of genotypes

For fecundity, among the genotypes, Borduar occupied a position closer to that of the ideal genotype with higher value than the mean performance which indicated that though Borduar had high fecundity, it showed moderate stability. On the other hand, genotype, Dhanubhanga, possessed high stability but with moderate fecundity value (Figure 4).

In the case of egg hatching of eri silkworm, genotype Borduar showed higher performance than the mean value with moderate stability followed by genotype Titabar positioned closer to the ideal genotype in the biplot (Figure 8). When the eri genotypes were compared with Borduar kept as reference, the generated biplot (Figure 9) indicated that Titabar was within concentric circle of Borduar and the remaining genotypes were placed far away with lower values than the mean performance besides less stability.

The earlier biometrical models (Eberhart and Russell, 1966; Perkins and Jinks, 1968; Freeman and Perkins, 1971) recommended three different parameters viz., high mean value ($\bar{X}$), regression co-efficient close to unity ($b=1$) and non-significant deviation from linear regression ($S^2_{di}=0$) to measure the stability and all the three parameters are to be simultaneously assessed by a breeder to select the genotypes for breeding programme. In the case of GGE biplot, mean value of characters and stability measures of genotypes were combined to generate an average environment coordination (AEC) which is the single indicator for stability performance and makes the breeder's job easy for selection programme. Among the 5 genotypes studied for larval weight, the performance of Borduar was close to the AEC indicating high value of stability (Figure 10).

Genotypes with less larval duration are preferred for commercial exploitation of eri silkworm. Accordingly, in our study, among the 5 genotypes tested in 5

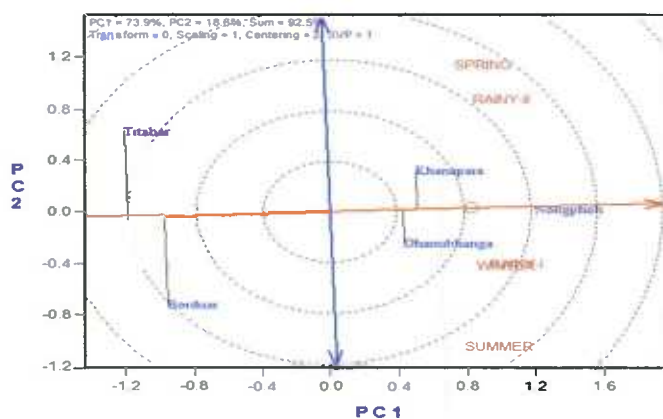


Figure 11: Larval Duration (Days) – Ranking of genotypes based on mean and stability

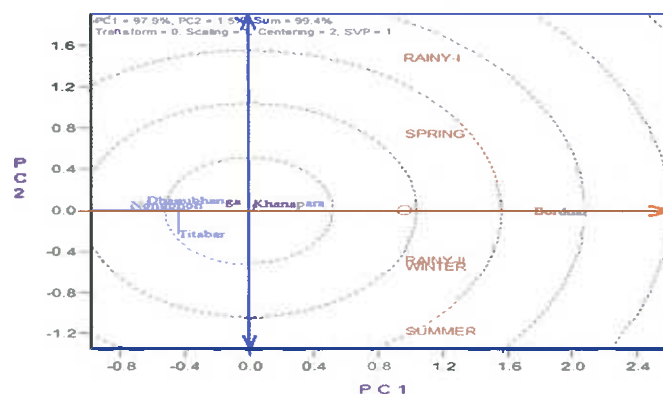


Figure 12: Effective rate of rearing (No.) – Ranking of genotypes based on mean and stability

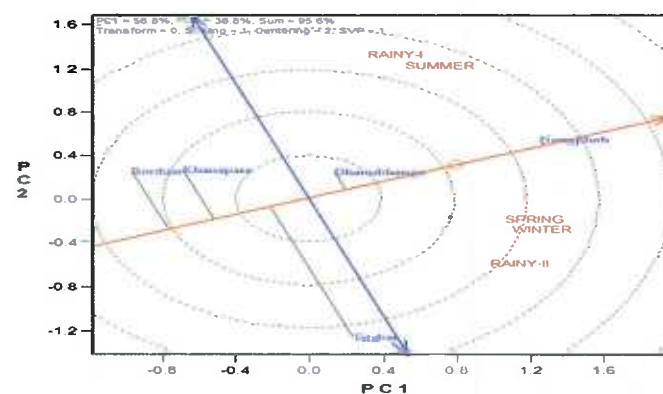


Figure 13: Cocoon weight (g) – Ranking of genotypes based on mean and stability

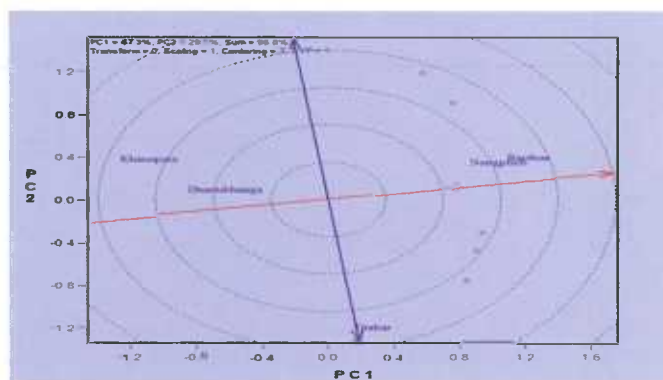


Figure 14: Cocoon shell weight (g) – Ranking of genotypes based on Ideal genotype

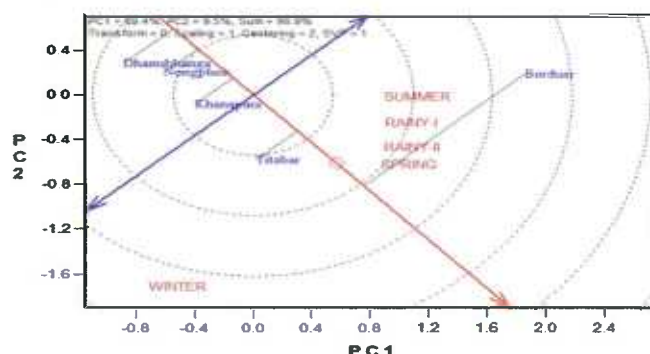


Figure 15: Cocoon shell percentage – Biplot showing stability performance of genotypes

environments, less larval duration was observed for genotype Titabar followed by Borduar but the latter had the disadvantage of less consistent performance in all the environments studied (Figure 11). The remaining genotypes viz., Dhanubhanga, Nongphoh and Khanapara possessed longer larval duration among which the latter two genotypes performed consistently in all the environments.

The effective rate of rearing (ERR) indicates the survival rate of eri silkworm from hatching till spinning of the cocoon which is also an indicator of more cocoon yield by a genotype. In our study, high ERR with average stability was observed only in the case of Borduar in all the 5 environments tested followed by average stable performance with average value of ERR observed for Khanapara (Figure 12). The remaining genotypes viz.,

Dhanubhanga, Nongphoh and Titabar had lower value of ERR besides less adaptation values.

Mean value and stability of genotypes were combined by introducing an average environment coordination (AEC) and generated GGE biplot for cocoon weight. The angle of AEC abscissa observed was $<45^\circ$. Among the genotypes, Nongphoh and Dhanubhanga were observed closer to the ideal genotype (Figure 13) among which the former was found to have high performance than the mean value in all the environments tested.

In the case of cocoon shell weight, genotypes viz., Borduar and Nongphoh were found closer to the AEC with high values of stability (Figure 14). The genotype viz., Titabar performed closer to the population mean value but showed less consistency among the environments. The remaining genotypes viz., Dhanubhanga and Khanapara showed less value of cocoon shell weight with average stability.

The abscissa of AEC of cocoon shell percentage observed was $>45^\circ$ which indicated wide variability for cocoon shell percentage among the eri genotypes. Genotype, Borduar had high cocoon shell percentage with high stability among the 5 genotypes studied (Figure 15). The genotype viz., Titabar performed closer to the population mean value with less consistency in all the environments studied. The remaining genotypes viz., Dhanubhanga, Khanapara and Nongphoh performed lower than the population mean value with average stability.

CONCLUSION

Our study demonstrated that the GGE biplot is an excellent tool for graphical presentation of genotype by trait data. Biplot analysis revealed that genotypes viz., Nongphoh and Borduar performed consistently well in all environments indicating their wider adaptability. The genotype, Dhanubhanga has shown tolerance to environmental changes and may be recommended for unfavourable environment to have high fecundity. Borduar and Titabar have shown linear performance

towards hatching per cent in all environments. Genotypes viz., Titabar and Dhanubhanga exhibited consistency in all environments but Borduar for favourable environment in terms of high larval weight. Titabar and Borduar were found to have less larval duration among which Borduar had high stability. Khanapara and Borduar had higher value of ERR than the population mean with moderate stability in all seasons. Among the 5 genotypes, only Borduar has shown stable performance for high cocoon yield in all seasons. Nongphoh and Dhanubhanga expressed high stability for cocoon weight in all environments. Similarly, Borduar and Nongphoh displayed high stability for cocoon shell weight in all environments. The genotypes, Borduar and Titabar had high stability for cocoon shell percentage in both favourable and unfavourable seasons, respectively.

When the genotypes were ranked based on their overall stable performances, Dhanubhanga was rated high for survival and Borduar, Nongphoh and Titabar for economic characters of cocoon. Therefore, these genotypes may serve as breeding resource to develop hybrids of eri silkworm characterised by high survival and superior cocoon traits in order to popularise ericulture in South India, especially Tamil Nadu, as a viable and sustainable industry.

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Article scientifique

EVALUATION DE GENOTYPES DU VER A SOIE ERI (*SAMIA RICINI* DONOVAN) PAR DES TECHNIQUES BILOT GGE

K. Velayudhan^{1*} and S. Masilamani²

¹Eri Silkworm Seed Production Centre, Central Silk Board, Hosur 635109, Tamil Nadu, India.

²Research Extension Centre, Central Silk Board, Krishnagiri 635115, Tamil Nadu, India.

*E-mail velayudhancsb@gmail.com

R É S U M É

Cinq génotypes du ver à soie eri (*Samia ricini* Donovan) soient Borduar, Dhanubhanga, Khanapara, Nongphoh et Titabarwere ont été étudiés dans le but de trouver le génotype productif et adapté aux conditions climatiques du Tamil Nadu. Les cinq génotypes sélectionnés ont été élevés en toutes saisons pendant une période de deux ans (2012-14). Les données ont été analysées par la technique biplot GGE (Yan, 2001) qui est une application biométrique récente largement utilisée dans les pays développés pour des essais multi-environnementaux (MET). Les résultats générés sous forme graphique délivrent trois aspects importants : i) profil qui-gagne-ou, ii) interrelations entre environnements et iii) performances moyennes et stabilité des génotypes. L'analyse révèle que le génotype Dhanubhanga présente une forte stabilité et une survie élevée et que Borduar, Nongphoh et Titabar possèdent des caractères économiques du cocon supérieurs. Ainsi, en utilisant ces génotypes comme parents, des techniques appropriées de breeding peuvent être mises en œuvre pour obtenir un hybride productif de ver à soie eri avec une survie élevée et des caractères du cocon améliorés afin de soutenir et développer l'élevage eri en Inde du sud, particulièrement au Tamil Nadu.

Mots-clés: Analyse biplot, ver à soie eri, interaction génotype-environnement, GGE biplot, *Samia ricini* (Donovan).



EVALUATION OF TECHNO-ECONOMICS AND THE IMPACT OF COMPUTER AIDED TEXTILE DESIGNING (CATD) UNITS

C. R. Nagaraj^{1*}, Jameela Khatoon¹, Bhanuprakash Raj¹, K. M. A. Kadhar²,
Prakash Y. Naik³, S. B. Murgod⁴ and Subhas V. Naik¹

¹Central Silk Technological Research Institute, Central Silk Board, Bengaluru 560068, India.

²Silk Conditioning and Testing House, Central Silk Board, Kancheepuram 631502, India.

³Silk Conditioning and Testing House, Central Silk Board, Dharmavaram 515671, India.

⁴Demonstration cum Technical Service Centre, Central Silk Board, Hindupur 515201, India.

*E-mail: nagaraj.csb@nic.in

ABSTRACT

A scheme was implemented during the XI and XII plan periods to support silk textile designers equipped with Computer Aided Textile Designing (CATD) package and consequently, more than 500 CATD units were established in the country. Hence, the study of techno-economics of these units and their impact in the field becomes pertinent. The units were found to be making adequate profits. The working capital requirement was quite substantial and there was no support forthcoming from the financial institutions. The units made tremendous impact in the field by eradicating child labour, reducing drudgery, improving design quality, reducing design cost, creating new designs and reducing delivery time. Major constraints faced by the units were lack of support from financial institutions and poor power supply, besides inadequacies in machinery maintenance, marketing and training.

Key words: Constraints, cost of production, impact, net profit, working capital.

INTRODUCTION

Much of the efforts involved in the process of design development can be minimized with the use of computers. Computer Aided Textile Designing (CATD) provides freedom to the textile designer to get the best out of his artistic ingenuity and better flexibility for his creativity. The use of CATD facilitates the artist with ease and quickness of operations besides making available innumerable options for incorporating changes in the designs. It also equips the textile designer with a tool to react to the changes in the market trend in a faster way. In view of the above advantages that the CATD offers as compared to the traditional designing, it was introduced as a scheme in the XI plan and continued in the XII plan as well. The CATD package for developing woven designs comprises of computer hardware, designing software and computerized card punching facility. Under the Catalytic Development Programme in the XI & XII Plans, more

than 500 CATD units were established in important weaving clusters of the country, mostly in Tamil Nadu, Karnataka and Andhra Pradesh. Hence, this study was undertaken with the aim of evaluating the techno-economics of the CATD units installed under the plan periods and their impact in the weaving clusters.

The specific objectives of the study were to evaluate the techno-economics of CATD units, assess the impact of these units in the field in terms of improvement in design quality, reduction in designing cost, delivery time and drudgery and to document the constraints faced by these units.

METHODOLOGY

Study area

Since more than 80 % of the units were established in

Tamil Nadu, Karnataka and Andhra Pradesh, these three states were selected for the study (Table 1). Kanchipuram, Coimbatore, Arni and Salem weaving clusters in Tamil Nadu, Bengaluru and Doddaballapur in Karnataka and Dharmavaram and Hindupur in Andhra Pradesh were the selected clusters.

Sampling distribution and data collection

Table 1: Sample distribution in the selected clusters

Cluster	No.	Per cent
Tamil Nadu	280	78.87
Karnataka	45	12.68
Andhra Pradesh	30	8.45
Total	355	100.00

A survey was conducted during 2013-14 in the above-mentioned weaving clusters and all relevant data were collected in the questionnaire-schedule prepared for the purpose. Random sampling was adopted and data from 355 CATD units were collected.

Analytical tools

The financial analysis was carried out on annual basis. All the cost and revenue components were calculated on 100 cards basis. The cost of production components were broadly categorized into card & lacing thread / wire cost, wages, power, overheads (repairs & maintenance, interest, administration, transportation) and depreciation. Revenue component included the sale of punched cards including the design component. Net profit / loss were calculated as the difference between total revenue and total cost of production. Impact and constraint analyses were carried out based on the opinion expressed by the designers and master weavers.

RESULTS AND DISCUSSION

Working of CATD units

The average number of working days per month of the

CATD units was found to be 24 days indicating that the units were operating at optimum capacity utilization in terms of working days and the range being 15 to 30 days. Out of the 355 units surveyed, 222 units (62.54 %) were working in their own premises while 133 units (37.46 %) operated in rented building. The CATD package mostly included card punching machine, computer and designing software, while a few units had laptop and card lacing machine. The average cost of the package was ₹ 1.91 lakhs. On an average, the units have been punching 1163 cards per day catering to various jacquard capacities viz., 120, 240, 400 and 600.

Economics of CATD Units

The cost and sales of the CATD units were calculated based on the data collected during the survey and the financial analysis is presented in Table 2.

Table 2: Financial analysis of CATD units

Particulars	Cost / Returns per / 100 cards (₹)	Percentage of total cost
Cost of Production (₹)		
Card & Lacing thread/wire	144.65	63.22
Wages	53.87	23.54
Power	4.02	1.76
Overheads	18.73	8.19
Depreciation	7.53	3.29
Total	228.82	100.00
Revenue (₹)		
Punched Cards	317.00	-
Profitability (₹)		
Net Profit / 100 cards	88.18	-
Net Profit / unit / annum	254888	-
Net Profit / unit / month	21241	-
Cash accrual (Net Profit + Depreciation) / 100 cards	95.71	-

Table 2 reveals that the spending on card and lacing thread / wire form the major cost component amounting to ₹ 144.65 (63.22 %) of the total cost of production per 100 punched cards. The purchase price of 100 cards varied

from ₹ 50 to 300 depending on the number of hooks and quality of cards. Wages formed the next major component in the cost of production with a share of 23.54 % which includes punching and lacing activities. Overheads contributed 8.19 % of the cost which includes transportation, repairs & maintenance and administrative expenses. The total cost of production per 100 cards worked out to be ₹ 229.

The average selling price of 100 punched cards was ₹ 317. The selling price of 100 punched cards varied from ₹ 150 to 600 depending on the number of hooks and the design content. Based on the cost and revenue, the net profit per 100 cards worked out to ₹ 88.18. The average net profit per unit per annum was ₹ 2.55 lakhs working out to about ₹ 21241 per month. Considering the type of units (cottage / micro) and the amount of investment (about ₹ 1.90 lakhs on machinery), the returns *i.e.*, the net profit seems to be adequate.

Working Capital of CATD units

The working capital of CATD units was calculated based on the information collected during the survey. The details of the working capital of CATD units are given in Table 3.

Table 3: Working capital of CATD units

Component	Holding period (days)	Amount (₹)
Raw material	17	20752
Work in process	3	6949
Finished goods	5	10923
Debtors	12	30865
Working expenses	24	15213
Working capital cycle	37	
Total working capital		84702

The working capital cycle of CATD units was found to be 37 days with the holding period of raw material (cards) being 17 days; work in process, 3 days; finished goods (punched cards), 5 days and debtors (credit sales) being 12 days (Table 3). The working capital of the units was found to be ₹ 84702 which was quite huge considering

the fact that most of the units were cottage / micro units with very little support from the banks. Out of the 355 units, only 26 (7.32 %) of them had availed institutional credit, while 45 (12.68 %) had accessed working capital loan from private sources at exorbitantly high interest rate ranging from 2 to 3 % per month. But majority of them, *i.e.*, 284 units (80.00 %) were found to be managing with their own source of finance.

Impact of CATD units in the field

To study the impact of the CATD units in the weaving clusters, an opinion poll was conducted among the master weavers / weavers, who are the customers of these units with a sample size of 30. All the respondents were of the view that the CATD had made a tremendous impact on the weaving industry and had brought in the following revolutionary changes:

- Eradication of child labour involved in manual card punching,
- Reduction in drudgery by removing manual card punching,
- Reduction in cost of designing due to computerization and by ending the monopoly of a few designers who were charging exorbitantly,
- Improvement in the quality of designs due to use of computer hardware and designing software,
- Creation of new designs due to the use of designing software along with the traditional designing skills and a development of huge library of designs (soft copy) and
- Reduction in delivery time of punched cards due to computerized designing and machine card punching.

Constraints faced by the CATD units

The constraints faced by the CATD units are grouped

under the heads of training, support from financial institutions, machinery maintenance / after sales services and power. The views of the respondents on these aspects are tabulated as below.

Training

Training plays an important role in optimum and efficient utilization of the new technology package given under schemes. The respondents were asked about the adequacy of training on card punching machine and computer aided designing.

A majority of the designers (59.15 %) expressed that the training given to them on card punching machine and designing was adequate which is helping them to run their units smoothly. 12.12 % of the respondents were of the opinion that the training was inadequate while there was no response from 28.73 % (Table 4).

Table 4: Adequacy of training

Particulars	Training	
	No.	Percentage
Adequate	210	59.15
Inadequate	43	12.12
No response	102	28.73
Total	355	100.00

Institutional financial support

Government support was given to establish the units in terms of subsidizing the cost of CATD package. But the units require substantial working capital for optimal capacity utilization. Hence, the respondents were enquired about the adequacy of financial support from institutional sources.

A majority of the designers (61.13 %) opined that the financial support in terms of working capital requirement is not forthcoming from the institutional sources. Hence, some of them have borrowed from private sources at high interest rates, ranging from 2-5 % per month whereas,

majority of them were found to manage it from their own sources (Table 5). Only 15.21 % expressed that the support was adequate, while there was no response from 23.66 % of them.

Table 5: Adequacy of institutional financial support

Particulars	Financial support	
	No.	Percentage
Adequate	54	15.21
Inadequate	217	61.13
No response	84	23.66
Total	355	100.00

Machinery maintenance and after sales service

Prompt machinery maintenance and after sales service by the manufacturers / suppliers play a vital role in proper utilization of the installed capacity and adhering to the delivery time to the customers. The designers were asked whether they faced problems in maintenance of the CATD package.

A majority of the respondents (79.15 %) expressed that they did not face any problems in machinery maintenance and the after sales service of the suppliers was good, while 14.37 % of them had problems such as sourcing the spare parts and poor response from the machinery suppliers *i.e.*, after sales service with respect to repair / breakdown of the system / machine (Table 6).

Table 6: Machinery maintenance and after sales service

Particulars	Machinery maintenance / After sales services	
	No.	Percentage
Problem	51	14.37
No problem	281	79.15
No response	23	6.48
Total	355	100.00

Power

Continuous supply of power is important for maintaining the delivery schedule of punched cards to the customers. The constraints in respect of the power supply were expressed as following (Table 7):

Table 7: Power supply

Particulars	Power	
	No.	Percentage
Problem	205	57.75
No problem	18	5.07
No response	132	37.18
Total	355	100.00

A majority of the designers (57.75 %) expressed that the units were facing power problems in terms of power cuts, irregular supply and low voltage which affected the working of the units and the delivery schedule given to the customers.

CONCLUSION

The CATD units established during the XI and XII plan periods have been working at optimum capacity utilization and catering to the needs of the silk weaving clusters in the states of Tamil Nadu, Karnataka and Andhra Pradesh. The units were generating adequate profits. The working capital cycle of the units was quite large, hence the working capital requirement was substantial. Most of the units did not have access to institutional sources of credit and hence, some of them had availed loan from private sources at high interest rates.

CATD units have made a tremendous impact in the field by eradication of child labour, reduction in drudgery, reduction in cost of designing, improvement in the quality of designs, development of new designs and reduction in delivery time of punched cards. Majority of designers

opined that lack of support from the banks for their financial requirements and poor power supply were the main constraints faced by them, besides machinery maintenance, marketing and training.

In the past few years, the CATD units have contributed significantly to the silk weaving sector and since it is an emerging sector, Government support to the new and existing units should continue in terms of subsidizing the machinery cost. The working capital requirements of the units being substantial, financial institutions should support these units by providing working capital loans.

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Article scientifique

EVALUATION TECHNICO-ECONOMIQUE ET IMPACT DES UNITES DE DESIGN TEXTILE ASSISTE PAR ORDINATEUR (CATD)

**C. R. Nagaraj^{1*}, Jameela Khatoon¹, Bhanuprakash Raj¹, K. M. A. Kadhar²,
Prakash Y. Naik³, S. B. Murgod⁴ and Subhas V. Naik¹**

¹Central Silk Technological Research Institute, Central Silk Board, Bengaluru 560068, India.

²Silk Conditioning and Testing House, Central Silk Board, Kancheepuram 631502, India.

³Silk Conditioning and Testing House, Central Silk Board, Dharmavaram 515671, India.

⁴Demonstration cum Technical Service Centre, Central Silk Board, Hindupur 515201, India.

*E-mail: nagaraj.csb@nic.in

RÉSUMÉ

Un schéma a été mis en oeuvre au cours des Xème et XIème plans pour assister les designers textiles utilisant un équipement de Design Assisté par Ordinateur (CATD) et en conséquence plus de 500 unités CATD ont été établies dans le pays. Aussi, l'étude technico-économique de ces unités et de leur impact dans le domaine est devenue pertinente. Ces unités font un bénéfice correct. Le besoin en capital est très substantiel et il n'y a pas de soutien provenant des institutions financières. Les unités ont eu un impact énorme dans le domaine en éradiquant le travail des enfants, en réduisant le travail pénible, en améliorant la qualité du design, en réduisant le coût, en créant des nouveautés et en réduisant les délais de livraison. Les contraintes majeures auxquelles font face ces unités sont le manque de soutien de la part des institutions financières et la médiocre fourniture d'énergie, ainsi qu'une maintenance des équipements, d'un marketing et d'une formation inadéquats.

Mots-clés: Contraintes, coût de production, impact, bénéfice net, capital d'investissement.



HETEROBELTIOSIS IN INDIAN TROPICAL TASAR SILKWORM, *ANTHRAEA MYLITTA* DRURY (LEPIDOPTERA: SATURNIIDAE) IN ASSOCIATION WITH CROP REARING SEASONS

R. Manohar Reddy^{1*}, M. Ramesh Babu² and P. Jayaprakash²

¹P₂ Basic Seed Farm, National Silkworm Seed Organization, Central Silk Board, Parigi 515261, India.

²National Silkworm Seed Organization, Central Silk Board, Bengaluru 560068, India.

*E-mail: dr.manohar1959@rediffmail.com

ABSTRACT

The semi-domesticated and wild ecoraces of tropical tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) contributes for vanya raw silk production in India. Presently, the commercial tasariculture depends on two semi-domesticated (Daba & Sukinda) ecoraces, and there is an imperative need for hybridization to exploit heterobeltiosis to make tasar activity more feasible. The viability of various general (parents with non-specific traits) and specific (parents with specific traits of commercial importance) F1 hybrid combinations developed among Daba (semi-domesticated), Jata and Raily (wild) ecoraces were evaluated during seed (July-August) and commercial (September-December) crop rearing seasons to assess the impact of rearing environment on extent of heterobeltiosis in commercial traits. The Jata x Daba general hybrid combination has shown relatively better performance among all the hybrids during both crop rearing seasons indicating their compatibility to varied environment over Raily x Daba general as well as specific hybrid combinations, which could only record better in shell weight and shell percentage. The heterobeltiosis was higher in all the hybrid combinations for larval span, shell weight and shell percentage than larval, cocoon and pupal weight, irrespective of the crop seasons. Higher heterobeltiosis in the larval span and silk related traits apart a little improvement in cocoon weight was recorded in Jata x Daba general hybrid combination in commercial crop rearing season followed by the same hybrid combination of seed crop season. The study infers that, there is scope to exploit the hybridization for heterobeltiosis in relation to the crop rearing seasons in tasar silkworm genotypes to augment silk yield through superior phenotypes for making tropical tasariculture commercially more sustainable.

Key words: *Antheraea mylitta*, ecoraces, F1 hybrid combinations, heterobeltiosis, rearing seasons.

INTRODUCTION

The cocoons of wild ecoraces, Raily (bivoltine) and Jata (univoltine) of tropical tasar silkworm, *Antheraea mylitta* Drury collected from nature grown host plants by tribes of Bastar (Chhattisgarh) and Thakurmunda (Orissa), respectively exhibit superior traits over the semi domesticated Daba (bi & trivoltine) and Sukinda (trivoltine) cocoons produced through commercial rearings. The commercial tasariculture currently depends

on only Daba and Sukinda and hence, a need is felt for developing high yielding hybrids to make the activity commercially viable. The least cost of production and better sustenance of sericulture industry basically depend on the quantity and quality of cocoon yield in tasar silkworm, *A. mylitta* and the combined impact of parameters viz., fecundity, hatching, effective rate of rearing (ERR), cocoon weight and silk content in addition to the rearing environment of crop seasons (Jolly *et al.*, 1971). But, the extent of heterosis and heterobeltiosis

expression in commercial traits depends on the interaction among the genetic variability of parental ecoraces along with the environment in which the hybrid grows (Jain *et al.*, 1981; Falconer, 1985). The heritability of quantitative parameters is found the most useful in silkworm breeding (Suryanarayana *et al.*, 1987; Siddiqui *et al.*, 1988) and however, the genotype - environment interaction influences the tasar insect life cycle and its absolute silk yield (Bhargava *et al.*, 2001; Reddy *et al.*, 2010). The use of phenotypic variability in parents can build up a hybrid, to exploit heterosis to augment productivity and quality (Singh *et al.*, 2002) and crossing of high x low, high x medium and medium x low yielding parents show positive heterosis (Naqvi *et al.*, 2004; Reddy *et al.*, 2010, 2011). The deviation among the tasar ecoraces is specific and large due to genetic variability and even after several generations, the Raily and Modal ecoraces are found differing from Daba in many parameters (Srivastava *et al.*, 2001). The potential phenotypic expression of genotype needs suitable climate due to its interaction with environment (Kumar *et al.*, 1999; Jong and Bijma, 2002; Mulder and Bijma, 2005) and the gene being the endogenic factor play a major role and environment being the exogenic factor influences the genes' expressivity to produce different phenotypes in different environments (Kumar *et al.*, 2008). The property of systematic development of different phenotypes in different environments is called phenotypic plasticity (Reddy *et al.*, 2008) and its importance in the tropics has been proven (Reddy *et al.*, 2009). Maximum heterosis was observed for fecundity, hatching and yield contributing traits in Daba x Modal and Raily x Laria combinations (Siddiqui, 1997; Naqvi *et al.*, 2004; Srivastava *et al.*, 2004; Zhao *et al.*, 2007). Presently, only two ecoraces are contributing for the major production of tropical tasar cocoons in India and the utilization of hybrids with season associated heterobeltiosis is essential in place of the present commercial ecoraces, Daba and Sukinda; to enhance silk yield in tasar culture. Hence, the present study was aimed to raise F₁ hybrid combinations among wild ecoraces, Jata (Orissa), Raily (Chhattisgarh) and cultivated Daba (Jharkhand) and also to evaluate their performances under different crop rearing seasons.

MATERIALS AND METHODS

Three parental ecoraces *viz.*, Daba, Jata and Raily of *A. mylitta* were initially reared in a randomized block design with three replications each during the seed crop rearing season (July-August) for three successive years, raised the non hibernating and hibernating seed cocoon stocks and kept in the tasar grainage house following integrated package of seed cocoons preservation (Narain *et al.*, 2001) and conducted grainage for seed production. The fresh parental moths emerged during June and September months of the respective year were used to produce disease free layings (DFLs) of F₁ hybrids *viz.*: Jata x Daba, Raily x Daba and their specific combinations such as high pupal weight female x high pupal weight male [PxP], high pupal weight female x high shell weight male [PxS], high shell weight female x high shell weight male [SxS], in addition to pure layings of parents through inbreeding. Three parental ecoraces along with eight F₁ hybrid combinations were reared simultaneously in a randomized block design with three replications each during seed (July-August) and commercial crop seasons (September-November) of three years on the economic plantation of *Terminalia tomentosa* (W&A) at field laboratory of Central Tasar Research and Training Institute, Ranchi, Jharkhand, India following integrated package of tasar silkworm rearing (Narain *et al.*, 2004). The larvae of one disease free laying (DFL) of parents as well as F₁ hybrid combinations were considered as one replication during rearing for recording observations. The average larval weight, larval span, single cocoon, pupal and shell weight and shell percentage were calculated based on the equal number of random samples. The data recorded on different parameters were subjected to statistical analysis.

RESULTS

The analysis of variance (ANOVA) revealed variations in respect of larval weight, larval span, single cocoon, pupal and shell weight and shell percentage among parents of seed crop, parents of commercial crop and F₁ hybrid combinations of seed and commercial crop rearing seasons (Table 1). However, the larval weight and span among the parents, pupal weight among the F₁ hybrid

Table 1: ANOVA for seed, larval and cocoon characters of parents and F₁ hybrids of seed (July-August) & commercial crop rearing seasons (October-November)

Source: Mean sum of squares							
Particulars	DF	Larval weight (g)	Larval span (days)	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Shell %
Replicates	02	11.4	5.6	0.19	0.39	0.07	8.4
Parents	02	5.2 NS	7.6 NS	8.6 **	6.0 **	0.32 **	7.1 *
F ₁ Hybrids	07	44.6 **	37.8 **	1.8 **	0.42 NS	0.91 **	42.6 *
Parents x F ₁ Hybrids	01	1.5 NS	79.9 **	1.9 NS	3.1 *	0.14 NS	26.4 **
Error	20	3.1	2.3	1.0	0.73	0.05	1.7

* - Significant at 5 % level, ** - Significant at 1 % level, NS - Non-significant

Table 2: Heterobeltiosis percentage of F₁ hybrids of wild and semi-domesticated ecoraces during seed crop rearing season (July-August)

F ₁ Hybrid combination	Larval weight (g)	Larval span (days)	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Shell (%)
J x D	+01.26	+17.38	+01.42	-0.580	+39.21	+30.80
R x D	+13.67	-03.22	+04.52	+0.320	+32.85	+24.53
J x D [PxS]	+03.96	+09.84	+02.84	+01.59	+34.31	+24.79
R x D [PxS]	-06.14	+12.10	-08.58	-09.40	-02.92	+04.19
J x D [PxP]	+03.49	+08.20	-0.920	+0.930	+03.92	+0.280
R x D [PxP]	-08.51	+14.52	-11.53	-10.98	-15.33	-06.13
J x D [SxS]	+07.69	+04.92	+07.80	+04.74	+55.88	+38.20
R x D [SxS]	+01.42	+03.22	-04.61	-08.45	+21.90	+19.49

J x D = Jata x Daba; R x D = Raily x Daba

[PxP] = High pupal weight female x High pupal weight male

[PxS] = High pupal weight female x High shell weight male

[SxS] = High shell weight female x High shell weight male

combinations and larval, cocoon and shell weight among parents versus F₁ hybrids were found non-significant. The variations in shell percentage among the parents and hybrids and pupal weight in parents versus hybrids were significant at 5 % level, while others are significant at 1 % level indicating the performance variation and impact of heterobeltiosis over different rearing seasons.

The performance levels of larval weight, larval span, single cocoon, pupal and shell weight and shell percentage in respect of F₁ hybrid combinations of seed crop season of rearing studied during the months of July

and August (Table 2) indicates the positive and negative levels of deviation in heterobeltiosis. Better performance was recorded in the general hybrid, Raily x Daba in all the parameters except for larval span, which is again a positive trend of completing the crop in shorter span with gain in all other commercial characters. Better pupal weight as noticed in this hybrid contributes to higher fecundity required in seed crop, while the other silk related parameters can contribute for the higher silk yield indicating the hybrids' suitability for silk production even in the seed crop season. However, the other general hybrid, the Jata x Daba combination has recorded positive results

Table 3: Heterobeltiosis percentage of F₁ hybrids of wild and semi-domesticated ecoraces during commercial crop rearing season (October-November)

F ₁ Hybrid combination	Larval weight (g)	Larval span (days)	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Shell (%)
J x D	-01.60	+04.12	+02.91	-01.51	+14.83	+12.58
R x D	+13.60	-0.790	-19.29	-19.26	+17.06	+35.55
J x D [PxS]	-0.300	+08.02	+07.28	+07.98	-02.20	-08.71
R x D [PxS]	-07.32	+15.41	-19.29	-21.32	-08.06	+13.92
J x D [PxP]	-12.69	+19.75	-06.10	-0.43	-40.11	-36.12
R x D [PxP]	-16.31	+23.81	-27.12	-24.83	-39.81	-17.45
J x D [SxS]	-03.13	+10.49	+04.18	-0.110	+20.33	+15.67
R x D [SxS]	+03.32	+04.08	-10.01	-16.87	+27.96	+42.16

J x D = Jata x Daba; R x D = Raily x Daba;

[PxP] = High pupal weight female x High pupal weight male

[PxS] = High pupal weight female x High shell weight male

[SxS] = High shell weight female x High shell weight male

in all the parameters except for pupal weight revealing its suitability for silk production than seed even during the seed crop rearing season. Among the other specific hybrids, the Jata x Daba combination has performed better in most of the parameters, while the Raily x Daba high shell weight hybrid stood better for shell weight and shell percentage.

The performance levels in respect of larval weight, larval span, cocoon, pupal and shell weight and shell percentage of F₁ hybrid combinations of commercial crop season of rearing conducted during October and November months (Table 3) indicate the variations in heterobeltiosis among different F₁ hybrid combinations, in spite the rearing season remaining the same. In terms of suitability to seed production with better cocoon and pupal weight, the Jata x Daba high pupal weight x high shell weight combination has recorded better cocoon and pupal weight, while the shell weight and shell percentage were inferior among all the hybrids reared in that rearing season. Among the hybrid combinations, the general and high shell weight combination have shown positive values for shell weight and shell percentage, while the high pupal weight x high shell weight and high pupal weight hybrid combinations expressed a negative effect for all the parameters except larval span.

DISCUSSION

The F₁ hybrid combinations prepared by crossing parental ecoraces with large genetic diversity can enhance the heterosis with genetic complementation, even on commercial prospective as heterobeltiosis (Jain *et al.*, 1981; Falconer, 1985). The phenotypic diversity though occurs due to genetic structure, the genotype - environment (G x E) interactions at times manifest considerable phenotypic variations in the selected and economically important traits (Kumar *et al.*, 1999, 2008; Jong and Bijma, 2002). Hence, the parental ecoraces from divergent eco-geographic areas (Jharkhand, Orissa & Chhattisgarh states of India) with higher phenotypic diversity have been chosen for the study. The ANOVA for the larval and cocoon characters of both seed and commercial crops showed highly significant variations among parents, hybrids and parents versus F₁ hybrids indicating the existence of genetic diversity among the parents. The parental genetic diversity might be the reason for the remarkably significant and positive variations in the performance levels as heterobeltiosis among the F₁ hybrids and also the basis for high variations of traits in parents versus F₁ hybrids. Though, the general attention in tasariculture was for silkworm seed from seed crop reared cocoons and silk from commercial crop reared cocoons,

the possible commercial exploitation is aimed at silk production even during seed crop rearing season. The suitable application of pure or F_1 hybrid genotypes irrespective of the crop rearing season can exploit the advantage of shorter crop span of seed crop rearing season than commercial crop season. Also, the availability of quality leaf and lesser prevalence of predators during seed crop rearing season will become added advantages for crop success and better yields. Further, the generation of higher number of tasar cocoons during seed crop season will have the choice of selection for better seed cocoon material and any way, the excess can be utilized for reeling raw silk besides creating employment to tasar practicing tribes round the year. The other promising indication from the study is that all the F_1 hybrids of the seed crop rearing season except Raily x Daba high pupal combination, have shown positive heterobeltiosis in shell weight and shell percentage, which clearly indicates the scope for their exploitation for silk production even during seed crop rearing season.

The goal of breeding must be to combine good characters of parents by setting up specific breeding environment as the environmental conditions have a great influence on the effectiveness of selection (Falconer, 1985; Kumar *et al.*, 2008). In most of the conventional heterosis breeding programmes, the geographical and phenotypic diversity is considered for available genetic distance among secluded populations (Bhargava *et al.*, 2001; Srivastava *et al.*, 2001). The other way of assessing the genetic diversity is with expression of heterosis in a particular hybrid as it demonstrates the existing degree of genetic diversity in parents. The reason for selecting Daba, Jata and Raily ecotypes was their varied yielding pattern in commercial trait(s) and their origin from semi domesticated and wild habitats. The scientific principle behind the selection of individual parents based on high pupal and high shell weight (for making varied F_1 hybrids) was the close association of bigger pupae with higher fecundity and higher shell weight with more silk yield. The performance of F_1 hybrids during commercial crop rearing season with extended larval span could not enhance larval, cocoon and pupal weight, but could

enhance the shell weight and shell percentage in general hybrids, while it was shell weight in specific hybrids. But, with the extended larval span of commercial crop season; proportionate increase in shell weight and shell percentage was not observed. These traits recorded improved heterobeltiosis during seed crop season in the same F_1 hybrid combinations. In spite of increase in the larval span during commercial crop season, a corresponding gain in pupal weight was not noticed and this might be due to allocation of larval assimilation mostly to shell content than pupa weight. This is an indication of tasar silkworm larva fore-casting of forth-coming prolonged winter and its predestined hibernation. Among the specific hybrid combinations, only high shell weight combinations have performed better over general hybrids. This further indicates the impact of the rearing season on the heterobeltiosis in silk related traits and however, the extended larval span might correlate the rearing season and higher silk content with favourable environment (Kumar *et al.*, 1999, 2008; Jong and Bijma, 2002) that prevailed during commercial crop rearing season in comparison to seed crop season.

CONCLUSION

Irrespective of crop seasons, the extent of heterobeltiosis was better for larval span, shell weight and shell percentage than larval, cocoon and pupal weight in all hybrid combinations. However, the higher positive heterobeltiosis in the larval span and silk related traits with improved cocoon weight recorded in Jata x Daba general hybrid combination in commercial crop rearing season followed by that in the same hybrid combination in seed crop rearing season indicates the scope of hybridization in relation to crop rearing seasons. Hence, the heterobeltiosis in tasar silkworm F_1 hybrid genotypes expressed as superior phenotypes can augment the silk yield and can make Indian tropical tasariculture commercially more sustainable.

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HETEROBELTIOSE CHEZ LE VER A SOIE TASAR TROPICAL INDIEN, *ANTHRAEA MYLITTA* DRURY (LEPIDOPTERA: SATURNIIDAE) EN RELATION AVEC LES SAISONS D'ELEVAGE

R. Manohar Reddy^{1*}, M. Ramesh Babu² and P. Jayaprakash²

¹P₂ Basic Seed Farm, National Silkworm Seed Organization, Central Silk Board, Parigi 515261, India.

²National Silkworm Seed Organization, Central Silk Board, Bengaluru 560068, India.

*E-mail: dr.manohar1959@rediffmail.com

RÉSUMÉ

Les races sauvages et semi-domestiquées du ver à soie tasar tropical, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae), contribuent à la production de soie grège vanya en Inde. Actuellement la production tasar commerciale dépend de deux races semi-domestiquées (Daba et Sukinda), et un important besoin d'hybridation existe pour exploiter l'hétérobeltiliose afin de rendre plus faisable l'activité tasar. La viabilité d'hybrides F1 à caractères commerciaux généraux (parents sans traits spécifiques) et spécifiques (parents ayant des caractères spécifiques d'intérêt commercial) développés à partir des races Daba (semi-domestiquée) et Jata et Raily (sauvages) a été évaluée au cours de la saison de grainage (Juillet-Août) et de la saison commerciale (Septembre-Décembre) afin de déterminer l'impact de l'environnement d'élevage sur l'étendue de l'hétérobeltiliose pour les caractères commerciaux. La combinaison hybride générale Jata x Daba a donné des performances relativement meilleures parmi tous les hybrides au cours des deux saisons ce qui montre une compatibilité aux environnements variés par rapport aux combinaisons générales et spécifiques Raily x Daba qui sont seulement meilleures pour le poids de la coque et la richesse soyeuse. L'hétérobeltiliose est plus forte, chez toutes les combinaisons hybrides, pour la durée de vie larvaire, le poids de la coque et la richesse soyeuse que pour le poids larvaire, du cocon et de la pupe quelles que soient les saisons. Une plus forte hétérobeltiliose pour la durée de vie larvaire et les caractères liés à la soie, sauf une petite amélioration pour le poids du cocon, a été enregistrée pour la combinaison générale chez Jata x Daba pendant la saison commerciale suivie par la même combinaison pendant la saison de grainage. Notre étude souligne qu'il y a une possibilité d'exploiter l'hybridation pour une hétérobeltiliose en relation avec les saisons d'élevage chez les génotypes du ver à soie tasar afin d'accroître le rendement en soie grâce à des phénotypes supérieurs pour rendre la culture commerciale du tasar plus durable.

Mots-clés: *Antheraea mylitta*, écoraces, combinaisons hybrides F1, hétérobeltiliose, saisons d'élevage.

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The Republic of Kenya has become the 17th Member Country of ISC with effect from 29th January, 2016. His Excellency, Willy Kipkorir Bett, Cabinet Secretary (Minister), Ministry of Agriculture, Livestock and Fisheries has signed the Instrument of Accession signalling Kenya's entry in ISC. The Government of Kenya has nominated Dr. Lusike Wasilwa who is the Director, Crop Systems in the Kenya Agricultural and Livestock Research Organisation as the National Delegate and the focal point of Kenya in all the matters of ISC. In addition to this Kenya has also nominated 5 delegates as mandated under the Statute of ISC.

On behalf of the ISC Member Countries, the ISC Secretariat cordially welcomes Republic of Kenya as the 17th Member Country of ISC.



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Sericologia

**International Sericultural Commission
CSB Complex**

**BTM Layout, Madiwala, Bengaluru - 560068
Karnataka, India**

Phone: +91-80-26282191

+91-80-26680162

+91-80-26681663

Fax: +91-80-26681663

Email: sericologia@gmail.com

Website: www.inserco.org