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PRODUCTION OF PHYTOALEXINS - A POST-INFECTIONAL DEFENSE MECHANISM IN MULBERRY (*MORUS* SPP.)

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ABSTRACT

Mulberry (*Morus* spp.) foliage is an exclusive source of nutrition for silkworms (*Bombyx mori* L.). The host plant is affected by many diseases that cause destruction of leaf tissue. Phytoalexins (PAs) produced in response to infection are known to impart resistance to the host. The current knowledge about PAs as one of the post-infectional defense mechanisms in mulberry and related plants, may help to secure the host plants from microbial attack. The PAs of mulberry consist of chemical compounds belonging to the class of benzofuran and stilbenes. Kuwanon C and ethyl β rescrylate were the antifungal compounds detected in mulberry roots, which completely inhibited the growth of pathogens- *Rosellinia necatrix* and *Helicobasidium mompa*. When mulberry was affected by 'dogare' and twig blight diseases, a few antimicrobial substances were identified as prenylflavon compounds viz., kuwanon C and E, morusin, albanin A~H and albafluran A~C in shoot epidermis. The cortex tissue produced moracin compounds A~Z, whereas, the xylem produced stilbene compounds viz., oxyresveratrol, 4'-prenyloxyresveratrol and moracin-M. Chalcomoracin, the benzofuran compound found in the mulberry leaves was observed to inhibit the growth of *Fusarium solani* f. sp. *mori*. This review provides an overview of phytoalexins in mulberry under pathogenecity.

Key words: Antifungal substances, moracin, mulberry, phytoalexin.

INTRODUCTION

Disease is caused whenever the vital functions of a plant are disrupted by a biotic stimulus. The biochemical dynamics of parasitism and pathogenesis are triggered and controlled by a series of interactions between host and pathogen. Plants have their own built-in defense mechanism(s) against the pathogen(s). But in some cases, these pathogens overcome the defense barrier with their offensive chemicals and cause disease. The pathogen(s) may invade the host by circumventing the recognition process of the host. The plant, upon recognizing the invader, synthesises compounds viz., "phytoalexin" as a defensive response and tries to inhibit the growth of microorganisms. The term 'phytoalexin' (PA) (phyton=plant; alexin=warding off substance), for the

first time was propounded by Muller and Borger (1940), to describe the substance that inhibits fungus development and formed when living plant tissues (potato tuber) are invaded by the parasite (*Phytophthora infestans*). Many experiments have been done in various plants across different families to understand antimicrobial substance (AMS) in defense mechanism which ultimately defined a concept of phytoalexins (Muller, 1956; Deverall, 1970). Phytoalexins (PAs) are defined as antibiotics which form as a result of an interaction of two different metabolic systems, where plants inhibit the growth of pathogenic microorganism(s) (Muller, 1956). PAs are formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors (Ingham, 1973). Phytoalexins [antimicrobial substance(s)] synthesis is an important chemical barrier of post-

infectious defense mechanism, which parallels the mechanism of defense through production of antibodies in the animal system (Cruickshank, 1963). However, recently a new definition for PAs was formulated: "PAs are low molecular weight substances produced by plants relatively rapid in high concentrations in response to infections by pathogen and physico-chemical stresses, which are active in a wide range of pH, temperature and nutrient conditions and confer protection against the infecting pathogen" (Paxton, 1981). PAs are presently known not only toxic to fungi (Bailey, 1974; Yoshikawa *et al.*, 1978) and bacteria (Lyon and Wood, 1975; Webster and Sequeira, 1977), but also, to plant and animal cells as well as nematodes (Smith and Banks, 1986). In some plants, PA has been implicated as an important component of natural resistance (Deverall, 1970). PAs are formed in the living cells of hypersensitive host in response to parasitic attack and inhibits further development of the parasite (Bose, 1989). They are fungistatic and bacteriostatic, active at very low concentration and do not exist in the healthy cells (Yoshikawa *et al.*, 1978). The antimicrobial properties of PAs have sought to establish that, the concentration, site of accumulation, and timing are consistent with the observed cessation of growth of the pathogen. PAs have been isolated and characterized in various plants, which are showing a wide structural complexity. PA(s) production seems to be a general process among higher plants during pathogenesis. The information regarding any remarkable work done in identifying and characterizing PAs in mulberry is very scanty. This review gives an overview of information about the antifungal substances in mulberry under pathogenesis.

Production of PAs in mulberry

Mulberry (*Morus* spp.), the sole food plant of silkworm (*Bombyx mori* L.) is often affected by large number of diseases caused by fungi, bacteria, viruses, mycoplasmas and nematodes. These diseases cause considerable amount of leaf loss qualitatively and quantitatively, eventually reducing the silk production. Bose (1989) made a preliminary study on role of

phytoalexin in natural resistance of mulberry plants to diseases. Subrata Biswas (1991) highlighted generally on mulberry phytoalexin, later, their role in disease control was emphasized by Subrata Biswas and Sengupta (1993). Zhu-Feng-Ping and Kaue Yuan Zhang (1994) conducted primary studies on mulberry PA. They inoculated *F. solani* f. sp. *mori* and observed varied antifungal activity (AFA) in different varieties of mulberry trees. Chen Pei-Geng *et al.* (1994) inoculated mulberry (YU-2 variety) bark with *F. solani* f. sp. *mori* and isolated four PAs viz., M₁, M₂, M₃ and M₄. Among them, M₂, M₃ and M₄ were identified as moracin C, G and N, respectively. The production of antifungal substances (AFS) in different parts of mulberry viz., root, shoot and leaf are briefly summarized as below:

I. Production of PAs in mulberry root and shoot parts

- i. Epidermis
- ii. Bark
- iii. Cortex
- iv. Xylem

II. Production of PAs in mulberry leaves

I. Mulberry root and shoot

a. Mulberry root

Root system plays a very important role in higher plants, helps in anchorage, absorption of water and minerals, storage of food and also involves significantly in various physiological processes. If the roots are prone to attack by the pathogens, there is interference in their usual functions, leading to the poor growth and development of aerial parts (shoot and leaves). In mulberry, the most commonly found root diseases are white root rot (caused by Ascomycetous fungus *Rosellinia necatrix* Berlesse) and violet root-rot (caused by Basidiomycetous fungus *Helicobasidium mompa* Tanaka). The stump region of the white root rot diseased mulberry plants shows a whitish mycelia mat as well as feeble growth of leaf buds, withering of leaves and weak growth of plants. Whereas, thick violet coloured mycelial mat can be seen in the case of violet root-rot. Due to this rotting, the water and mineral(s) uptake is hindered; as a result, all other

physiological functions of the host are altered. Ultimately, in both the cases, the roots rot and the plants die off.

Several investigations were conducted (Shirata and Takahashi, 1982 a; Shirata *et al.*, 1983) on the production of antifungal substances (AFS) in the roots of mulberry and moraceae plants using phytopathogenic fungi including *R. necatrix*, *H. mompa*, *Bipolaris leersiae*, *Diaporthe nomurai*, *Fusarium roseum*, *F. lateritium* f. sp. *mori* and *F. solani* f. sp. *mori*. The AFS were extracted from treated root tissues with water, chloroform, ethyl ether and n-hexane. AFA observed in various parts of mulberry roots (epidermis, bark, cortex and xylem tissues) are detailed below:

i. Epidermis: In the epidermis and / or periderm of root, the amount and number of active substances varied with different cultivars and species of moraceae plants when inoculated with *B. leersiae*. The acetone extract inhibited the growth of many phytopathogenic fungi (eg: *R. necatrix*). The antimicrobial activity (AMA) was more in roots (*i.e.*, less at the younger tip regions than the old regions) when compared to shoots. The activities were tested and observed on the spot of Rf values from 0.12 to 0.15 by Shirata *et al.* (1983).

ii. Bark: Kuwanon C (Nomura *et al.*, 1981; Takasugi, 1981) and ethyl β -rescrylate (Uno *et al.*, 1981) extracted from the bark of mulberry roots exhibited antimicrobial activity. Kuwanon C inhibited the growth of *R. necatrix* completely at a concentration of 14 ppm; thus exerting a control on white root-rot disease (Takasugi, 1981).

iii. Cortex and xylem tissues: Antifungal compounds (AFC) were detected from the browning cortex and xylem tissues of mulberry root, when infected with *R. necatrix* and *H. mompa* (Takahashi and Shirata, 1982). But these substances were not detected in healthy tissues. Here, the AFA was detected within a day and the intensity of the activity increased for the next 10 days. But, when the cortex and xylem tissues were inoculated with *F. solani* f.

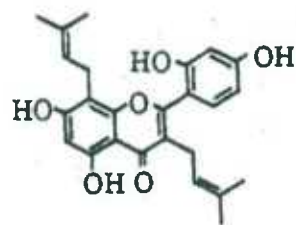
sp. *mori*, a surge in the AFA was noticed for 2 days which declined soon and was recorded nil after 10 days. The optimum temperature for the production of AFS was found to be 15-25 °C in cortex and more than 35 °C in xylem tissues. However, the activity reached peak within 5-10 days of inoculation.

b. Mulberry shoot

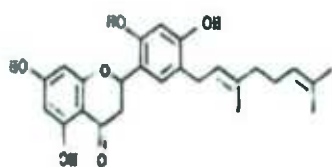
Mulberry shoot rearing is widely popularized as labour saving technique in countries where sericulture is practiced. This method not only reduces the labour but also makes bed cleaning easy, saves time and energy. As mulberry plants are subjected to periodical pruning, a severe epidemic of shoot-killing disease is caused due to the entry of pathogens through their cut surfaces or wounds. In order to overcome the attack of infectious pathogens, PAs are produced (Takahashi and Shirata, 1982).

Details of the structures and sources of many antimicrobial plant natural products have been compiled by Van Etten *et al.* (1994) and Richard (2001). The simplest functional definition recognizes PAs as compounds that are synthesized *de novo* (as opposed to being released by, for example hydrolytic activity) and phytoanticipins are pre-formed infectional inhibitors. However, the distinction between PAs and phytoanticipins is not always obvious. Some compounds may be PAs in one species and phytoanticipins in others (Takasugi *et al.*, 1982 b).

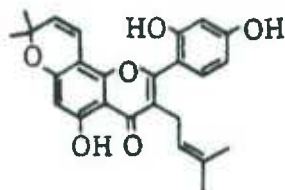
i. Prohibitin(s) in shoot epidermis: The pre (=first) formed inhibitory substances that confer some degree of protection to the host plants against microorganisms are called "prohibitins". These belong to different groups of secondary plant metabolites, namely prenylflavon compounds, kuwanon C and E, morusin, albanin A~H and albafluran A~C (Takasugi *et al.*, 1980 a; Takasugi, 1981; Takasugi *et al.*, 1982 a).



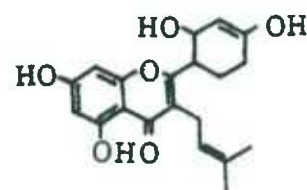
kuwanon C



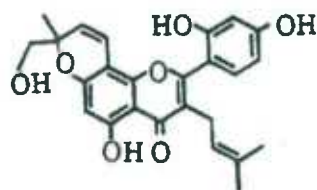
kuwanon E



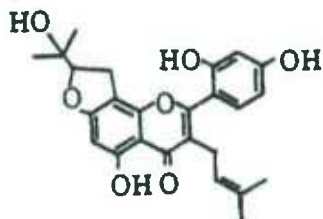
morusin



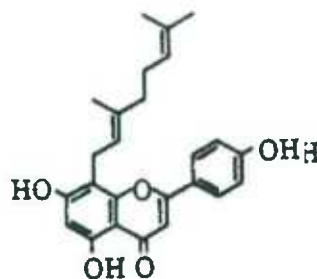
albanin A



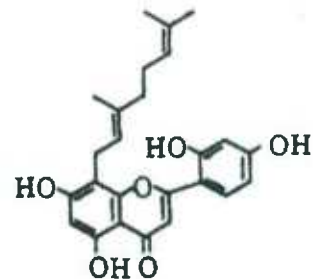
albanin B



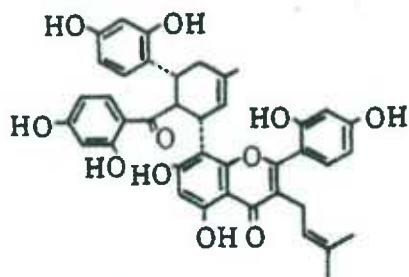
albanin C



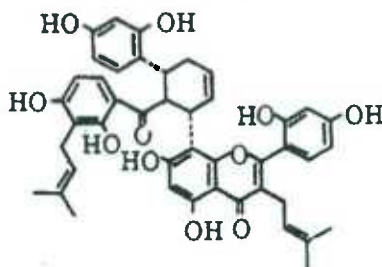
albanin D



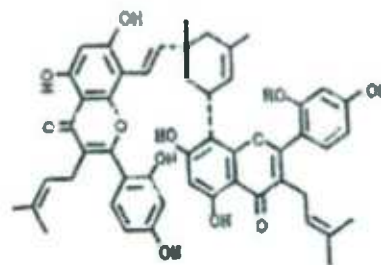
albanin E



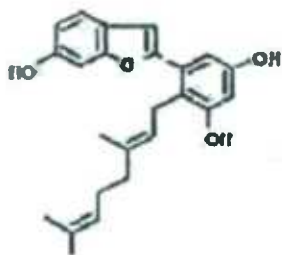
albanin F



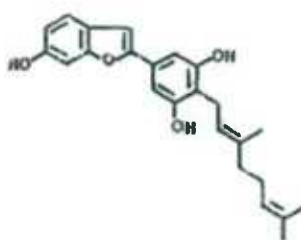
albanin G



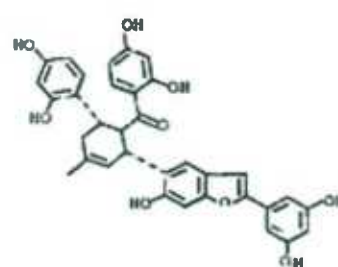
albanin H



albafrican A



albafrican B



albafrican C

(Credit: Takasugi *et al.*, 1980 a; Takasugi, 1981; Takasugi *et al.*, 1982 a)

A few studies were made in the mulberry shoot (Shirata *et al.*, 1979; Yoshida and Shirata, 1994). Twig blight disease is common where mulberry cultivation is practiced. It is also known as “Megare” or “Bud blight” disease. It is caused by a fungus of *Fusarium* species. Reddish brown lesions appear around the diseased twigs.

When the lesions coalesce and expand, the sap movement is hindered in the twigs. The buds located above the infected area starts to wither off suddenly as they do not receive proper nutrition due to interference in sap movement. Mulberry is mainly propagated vegetatively through buds. Thus, a disease of this stature affecting the

buds causes great damage. In order to know the effect of temperature on the hyphal growth and lesion development in mulberry shoot, four species of *Fusarium* causing twig blight (*F. lateritium* f. sp. *mori*, *F. roseum*, *F. solani* and *F. moniliformae*) were isolated and inoculated at the site of wound in different seasons by Shirata *et al.* (1979). They found that the rate of lesion(s) development at various temperatures differed with fungal species. In *F. roseum*, lesions were formed in summer at 30 °C. Lesions were developed at 5-25 °C in winter by *F. lateritium*. But, in the case of *F. solani*, lesions were formed both in summer and winter between 5 and 30 °C. No pathogenicity was noticed for *F. moniliformae*. Only three isolates (*F. roseum*, *F. solani* and *F. lateritium*) showed AFA, amongst these, *F. roseum* exhibited the strongest inhibition. Tolerance of isolates was grouped and the order was *F. solani* > *F. lateritium* > *F. roseum*. There may be an intimate relationship between the tolerance and pathogenicity of isolates (Takahashi and Shirata, 1982).

Another common fungal disease of mulberry, Dogare blight is caused by *Diaporthe nomurai* (Ascomycetous). Here, the bud formation is suppressed, shoot wither off suddenly and several brown coloured eruptions are noticed on the surface of the stem bark. Yoshida and Shirata (1994) studied the differences in the formation of lesions on winter mulberry (*Morus*) varieties (260) affected by fungal diseases viz., dogare blight (caused by *D. nomurai* - D₈) and twig blight (caused by *F. lateritium* f. sp. *mori* - L₁₇ and *F. solani* f. sp. *mori* - S₂₆). In most of the mulberry varieties, expression of resistance to both the diseases varied with the incubation temperatures (10 °C and 20 °C). However, 'Kumonryu' and 'Kurodogi' showed a remarkable susceptibility while, 'Seisuke' showed resistance to the D₈ isolate and Siwasuguwa', 'Murasakiwase', 'Yanagiha' and 'Tosawase' were resistant to L₁₇ at both the temperatures. When the relationship between virulence and the incubation temperature for each fungus isolate was analysed, the D₈ isolate showed a high virulence even at 10°C with slight variability, virulence of the L₁₇ isolate tended to be higher at 10°C than at 20°C. S₂₆ isolate showed an opposite reaction. The accumulation of PAs in various parts of the lesions

developed by the D₈ isolate was detected, with their large production occurring in the yellowing areas and the zone between the yellowing and browning area.

ii. Shoot bark

It is well known that plant tissues when wounded or infected with a pathogen often turn brown and the activities of enzymes peroxidase (PO) and polyphenoloxidase (PPO) increase (Stahmann and Demorest, 1973). These changes are associated with common metabolic alterations induced by mechanical injury or infection by pathogens and often considered to be involved in the mechanism of resistance (Tomiya, 1963; Kosuge, 1969; Tomiyama, 1977). In many plant diseases, PAs are known to accumulate in the brown infections and to play an important role in disease resistance (Tomiya, 1963; Deverall, 1970; Ingham, 1972; Kuc, 1972). Shirata *et al.* (1978 b) analysed qualitative and quantitative changes in the activities of PO and PPO and the production of PA like substances in mulberry under fungal infection. The mulberry shoots were inoculated by pathogens, *F. solani* and *D. nomurai* (causal organisms of twig and dogare blight diseases, respectively) at the wounded surface. The activity of PO and PPO was increased immediately (within one minute) at the cut surface tissue of the wound made on the bark. The antifungal or PA-like substances were also formed. Browning of the bark segments cut from the shoots was inhibited by treating with blasticidin - S, an inhibitor protein, which also stopped further increase of PO activity. There was also an increase in PO activity in the neighbouring tissues of the wound. The highest peaks of PO and PPO activities were detected at the border of the diseased lesion. The PO activity was found to be due to PO present in latex, which flowed out from laticiferous tissue next to the cut surface. Bands C-1, A-1, A-2, A-3 and A-4 were found by the gel isoelectric focusing on PO activities. The latex secreted from the wound showed A-2, A-3 and A-4 bands. In the case of healthy tissue, C-1, A-2 and A-3 bands were formed (Figure1). This study points out that the oxidative enzymes such as PO and PPO are partly involved in the entire sequence of plant response to wound

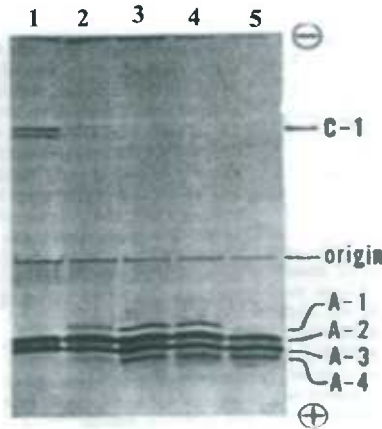


Figure 1: Electrophoregrams of peroxidase activities of extracts obtained from mulberry shoots inoculated with *F. solani* and *D. nomurai*, and that of peroxidase contained in latex: Samples were made 12 days after inoculation.

1. Healthy part of the intact cortex and phloem tissue,
2. Healthy tissue adjacent to the girdled zone, 12 days after cutting,
3. Browned tissue infected by *F. solani*,
4. Browned tissue infected by *D. nomurai*,
5. Latex secreted from wound made on the intact shoot. (Credit: Shirata *et al.*, 1978 b)

or infection.

Shirata (1982) noticed antifungal activity in pieces of bark cut from one year old mulberry shoot (*M. alba* L.) of cultivar 'Ichinose'. The intensity of AFA increased at lower temperature (5 and 10 °C) but decreased after an initial increase at higher temperatures (15, 20, 30 and 35°C). These changes were more conspicuous at the upper regions of the shoot than in the middle and lower regions. The activity of AFS increased with time and temperature.

iii. Shoot cortex

When pathogenic fungi invade mulberry shoots through lenticels or wounds, the cortical tissue beneath the epidermis turns brown in colour. This brown coloured portion gradually expands, later ceases to enlarge, so that the death of whole plant is avoided. A situation similar to hypersensitive defense mechanism is seen and this phenomenon was studied by Shirata (1978) and Takasugi *et al.* (1978 a, b) to detect the production of PA. Shirata *et al.* (1978 b) conducted an experiment to find out antifungal activity in 7 plant species of Moraceae family (*Morus bombycis* Koidz., *Cudrania tricuspidata* Bureau.,

Table 1: Inhibition zone of mycelial growth of *C. miyabeanus* caused by the cup method using acetone extracts from the cortices treated with water or spore suspension of *F. solani* f. sp. *mori*

Plant	Inhibition zone						Antifungal substance production
	Water		Spore suspension				
<i>Morus bombycis</i> Koidz		-	-	++	++	++	High
<i>Cudrania tricuspidata</i> Bureau		-		++	++	++	High
<i>Broussonetia papyrifera</i> Vent..	-	-	-	++	++	+	High
<i>B. kazinoki</i> Sleb		-		++	++	+	High
<i>Cudrania cochinchinensis</i> Kudo et. Masam var. <i>gerontogea</i>	-	-		+	+	+	Relatively high
Kudo et. Masam							
<i>Ficus carica</i> L.				+	±	±	Relatively low
<i>Ficus erecta</i> Thunb.	-	-	-	+	±	±	Relatively low

(Credit: Shirata *et al.*, 1978 b)

The inhibition zones were scored and classified into 4 grades -, ±, +, ++ indicating diameter of 8, 8.1-10.0, 10.1-15.0 and over 15.1 mm, respectively.

Broussonetia kazinoki Sieb., *B. papyrifera* Vent., *C. cochinchinensis* Kudo et. Masam. var. *gerontogea* Kudo et. Masam., *Ficus carica* L. and *F. erecta* Thunb.), wherein they inoculated cortical tissues of the shoots with *F. solani* f. sp. *mori*. Most of the inoculated cortical tissues became brown, whereas, the water-treated, control ones showed only slight discolouration. Later, the cortical tissue was placed on potato dextrose agar medium (PDA) seeded with spores of *Cochliobolus miyabeanus*. After incubation at 28 °C for 2 days, the inhibition zones were scored and classified into 4 grades -, ±, +, ++ indicating diameter of 8, 8.1-10.0, 10.1-15.0 and over 15.1 mm, respectively (Table1). These grades were used as standard criteria to evaluate the production of AFS. About 75 % of the species were assessed to produce AFS at various rates of intensities and remaining 25 % species showed a little or no production.

Another experiment was performed by Shirata *et al.* (1978 a) to investigate the intensity of AFA. Two fungi viz., (*C. miyabeanus* and *F. solani* f. sp. *mori*) were inoculated to the cortex after removing the epidermis and periderm of the shoot. The high rate of AFS was found



Figure 2: Inhibition zone of mycelial growth of *C. miyabeanus* appeared around the cup on spore seeded PDA: Left: acetone extract from mulberry cortex treated with *F. solani* f. sp. *mori* was placed in the cup; Right: acetone extract from non-treated shoot was placed in the cup (Credit: Shirata *et al.*, 1978 a).

immediately after inoculation, indicating that AFS were already present in these tissues before wounding *i.e.*, prohiçitin. AFS in the shoot of 7 species of Moraceae (mentioned above) were examined for Rf values by thin layer chromatography (TLC) method. After evaporating the solvent, the plate was sprayed with PDA broth containing spores of *C. miyabeanus* and incubated at 25°C under humid conditions for two days. The transparent inhibition zones appeared in striking contrast to the black background where fungus developed vigorously (Figure 2). The occurrence of inhibition zones was taken as an indication of presence of AFS. The Rf values of the main inhibition zone were almost same in all the Moraceae plants studied.

Shirata (1978) selected the cortex tissue of one year-old shoot of mulberry cultivar Ichinose and inoculated with spore suspension and mycelia of *F. solani* f. sp. *mori*. Antimicrobial activity was examined using spores of *B. leersiae*. The extract from the infected tissue of summer and winter shoots showed AFA. To induce AFA on cortex surface, 100 spores/cm² were required. AFA was tested under a wide range of temperature, from 5 to 30 °C in the extract of browned parts of cortex. AFA was also induced in the cortices by treatments with culture filtrates of seven plant pathogenic fungi *viz.*, *F. lateritium* f. sp. *mori*, *F. roseum*, *F. solani* f. sp. *mori*, *D. nomurai*, *B. leersiae*, *R. necatrix* and *Sclerotinia sclerotiorum*.

Antimicrobial compounds (Shirata, 1978) (AMC)

were extracted from the cortex tissues inoculated with *F. solani* f. sp. *mori* with methanol, acetone, ethyl acetate and ethyl ether, but could not be extracted with water, n-hexane, and carbon tetrachloride (Figure 3). The obtained AMC were stable for one hour at 12°C or for more than six months at 20 °C and they were dialyzable. Rf values of AMC on Silica gel-G TLC developed with ethyl ether were 0.20 and 0.63. From these results, it was concluded that AMC were PAs of mulberry. Chemical structures of the AMC of Rf value 0.63 were determined and these new compounds were named as moracin A and B.

Shirata and Takahashi (1982 b) investigated the presence of AFA in arboreal plant tissues. Cortical disks of one to two-year-old shoots of six species belonging to Moraceae family including mulberry were placed on PDA media seeded with fungi and bacteria, and assayed at 25–30°C for 1 - 2 days. Inhibition zones of microbial growth were observed (direct method). The disks of mulberry and persimmon showed inhibition zones, indicating AFA against *B. leersiae* and *F. roseum*. The cortical disks of mulberry shoots showed AFA in neutralized PDA, while those of persimmon showed AFA only on acidified PDA. The control disks of mulberry shoots treated at –20 °C for 60 min or in hot water at 60°C for 10 min failed to display any AFA. Pieces of various organs of mulberry plant, *i.e.*, leaf and petiole, cortical tissues of shoot and root

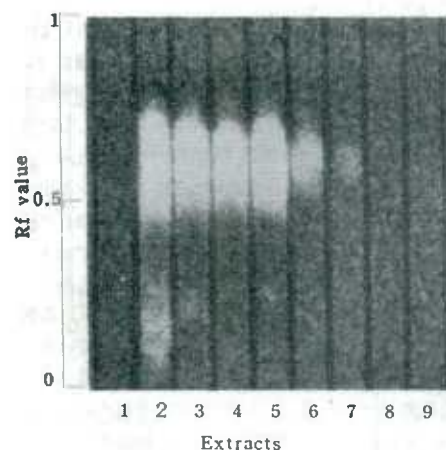


Figure 3: TLC showing the inhibitory effects against *B. leersiae* of the extracts from cortices inoculated with *F. solani* f. sp. *mori*, which were obtained by using various organic solvents. (Credit: Shirata *et al.*, 1978 a).

displayed AFA, but it could not be detected in xylem tissues of either shoot or root by this method. On the other hand, the acetone extract from all these pieces assayed by the direct method showed AFA against *B. leersiae*. AFA could not be spotted in browned cortical tissues infected with pathogen by using the direct method whereas, acetone extract showed AFA.

Takasugi *et al.* (1980 b) analysed the cortical tissues of one year old shoots inoculated with 10^8 cells / ml spore suspension of *F. solani* f. sp. *mori* and 10^9 cells / ml of *Pseudomonas cichorii*. The shoots were incubated in a moist condition at 20-25 °C for 5 days. The cortex turned brown within 1-2 days after inoculation. Acetone extract from brownish cortex showed AMA to many phytopathogenic fungi and bacteria. The AMS were identified as broussonin A-F, broussinol and marmesin in the browned cortex of paper mulberry. Further, they observed that Broussonin A and B possessed antibacterial activity to 7 genera. *Agrobacterium* and *Streptomyces*

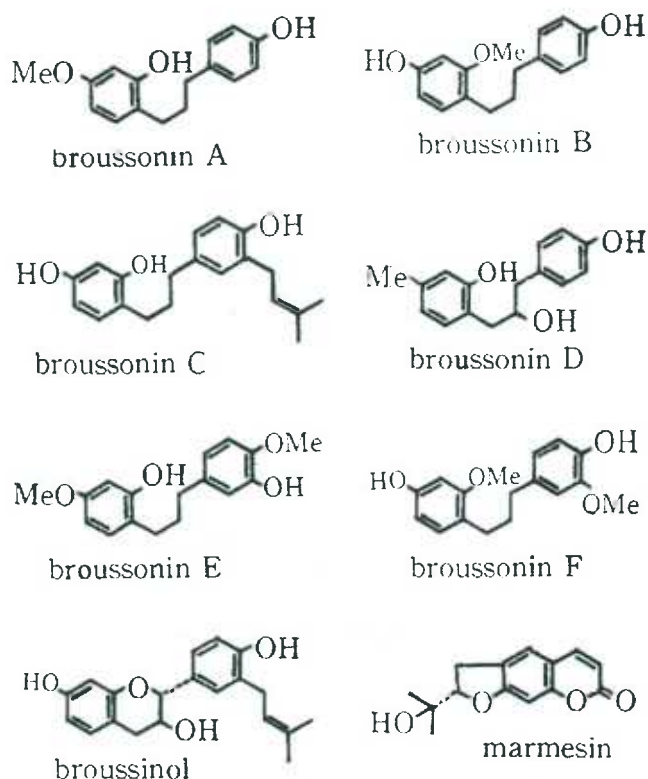
were inhibited strongly and *Bacillus*, *Corynebacterium*, *Xanthomonas* and *Erwinia* were inhibited weakly, *Pseudomonas cichorii* was tolerant and Marmesin did not show any antibacterial activity (Table 2).

Table 2: Antibacterial activity of phytoalexins tested with drop method

Bacteria	BA ^a	BB	M
<i>Agrobacterium tumefaciens</i>	++ ^b	++	-
<i>Bacillus subtilis</i>	+	+	±
<i>Corynebacterium michiganense</i> pv. <i>michiganense</i>	+	+	±
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	±	±	-
<i>Pseudomonas cichorii</i>	±	±	-
<i>Streptomyces</i> sp.	++	++	-
<i>Xanthomonas campestris</i> pv. <i>alfalfa</i>	+	+	-

^aOne drop of 50 % aqueous acetone including each phytoalexin and prohibitin was spotted onto Wakimoto agar medium seeded with bacteria. BA: broussonin A; BB: broussonin B; M: marmesin.

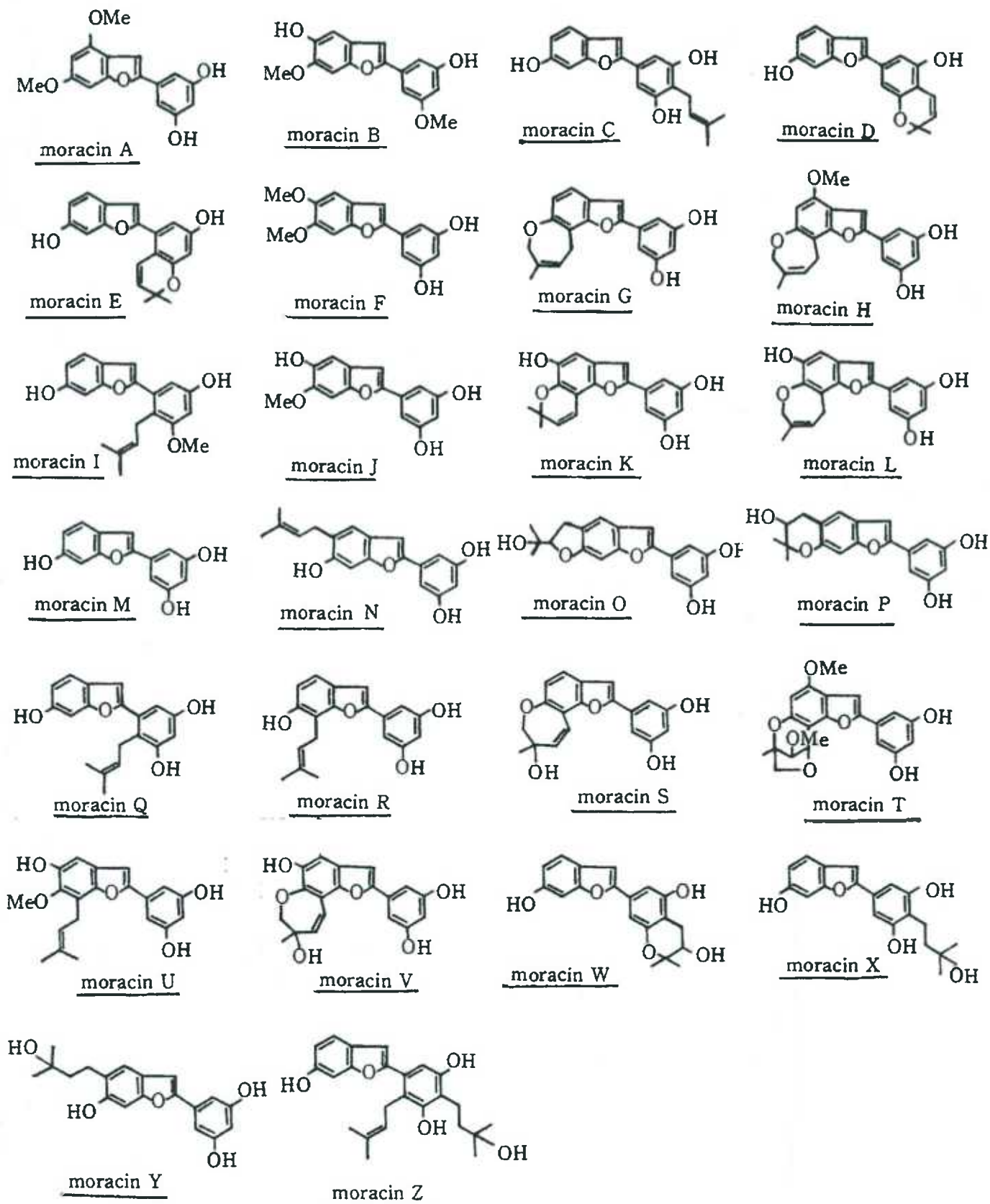
^bInhibition grade was rated on the following scale; ++: diameter of inhibition zone was larger than spotted zone; +: inhibition zone covered only the spotted zone; ±: spotted zone was clouded uniformly by bacterial growth but detectable, or small moderate inhibition zone was observed in the center of spotted zone, -: no inhibition. (Credit: Shirata *et al.*, 1983)



PAs from shoot cortex tissue of paper mulberry
(Credit: Takasugi *et al.*, 1980 a)

AFA of moracin A-H against pathogenic and non-pathogenic fungi was observed (Takasugi *et al.*, 1978 a, b; Takasugi *et al.*, 1979) (Table 3). PAs produced in shoot cortex were chemically identified as 26 new compounds, which have 2-phenyl-benzofuran skeleton, a rare example in natural products and were named moracin A-Z (Shirata *et al.*, 1983).

Murakami *et al.* (1998) detected PAs in mulberry shoot by Vanillin reagent method. The colour and R_f values of mulberry PAs were developed on TLC plate (Table 4). Takasugi *et al.* (1978 a, b; 1979) reported the molecular formulae as well as yield (%) of seven 2-phenylbenzofuran PAs (moracin A~H) isolated from shoot cortex and phloem tissues of mulberry (*Morus alba* L.) infected by *F. solani* f. sp. *mori* as following.



(Credit: Takasugi *et al.*, 1978 a,b; Takasugi *et al.*, 1979; Shirata *et al.*, 1983)

Table 3: Antifungal activity** of moracins A-H

Fungus	MORACIN							
	A	B	C	D	E	F	G	H
<i>Fusarium roseum</i>	6 - 12	12 - 25	3.5 - 7	7 - 14	15	50	15	15
<i>F. lateritium</i> f. sp. <i>mori</i>	25 - 49	49	7 - 14	28 - 56	50	200	500	100
<i>F. solani</i> f. sp. <i>mori</i>	-	-	224	112	200	1000	500	1000
<i>Diaporthe nomurai</i>	6 - 12	12	14 - 28	7 - 14	15	1000	50	50
<i>Stigminia mori</i>	6 - 12	12 - 25	112 - 224	56 - 112	200	50	50	50
<i>Sclerotinia sclerotiorum</i>	-	-	-	-	50	50	500	200
* <i>Cochliobolus miyabeanus</i>	12 - 25	49	< 3.5	< 3.5	100	100	50	50
<i>Rosellinia necatrix</i>	-	-	28 - 56	14 - 28	15	50	15	15

* A non-pathogenic fungus; ** minimum concentration (µg/ml) required for complete inhibition of fungal growth.

Table 4: Colour and Rf value of mulberry phytoalexins on

Phytoalexin	TLC plate		
	Colour ^a	1 ^b	2
Moracin A	dark blue	0.50	0.64
Moracin B	blue	0.50	0.67
Moracin C	red	0.62	0.62
Moracin D	purple	0.66	0.65
Moracin E	navy blue	0.66	0.65
Moracin F	blue	0.34	0.62
Moracin G	blue	0.55	0.64
Moracin H	blue	0.52	0.65
Moracin I	red	0.57	0.65
Moracin J	blue navy	0.36	0.56
Moracin K	blue	0.41	0.58
Moracin L	blue	0.39	0.58
Moracin M	purple	0.43	0.53
Moracin N	blue	0.50	0.58
Moracin O	blue	0.30	0.58
Moracin P	blue	0.27	0.58
Moracin Q	purple	0.48	0.58
Moracin R	purple	0.53	0.58
Moracin S	blue	0.30	0.51
Moracin T	blue	0.31	0.61
Moracin U	blue	0.45	0.58
Moracin V	blue	0.18	0.51
Moracin W	red	0.32	0.58
Moracin X	red	0.29	0.53
Moracin Y	blue	0.18	0.53
Moracin Z	red	0.45	0.63
Chalcomoracin	red	0.05	0.51
Kuwanon C	yellow	0.48	0.65
Morusin	green	0.46	0.73
Oxyresveratrol	red	0.20	0.45
4 prenyloxyresveratrol	red	0.52	0.58

^a Color of Phytoalexin was induced by Vanillin reagent.

^b Developing solvent: diethyl ether (1), chloroform: methanol: water-20:7:1 (2)

Phytoalexin

Molecular
formula

Yield (%)

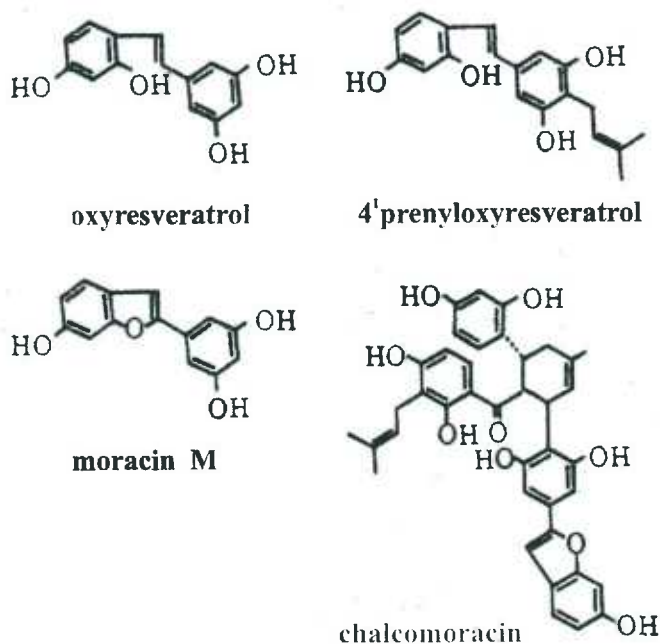
moracin A	C ₁₆ H ₁₄ O ₅	0.012
moracin B	C ₁₆ H ₁₄ O ₅	0.322
moracin C	C ₁₉ H ₁₈ O ₄	0.020
moracin D	C ₁₉ H ₁₆ O ₄	0.010
moracin E	C ₁₉ H ₁₆ O ₄	0.020
moracin F	C ₁₆ H ₁₄ O ₄	0.002
moracin G	C ₁₉ H ₁₆ O ₅	0.001
moracin H	C ₂₀ H ₁₈ O ₄	0.009

may cause die-back disease. However, when a lateral shoot below is grown to bear many leaves, the lesion stops expanding at the portion of the xylem adjacent to the base of the lateral shoot. It was found that an extremely large amount of PA was accumulated in that portion (Shirata and Takahashi, 1979; Takasugi *et al.*, 1980 a; Takahashi and Shirata, 1982; Shirata *et al.*, 1983). The production of antifungal substances in xylem tissues of mulberry shoots (*M. alba* L.) was studied. The xylem blocks of shoot inoculated with *F. solani* f. sp. *mori* produced antifungal substances, which were not detected in those of intact shoots. The xylem blocks of uninoculated winter shoot incubated in moist condition also showed a similar effect.

iv. Xylem tissues of mulberry shoot

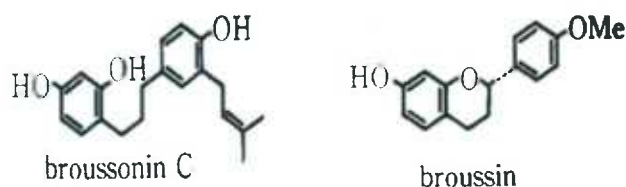
When mulberry shoots are cut for harvesting, the xylem is directly exposed to the entrance of fungi, and

These substances seemed to be produced in living tissues, because when the xylem blocks were treated at 60 °C for 60 minutes or 55 °C for 10 minutes in water or chipped too fine, it failed to produce antifungal substances. These substances inhibited the growth of fungi and bacteria, the pathogens causing mulberry diseases and were concluded as phytoalexins. Further, phytoalexins were detected in xylem tissues taken from one year old shoot of Moraceae plants belonging to 7 species (Shirata and Takahashi, 1979). They were identified as oxyresveratrol, 4'-prenyloxyresveratrol, moracin M and chalcomoracin.



(Credit: Takahashi and Shirata, 1982; Shirata *et al.*, 1983)

Takasugi *et al.* (1980 a) purified two classes of PAs, broussonin C and broussin from xylem tissue of paper mulberry shoot.



II. Mulberry leaves

Mulberry leaves were inoculated with *F. solani* f. sp. *mori*, which induced the formation of a different type of antifungal compound, chalcomoracin (Takasugi *et al.*, 1980 b; Shirata and Takahashi, 1982 c; Takahashi and Shirata, 1982) - a major PA of the leaves. The production of PA began from 12 hours after inoculation, reaching peak in 2-4 days and then decreased. The PA production was noticed less in the upper but more in middle / lower leaves. The chalcomoracin completely inhibited the germination of *F. roseum* and *B. leersiae* at 10^{-4} - 10^{-5} M concentrations and later formulated as $C_{39}H_{36}O_9$ (Takasugi *et al.*, 1980 b).

In another study (Shirata and Takahashi 1982 c), non-infected leaves of *M. alba*, *C. tricuspidata*, *C. cochinchinensis* var. *gerontoger*, *B. kazinoki*, *B. papyrifera*, *F. carica* and *F. erecta* showed antibacterial activity to *Staphylococcus* sp. But, only *F. carica*, showed antifungal activity to *F. solani* f. sp. *mori*, *D. nomurai*, *B. leersiae*, *F. lateritium* f. sp. *mori* and *F. roseum*. A novel terpenoid-type (Kim-Kyung-Hee and Matsuyama Nobuaki, 1998) PA was isolated from mulberry leaves infected with *Phloeospora maculans*, causal agent of leaf spot disease. The molecular formula and weight of this substance were decided as $C_{21}H_{24}O_4$ (M. wt. 340) by HR/ (+) FAB mass spectrometry. Other kinds of substances, which showed AFA, were also detected on TLC in diseased mulberry leaves.

Antimicrobial spectra of the compounds from mulberry

The PA and prohibitins isolated from mulberry were tested for inhibitory activity against spp. of pathogenic fungi by Shirata *et al.* (1983) (Table 5). Totally, 17 compounds were tested, which comprised of:

Five prohibitins from shoot epidermis – Albanin A-C, Kuwanon C, Morucin

Eight PAs from shoot cortex – Moracin A~H

Three PAs from shoot xylem tissue – Oxyresveratrol,

Table 5: Antifungal activity of phytoalexins and prohibitins of mulberry tested with holeside method

	SHOOT																Leaves
	Epidermis					Cortex								Xylem			
	^a AA	AB	AC	KC	M	MA	MB	MC	MD	ME	MF	MG	MH	O	PO	MM	CM
	1234 ^b	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234
<i>Diaporthe nomurai</i>	00.. ^c	000.	000.	00..	0000	0...	00..	00..	0...	0...	00..	00..	00..	000.	0000	000.	0...
<i>F. lateritium f. sp. mori</i>	000.	0000.	0000	0000	0000	00..	00..	0...	00..	000.	000.	000.	0000	0000	000.	000.	0000
<i>Fusarium roseum</i>	00..	00..	00..	00..	0000	00..	00..	0...	0...	0...	00..	0...	0...	00..	00..	00..	00..
* <i>F. solani f. sp. mori</i>	00..	0000	0000	0000	0000	000.	0000	000.	000.	000.	0000	0000	0000	0000	0000	000.	0000
<i>Rosellinia necatrix</i>	00..	00..	00..	0...	000.	00..	00..	0...	0...	0...	00..	0...	0...	0000	00..	000.	0000
<i>Sclerotinia sclerotiorum</i>	00..	0000	0000	000.	0000	00..	00..	00..	00..	0...	00..	0000	000.	0000	000.	000.	000.
<i>Stigminia mori</i>	00..	00..	00..	0...	0000	00..	00..	000.	000.	00..	00..	00..	00..	0000	00..	000.	0...

^aAA: albanin A, AB: albanin B, AC: albanin C, KC: kuwanon C, M: morusin, MA: moracin A, MB: moracin B, MC: moracin C, MD: moracin D, ME: moracin E, MF: moracin F, MG: moracin G, MH: moracin H, O: oxyresveratrol, PO: 4^l prenyloxyresveratrol, MM: moracin M, CM: chalconmoracin. ^bFungal growth was observed 1-2 days after incubation at 20-25 °C in the solution PDA broth including each compound. 1: 3.5 ppm, 2: 14 ppm, 3: 56 ppm, 4: 224 ppm. ^cIntensity of antifungal activity was scored and classified into four grades; 0: normal growth, 1: about half of the growth, 2: a little growth, 3: no growth. * Pathogen of mulberry (Credit: Shirata *et al.*, 1983).

One PA from mulberry leaves
 Moracin M,
 4^l - Prenyloxyresveratrol
 – Chalconmoracin

a) Fungi: Intensity of AFA of each compound changed with the fungal species. PA from cortex was stronger when compared to those from xylem. Moracin C and moracin D completely inhibited the growth of *R. necatrix* and *S. trifoliorum*. The pathogens of mulberry were more tolerant than non-pathogens.

b) Bacteria: Twenty four species of bacteria including two species pathogenic on mulberry were tested. *Agrobacterium*, *Bacillus*, *Corynebacterium* and *Streptomyces* were sensitive to many compounds. Weak sensitivity was observed in 10 species of *Xanthomonas*, whereas, *Pseudomonas* and *Erwinia* were tolerant to the compounds tested. Among the 17 compounds, moracin-M showed strong antibacterial activity (Table 6).

Nagaveni and Shree (2003) studied post inflectional changes in total proteins and orthodihydric (OD) phenolic contents in 13 popular mulberry (*Morus* spp.) varieties for resistant and susceptible reaction towards the pathogen,

Cercospora moricola Cooke causing leaf spot disease (artificial induction). They categorized mulberry varieties into resistant (RVs) - BC₂₅₉, MR₂, RFS₁₃₅, V₁, S₁₃ and S₃₄; moderately resistant (MRVs) - DD, M₅ and S₃₆; moderately susceptible (MSVs) - Mysore local and S₄₁ and highly susceptible varieties (SVs) - S₅₄ and S₁₆₃₅. A significant increase in the total protein content of RVs was noticed. On the contrary, there was a drastic reduction in MRVs, MSVs and SVs. The changes in the leaf protein content and phenolic compounds have been reported as a result of almost every infection and are implicated as resistant factors in many host-pathogen combinations (Kuc, 1957; Kosuge, 1969). The increased protein content in RVs could be one of the contributing factors for the observed resistance (Stintzi *et al.*, 1993). The total phenolic content in RVs and MRVs was significantly increased; but it was decreased in the MSVs and SVs. The OD phenols were significantly increased in RVs and MRVs. In MSVs, it showed a significant increase as well as decrease. However, in the SVs, it was significantly decreased. The presence of high phenolic content in RVs indicate the synthesis of phytoalexins (Nagaveni *et al.*, 2003 b). The phenols synthesized by plant tissues after infection appear to have a broad antifungal spectrum (Kosuge, 1969). Nagaveni *et al.* (2003 a) studied spore (*Cochliobolus*

Table 6: Antibacterial activity of phytoalexins and prohibitins of mulberry tested with drop method^a

Bacteria ^b	SHOOT																	Leaves
	Epidermis					Cortex								Xylem				
	AA	AB	AC	KC	M	MA	MB	MC	MD	ME	MF	MG	MH	O	PO	MM	CM	
<i>Agrobacterium tumefaciens</i>	++	±	±	-	-	++b)	+	+++	++	++	+	+	+		+	+++	-	
<i>Bacillus subtilis</i>	+++	++	++	++	-	++	+	+++	++	++	++	++	++	-	±	++	+	
<i>Corynebacterium michiganense</i> pv. <i>michiganense</i>	+++	++	++	+++	++	++	+	+++	++	+++	+	+++	+++		±	++	+	
* <i>Erwinia carotovora</i> subsp. <i>carotovora</i>	±			±	-	±			-	±	±	±	-	-	-	+	-	
<i>Pseudomonas avenae</i>	±	-	±	-	-	-	-	±	±	++	±	±	±	-	±	++		
<i>P. cichorii</i>		-	-	-	-													
<i>P. marginalis</i> pv. <i>marginalis</i>							-	-	-	-	-	-			-	-	-	
<i>P. syringae</i> pv. <i>erobotryae</i>	-	-		-					-	-		-	-		-	-		
<i>P. syringae</i> pv. <i>japonica</i>	++	+	+	++		±	±	++	++	++	+	++	++	-	±	+++	-	
* <i>P. syringae</i> pv. <i>mori</i>		-	-	-	-	-	-	±	±	-	-			-	-	-		
<i>P. syringae</i> pv. <i>syringae</i>	-											-	-			+		
<i>P. syringae</i> pv. <i>tabach</i>		±		±				-	-	+	-	+	+	-	-	++		
<i>P. viridiflava</i>	-	-	-	-	-	-	-						-		-	-		
<i>Streptomyces</i> sp.	+++	++	++	+++	++	++	+	+++	+++	+++	++	+++	+++	-	+	+++	++	
<i>Xanthomonas campestris</i> pv. <i>alfalfa</i>	+	±	±	+	-	+	±	±	+	+	±	±	±		-	++	-	
<i>X. campestris</i> pv. <i>campestris</i>	+	±	±	+		+	±	±	±	++	+	±	±		±	++	-	
<i>X. campestris</i> pv. <i>citri</i>	+	+	+	+	-	+	±	±		+	±	±	±	-	±	++	-	
<i>X. campestris</i> pv. <i>cuculbitae</i>	+	±	±	+	-	++	±	+	++	+	+	+	+		±	++		
<i>X. campestris</i> pv. <i>glycines</i>	±	±	±	±		+	±	±	+	±	±	±	±		±	++	-	
<i>X. campestris</i> pv. <i>hyacinthi</i>	+	±	±	+		+	±	±	±	+	±	+	+	-	±	++	-	
<i>X. campestris</i> pv. <i>oryzae</i>	++	+	+	+		+	±	-	++	++	±	+	+		±	+++	+	
<i>X. campestris</i> pv. <i>pisi</i>	+	±	±	+		+	±	±	-	±	±	±	±		±	++	-	
<i>X. campestris</i> pv. <i>pruni</i>	+	±	±	+		+	±	±	±	+	+	+	+		±	++		
<i>X. campestris</i> pv. <i>vitians</i>	++	±	±	++	-	±	±	±	±	+	±	+	+	-		++		

^aOne drop of 50 % aqueous acetone including each phytoalexin and prohibitin was spotted onto Wakimoto agar medium seeded with bacteria.

^bInhibition grade was rated on the following scale; +++: diameter of inhibition zone was larger than spotted zone; ++: inhibition zone covered only the spotted zone; +: about half inhibition zone of ++; ±: spotted zone was clouded uniformly by bacterial growth but detectable, or small moderate inhibition zone was observed in the center of spotted zone, -: no inhibition. Pathogen of mulberry (Credit: Shirata *et al.*, 1983).

miyabeanus) germination and inhibition (%) at hourly intervals for five hours to know the presence of PAs in different mulberry (*Morus* spp.) cultivars. Acetone leaf extracts from healthy and *C. moricola* infected regions were taken from popular indigenous cultivars belonging to the above mentioned categories. The inhibition percentage (%) of spore germination was more in RVs when compared to MRVs, MSVs and S variety. This indicates that PA synthesis was more in RVs and therefore, germination was slow when compared to others. MRVs also inhibited germination of spores, but not as much as RVs. MSVs although inhibited germination initially, promoted it later. Here, PA was

synthesized at a lower rate. However, S variety (S₁₆₃₅) being highly susceptible, allowed and even enhanced germination of spores indicative of less synthesis of PAs (Nagaveni *et al.*, 2003 a).

Conclusion

Mulberry plants are exposed to various pathogenic microorganisms in their surroundings. Generally, the plants may have some kind of protective mechanisms in response to attack by pathogens. Many studies revealed the presence of antifungal and antibacterial substances in different parts of mulberry plants. These substances afford

protection and fight out the pathogens causing diseases. Hence, such compounds which are usually produced post-infectionally are appropriately named as “phytoalexins”. The chemical nature of mulberry PA indicates a considerable range of benzofuran and stilbenes. In mulberry root, kuwanon C and ethyl rescrylate were the AMS which effectively inhibited the growth of mulberry root pathogens. Mulberry shoot bark produced PO, PPO and phytoalexin-like substances when infected by pathogens. In mulberry shoot epidermis, antifungal components were chemically identified as prenylflavon compounds, namely kuwanon C and E, moracin, new compounds – albanin A~H and albafuran A~C. Thus, it is inferred, that the epidermis not only acts as a physical barrier but also protects the plant from pathogenic fungal invasion by producing AFS in response to infection, thus, checking further spread of the disease. The cortex produced benzofuran compounds, moracin A~Z. The mulberry pathogens (fungi causing twig blight disease), *F. solani* f. sp. *mori*, *F. lateritium* f. sp. *mori* and *F. roseum* were more tolerant to moracin-A and moracin-B. Moracin-C and moracin-D inhibited the complete growth of the fungi, *R. necatrix* and *S. trifoliorum*. The xylem produced stilbene compounds oxyresveratrol, 4'-prenyloxyresveratrol and moracin-M, when infected by fungi. Oxyresveratrol did not show AFA. However, moracin-M acted as a strong antibiotic against pathogens. It is said that mulberry shoots protect themselves by producing and accumulating PAs in the browned tissues before invasion of fungi and later use them to defend themselves against invasion by fungal pathogens. Chalcomoracin, a benzofuran compound was the PA found in the leaf blade of mulberry, which also contributed to the biochemical defense mechanism against pathogenic invasion.

PAs are antifungal stress metabolites formed in plants, which resist or suppress the activity of the invader. They play a role in disease resistance and in the control of plant disease to a certain extent. PAs are natural products and thus their use in controlling plant diseases are more reliable, harmless and has no chance of residual effects which is very common in the case of fungicides and

pesticides. Activation of PA synthesis by application of biotic and abiotic elicitors may enhance field resistance of plants to some extent. Treatment of the plants as well as seeds with PA elicitors can also be effective. Therefore, it seems reasonable to speculate that more potential PAs can be made available in the near future to control various diseases of mulberry.

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Revue

PRODUCTION DE PHYTOELAXINES – UN MECANISME DE DEFENSE POST INFECTION CHEZ LE MURIER (*MORUS* SPP.)

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RESUME

Le feuillage du mûrier (*Morus spp.*) est une source exclusive de nourriture pour les vers à soie (*Bombyx mori* L.). De nombreuses maladies détruisent le tissu foliaire. Les phytoelaxines (Pas) produites en réponse à l'infection sont connues pour apporter de la résistance à l'hôte. La connaissance sur les PAs comme l'un des mécanismes de défense post infection chez le mûrier et d'autres plantes peut aider à protéger les plantes hôtes des attaques microbiennes. Les Pas du mûrier sont des composés chimiques de benzofurane et de stilbènes. Le Kuwanon C et l'éthyl β rescrylate sont des antifongiques détectés dans les racines du mûrier qui inhibent complètement la croissance des pathogènes - *Rosellinia necatrix* et *Helicobasidium mompa*. Lorsque le mûrier est affecté par le « dogare » et la rouille des tiges, quelques substances antimicrobiennes comme les composés de flavonesprénylés, le kuwanon C et E, la morusine, l'albanine A-H et l'albafurane A-C sont détectés dans l'épiderme des tiges. Le cortex produit les moracines A-Z alors que le xylème produit les composants de stilbène comme l'oxyresvératrol, le 4'-prényloxyresvératrol et la moracine-M, la chalcomoracine, les composés de benzofurane sont dans les feuilles, ce qui inhibe la croissance de *Fusarium solani* f. sp. *mori*. Cette revue donne un panorama des phytoelaxines chez le mûrier lors de pathologies.

Mots-clés: Substances antifongiques, moracine, mûrier, phytoelaxine.



GENETIC CHARACTERIZATION OF AN ALPHABACULOVIRUS CAUSING TIGER BAND DISEASE IN THE OAK TASAR SILKWORM, *ANTHRAEA PROYLEI* J (LEPIDOPTERA: SATURNIIDAE)

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ABSTRACT

In the recent decade, a dreadful viral disease commonly known as tiger band disease affecting the oak tasar silkworm, *Antheraea proylei* has caused severe loss in the production of silk. The virus was isolated and the complete viral genome was determined, unveiling that a group I alphabaculovirus i.e., *Antheraea proylei* nucleopolyhedrovirus (AnprNPV) is the causal agent of this disease. The genome is 126,930 bp in length with 53.46 % G+C content. It encodes 147 open reading frames (ORFs), consisting of 37 core genes, 25 lepidopteran baculovirus conserved genes, 11 lepidopteran NPVs genes, 41 genes found in all Group I NPVs, 27 genes found in some Group I NPVs, 5 genes found specifically in NPVs of saturniid silkworms and also with 1 additional unique gene, *he65-like* (Anpr35). There are 109 intergenic regions distributed ranging from 1 to 454 bp, contributing to 7.33 % of the whole genome. Within intergenic regions, 239 microsatellites (SSRs) motifs having mono to hexa-nucleotide repeats were distributed where trinucleotides had maximum abundance (47.89 %). Six homologous regions (Hr1 to Hr6) present in the genome have perfect / imperfect palindromes of 30 bp sequence, CGBTTTTTCNAGNHTGDYYRTNCTYGAAAANC associating with a direct repeat, TCRGCGCTGA. Phylogenetic analysis showed the clustering of AnprNPV in the same clade as the alphabaculovirus group I of the saturniid silkworms *A. pernyi* and *Philosamia cynthia ricini* indicating a close relationship with those viruses. Furthermore, AnprNPV and *A. pernyi* nucleopolyhedrovirus (AnpeNPV) isolates had a nucleotide sequence identity above 95 %, which strongly suggest that AnprNPV is a regional variant of AnpeNPV.

Key words: *Antheraea proylei*, nucleopolyhedrovirus, saturniid silkworm, viral genome.

INTRODUCTION

Antheraea proylei, the oak tasar silkworm, is one of the economically important silkworms reared for the production of tasar silk. It is reared extensively along the Sub-Himalayan oak belts of India extending from Jammu and Kashmir in the North-West to Manipur in the North-East. In Manipur, the host plants viz., *Quercus acutissima*, *Q. dealbata*, and *Q. serrata* are abundantly grown and

used for tasar culture. The tasar silk production in this region contributes to a substantial part of the yearly income for almost 10,000 families actively rearing silkmoths in 7500 ha of oak-tasar grown areas. Of the 9.5 tons of north-east Indian oak-tasar raw silk produced, 7.3 tons are from Manipur. However, as it is reared under outdoor conditions, silkworms are readily exposed to various microbial diseases caused by bacteria, viruses, protozoans and fungi. In the recent past, a new viral

disease, dubbed the tiger band disease was encountered in *A. proylei*, manifesting symptoms of loss of appetite, black spots, and a black band pattern similar to tiger stripes and motionless appearance, associated with the larva. In the North-eastern India, the extent of damage has contributed up to 70-80 % of larval and pupal mortality causing huge amount of crop losses. Since then, tiger band disease has become a great threat to the oak tasar growers both in the North-eastern and North-western regions of India. Even though favourable climatic conditions prevailing in North-east India makes it an ideal home for many wild sericigenous insects such as muga, eri, tropical tasar, temperate tasar and fagara silkmoths (Reeta *et al.*, 2016), infestation of tiger band disease in *A. proylei* poses a significant threat in successful oak tasar rearing. However, detailed work on identification of the causal organism of the tiger band disease of *A. proylei* and its genetic characterization are still a lacuna. In this study, we have identified and characterized the causal organism of tiger band viral disease of *A. proylei* as being an alphabaculovirus, AnprNPV.

MATERIALS AND METHODS

Virus isolation and purification

Virus-infected *A. proylei* larvae were collected in April, 2016 from the Regional Tasar Research Station, Imphal, Manipur, India. The larvae were frozen and thawed to break down the infected cells and tissues. It was suspended in double-distilled water, homogenized, and filtered through cheese cloth. The filtrate was subjected to differential centrifugations of 600 rpm for 5 min and 1300 rpm for 1 min. The collected supernatants were centrifuged at 7000 rpm for 20 min. After treating the collected pellet containing polyhedral occlusion bodies (OBs) for 2 h in 2 % SDS, it was centrifuged at 5000 rpm for 10 min. The pellet collected was suspended in nuclease-free water for future use. OBs were further purified using a 50 % w/v (sucrose in TE buffer) cushion and ultracentrifugation at 100,000 rpm for 30 min. The purified OBs were suspended in nuclease-free water and stored at -20 °C.

Electron microscopy

Electron microscopy images of OBs were taken using a JEOL 2011 microscope at the Integrated Imaging Facility, University of Notre Dame, IN, USA.

DNA extraction

OB suspension (100 µl) was dissolved in 500 µl of lysis buffer (10 mM Tris pH8, 100 mM NaCl, 10 mM EDTA pH8 and 0.5 % SDS) and incubated at 37 °C for 30 min with 4 µl of RNase (4 µg/µl). Proteinase K (20 mg/ml) was added and incubated for 150 min at 55 °C. The lysed suspension was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by ethanol precipitation. DNA concentration and quality was determined using the Qubit 2.0 Fluorometer and High Sensitivity DNA Assay (Life Technologies Corp., Carlsbad, CA) along with gel electrophoresis (0.8 % agarose gel).

DNA sequencing, assembly and annotation

DNA sequencing was performed using the Illumina MiSeq platform (Illumina, Inc.) in the Genomic Core Facility, University of Notre Dame, IN, USA, using the MiSeq Reagent Nano Kit v2 (500 cycles) by following the manufacturer's instructions. A total of 587 Mbp of data were obtained using 251 bp paired end reads. The *de novo* assembly was generated using SPAdes (Nurk *et al.*, 2013) and the resulting genome assembly was compared with the *Antheraea pernyi* nucleopolyhedrovirus (AnpeNPV) genome (Fan *et al.*, 2007) using dnadiff (Kurtz *et al.*, 2004). Out of the 206 contigs, 55 were observed to be the virus sequence in question and the contigs were ordered with Abacas version 1.3.1 (Fan *et al.*, 2007; Assefa *et al.*, 2009). Using Mummer version 3.23, only four out of the 55 contigs were used to assemble the complete genome and the rest were shown as overlapping repeats (Kurtz *et al.*, 2004). The probable ORF regions were detected using TransDecoder, version 3.0.1 (Haas *et al.*, 2013). Annotation of ORFs was done using BLASTx and BLASTp with non-redundant protein sequences (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Five gaps with the size of 27 to 798 bp were found within the assembled genome. The gap sequences were filled and compiled by

PCR and sequencing. The circular map of the complete annotated genome of *A. proylei* nucleopolyhedrovirus (AnprNPV) was generated using SnapGene Viewer 4.0.3 (http://www.snapgene.com/products/snapgene_viewer/) and edited with Adobe Photoshop 7. The homologous regions (hrs, 1-7) were identified using Palindrome Finder (<http://emboss.bioinformatics.nl/cgi-bin/emboss/palindrome>) and Tandem Repeat Finder (<https://tandem.bu.edu/trf/trf.html>) and confirmed by comparing with the *hr* consensus region of the AnpeNPV genomes (AnpeNPV-L2 and AnpeNPV-Z) (Fan *et al.*, 2007; Nie *et al.*, 2007). Microsatellite repeats in intergenic regions were detected with Microsatellite Repeats Finder (http://insilico.ehu.es/mini_tools/microsatellites/). ORFs with signal peptides were predicted using Signal-BLAST in CAME (Frank and Sippl, 2008) (<http://sigpep.services.came.sbg.ac.at/signalblast.html>).

Phylogenetic analysis

Phylogenetic analysis was carried out for 55 baculovirus species based on the nucleotide sequences of 37 concatenated core genes. Sequences were aligned based on amino-acid sequences using the Muscle algorithm as implemented in MEGA v 6.0 (Tamura *et al.*, 2013). The phylogeny was inferred by the maximum likelihood (ML) method along with the JTT substitution model of DNA evolution and estimated with the JmodelProtest 2.4 software program (Posada, 2008). The parameters for ML analysis were estimated during the analysis and the node support values were assessed by bootstrap with 1000 replicates.

RESULTS AND DISCUSSION

Polyhedral structure

Phase-contrast microscopic images of the purified viral OBs of *A. proylei* alphabaculovirus (AnprNPV) showed the presence of a large number of rounded particles of typical baculovirus nature (Figure 1). Electron microscopy of these viral OBs revealed the presence of multiple bacilliform nucleocapsids within one virus envelope indicating group I alphabaculovirus (Kumar *et*

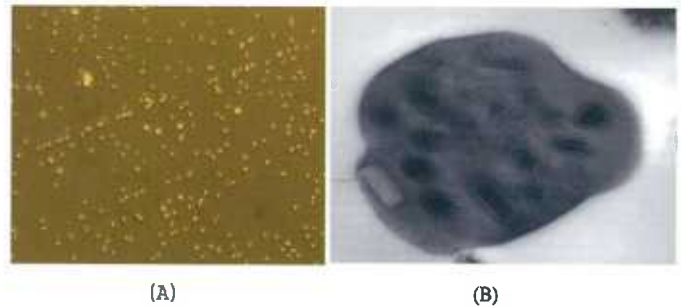


Figure 1: Phase contrast and electron microscopic images of purified viral ocular bodies (OBs) of *Antheraea proylei* nucleopolyhedrovirus. Phase contrast microscopic image observed at 40X magnification showed numerous rounded particles of OBs, a typical character of baculoviruses (A). Electron microscopic image at 400 μ m magnification showed multiple bacilliform nucleocapsid virions within the virus envelope indicating an alphabaculovirus (B).

al., 2011; Nurk *et al.*, 2013).

Characteristic features of the AnprNPV-TS genome

The complete genome of AnprNPV was 126,930 bp in size having 53.46 % G+C content (Accession number: KY979487). The AnprNPV genome encoded 147 ORFs of putative proteins having at least 50 amino acids in length. Of these, 70 ORFs were distributed in a clockwise direction and 77 ORFs in an anti-clockwise orientation. The AnprNPV was compared with eight other homologous baculovirus genomes: isolates of *A. pernyi*, AnpeNPV-L2 (Fan *et al.*, 2007) and AnpeNPV-Z (Nie *et al.*, 2007), *Philosamia cynthia*, PhcyNPV (Qian *et al.*, 2013), *Bombyx mori*, BmNPV (Xu *et al.*, 2010), *B. mandarina*, BomaNPV (Xu *et al.*, 2010), *Autographa californica*, AcMNPV (Ayres *et al.*, 1994), *Orgyia pseudotsugata*, OpMNPV (Ahrens *et al.*, 1997) and *Lonomia obliqua*, LoobNPV (Aragão-Silva *et al.*, 2016) (Table 1). The size and G+C content of AnprNPV were similar to that of the nucleopolyhedroviruses of saturniid silkworms, AnpeNPV-Z, AnpeNPV-L2 and PhcyNPV, but was different from those of the Bombycidae family, BomaNPV and BmNPV. Most of the ORFs shared maximum similarity with the two isolates of the *A. pernyi* NPV genomes (AnpeNPV-L2 and AnpeNPV-Z). Three

Table 1: Orientation of 37 core genes of AnprNPV genome compared with homologous baculovirus genomes

AnprNPV core gene Orf	Gene	Position (bp)	AnpeMNPV -L2	bp & simi- arity (%)	AnpeMNPV -Z	bp & simi- arity (%)	PhcyNPV	bp & simi- arity (%)	BmNPV	bp & simi- arity (%)	BomaNPV	bp & simi- arity (%)	AcMNPV	bp & simi- arity (%)	OpMNPV	bp & simi- arity (%)	LoobNPV	bp & simi- arity (%)
10 (+)	<i>odv-e56</i> (occlusion-derived virus envelope protein E56)	7182→8306 (1125)	10 (+)	1125 (99)	139 (-)	1125 (99)	9 (+)	1125 (99)	132 (-)	1128 (67)	131 (-)	1128 (66)	148 (-)	1131 (70)	146 (-)	1124 (85)	20 (-)	1128 (67)
14 (-)	<i>odv-ec27</i> (occlusion-derived virus envelope / capsid protein E27)	11318→12208 (891)	14 (-)	891 (100)	135 (+)	927 (90.5)	13 (-)	891 (99)	128 (+)	873 (69)	127 (+)	873 (69)	144 (+)	873 (69)	141 (+)	894 (73)	16 (+)	861 (63)
15 (-)	<i>odv-e18</i> (occlusion-derived virus envelope protein E18)	12224→12487 (264)	15 (-)	264 (100)	134 (+)	207 (68.96)	14 (-)	264 (98)	127 (+)	306 (63)	126 (+)	306 (63)	143 (+)	189 (90)	140 (+)	258 (86)	15 (+)	273 (72)
16 (-)	<i>p49</i>	12484→13932 (1449)	16 (-)	1449 (100)	133 (+)	1449 (100)	15 (-)	1407 (99)	126 (+)	1431 (71)	125	1431 (71)	142 (+)	1434 (71)	139 (+)	1455 (87)	14 (+)	1443 (68)
20 (+)	<i>p74</i>	18373→20295 (1923)	20 (+)	1923 (99)	129 (-)	1923 (99)	20 (+)	1923 (99)	123 (-)	1938 (74)	120 (-)	1938 (78)	138 (-)	1938 (78)	134 (-)	1935 (88)	10 (-)	1950 (75)
25 (-)	<i>alkaline exonuclease</i>	23686→25083 (1398)	25 (-)	1398 (99)	124 (+)	1398 (100)	25	1398 (99)	117	1263 (53)	116 (+)	1263 (53)	133 (+)	1260 (53)	131 (+)	1275 (78)	7 (+)	1347 (45)
38 (-)	<i>per os infection factor-1 (pif-1)</i>	35371→36966 (1596)	38 (-)	1596 (100)	111 (+)	1596 (100)	36 (-)	1596 (98)	103 (+)	1584 (72)	102 (+)	1584 (71)	119 (+)	1593 (76)	119 (+)	1590 (81)	41 (+)	1602 (70)
42 (+)	<i>per os infection factor-3 (pif-3)</i>	38578→39193 (618)	42 (+)	618 (99)	107 (-)	618 (100)	39 (+)	795 (99)	100 (-)	615 (65)	99 (-)	615 (65)	115 (-)	615 (63)	115 (-)	617 (75)	40 (-)	654 (57)
48 (+)	<i>odv-ec43</i>	41551→42726 (1176)	47 (+)	1176 (99)	101 (-)	1176 (99)	44 (+)	1176 (99)	96 (-)	1176 (65)	95 (-)	1176 (65)	109 (-)	1173 (66)	109 (-)	1173 (83)	33 (-)	1146 (63)
55 (+)	<i>p48</i>	50528→51691 (1164)	53 (+)	1164 (99)	95 (-)	1164 (99)	50 (+)	1164 (99)	91 (-)	1164 (67)	90 (-)	1164 (67)	103 (-)	1164 (68)	104 (-)	1235 (69)	44 (+)	1173 (67)
57 (+)	<i>p40</i>	52022→53089 (1068)	55 (+)	1068 (100)	93 (-)	1065 (99)	52 (+)	726 (99)	89 (-)	1089 (63)	88 (-)	1089 (63)	101 (-)	1086 (64)	102 (-)	1065 (79)	46 (+)	1140 (52)
58 (+)	<i>p6.9</i> (basic DNA binding protein)	53127→53366 (240)	56 (+)	240 (100)	92 (-)	240 (100)	54 (+)	240 (99)	88 (-)	198 (58)	87 (-)	198 (70)	100 (-)	164 (77)	101 (-)	156 (87)	47 (+)	-
59 (-)	<i>lef-5</i> (late expression factor 5)	53363→54142 (780)	57 (-)	780 (100)	91 (+)	780 (100)	55 (-)	780 (99)	87 (+)	789 (60)	86 (+)	798 (60)	99 (+)	798 (61)	100 (+)	792 (73)	48 (-)	810 (53)
60 (+)	<i>3aK</i>	54089→55030 (942)	58 (+)	942 (100)	90 (-)	942 (100)	56 (+)	942 (99)	86 (-)	963 (57)	85 (-)	963 (58)	98 (-)	963 (57)	99 (-)	942 (84)	49 (+)	957 (55)
62 (-)	<i>hypothetical protein "pif-4/19k/odv-e28"</i>	56114→56632 (519)	60 (-)	519 (100)	88 (+)	519 (100)	58 (-)	519 (100)	83 (+)	549 (71)	83 (+)	549 (72)	96 (+)	522 (71)	97 (+)	519 (87)	50 (-)	510 (96)

Genetic characterization of tiger band disease causing virus of oak tasar silkworm

63 (+)	<i>helicase</i>	56622→60260 (3639)	61 (+)	3639 (99)	87 (-)	3639 (99)	59 (+)	3639 (99)	82 (-)	3669 (59)	82 (-)	3669 (59)	95 (-)	3666 (59)	96 (-)	3672 (79)	51 (+)	3717 (50)
64 (-)	<i>adv-e25</i> (occlusion-derived virus envelope protein E25)	60300→60986 (687)	62 (-)	687 (100)	86 (+)	687 (100)	60 (-)	687 (100)	81 (+)	687 (61)	81 (+)	687 (61)	94 (+)	687 (64)	95 (+)	690 (81)	52 (-)	687 (56)
65 (-)	<i>p18</i>	60991→61467 (477)	63 (-)	477 (100)	85 (+)	477 (100)	61 (-)	477 (99)	80 (+)	486 (72)	80 (+)	486 (72)	93 (+)	486 (71)	94 (+)	480 (89)	53 (-)	492 (66)
66 (+)	<i>p33</i>	61466→62227 (762)	64 (+)	762 (100)	84 (-)	762 (100)	62 (+)	762 (99)	79 (-)	780 (77)	79 (-)	780 (77)	92 (-)	780 (78)	93 (-)	849 (71)	54 (+)	762 (74)
68 (-)	<i>lef-4</i> (late expression factor 4)	62788→64164 (1377)	66 (-)	1377 (100)	82 (+)	1377 (100)	64 (-)	1377 (99)	77 (+)	1398 (51)	77 (+)	1398 (51)	90 (+)	1395 (53)	91 (+)	1374 (74)	56 (-)	1467 (49)
69 (+)	<i>vp39</i> (major viral capsid protein)	64177→65190 (1014)	67 (+)	1014 (100)	81 (-)	1014 (100)	65 (+)	1008 (95)	76 (-)	1053 (61)	76 (-)	1044 (60)	89 (-)	1044 (61)	90 (-)	1056 (83)	57 (+)	1053 (66)
72 (-)	<i>vp91</i> (viral capsid associated protein)	66893→68460 (2568)	70 (-)	2568 (99)	79 (+)	2568 (99)	68 (-)	2592 (98)	73 (+)	2520 (61)	72 (+)	2514 (61)	83 (+)	2544 (61)	86 (+)	2460 (76)	62 (-)	2508 (54)
74 (+)	<i>hypothetical protein</i>	69676→70320 (645)	72 (+)	645 (100)	77 (-)	645 (100)	69 (+)	645 (100)	71 (-)	705 (71)	70 (-)	705 (71)	81 (-)	702 (72)	84 (-)	657 (87)	64 (+)	723 (69)
75 (+)	<i>gp41</i> (occlusion-derived virus envelope glycoprotein)	70313→71401 (1086)	73 (+)	1086 (100)	76 (-)	1089 (100)	70 (+)	1089 (100)	70 (-)	1212 (60)	69 (-)	1212 (60)	80 (-)	1230 (62)	83 (-)	1104 (78)	65 (+)	1326 (65)
77 (+)	<i>Hypothetical protein</i>	71700→72011 (312)	75 (+)	312 (100)	74 (-)	312 (100)	72 (+)	312 (100)	68 (-)	333 (52)	67 (-)	333 (52)	78 (-)	330 (56)	81 (-)	318 (69)	66 (+)	357 (51)
78 (+)	<i>vlf-1</i> (very-late factor 1)	72016→73128 (1113)	76 (+)	1113 (100)	73 (-)	1113 (100)	73 (+)	1113 (100)	67 (-)	1140 (81)	66 (-)	1140 (81)	77 (-)	1140 (81)	80 (-)	1124 (87)	67 (+)	1170 (78)
86 (-)	<i>adv-nc42/ ac68/pif-6</i>	76251→76661 (411)	84 (-)	411 (100)	64 (+)	411 (100)	79 (-)	411 (98)	59 (+)	405 (62)	58 (+)	405 (64)	68 (+)	405 (61)	73 (+)	396 (78)	73 (-)	402 (63)
88 (-)	<i>desmoplakin</i>	77769→80246 (2478)	86 (-)	2478 (100)	62 (+)	2490 (98)	81 (-)	2466 (94)	57 (+)	2418 (30)	56 (+)	2418 (30)	66 (+)	2427 (29)	71 (+)	2628 (37)	75 (-)	2364 (28)
89 (+)	<i>DNA polymerase</i>	80365→83126 (2871)	87 (+)	2871 (99)	61 (-)	2763 (99)	82 (+)	2871 (98)	56 (-)	2961 (61)	55 (-)	2970 (61)	65 (-)	2955 (61)	70 (-)	2958 (78)	76 (+)	3024 (56)
91 (-)	<i>lef-9</i> (late expression factor 9)	84308→85795 (1488)	89 (-)	1488 (100)	59 (+)	1479 (99)	84 (-)	1488 (99)	53 (+)	1473 (75)	52 (+)	1471 (75)	62 (+)	1551 (74)	65 (+)	1470 (83)	80 (-)	1479 (73)
98 (-)	<i>vp1054</i> (viral capsid associated protein)	88998→90128 (1131)	96 (-)	1131 (100)	53 (+)	1131 (100)	90 (-)	1131 (100)	46 (+)	1098 (52)	45 (+)	1098 (52)	54 (+)	1098 (52)	58 (+)	1137 (78)	88 (-)	1104 (53)

100 (-)	hypothetical protein	90191←90631 (441)	98 (-)	441 (100)	51 (+)	441 (100)	91 (-)	441 (99)	44 (+)	420 (60)	43 (+)	420 (60)	53 (+)	420 (58)	56 (+)	441 (77)	90 (-)	432 (58)
102 (+)	<i>lef-8</i> (late expression factor 8)	91878→94499 (2622)	100 (+)	2622 (99)	49 (-)	2625 (98)	94 (+)	2621 (99)	41 (-)	2634 (69)	40 (-)	2634 (69)	50 (-)	2631 (69)	54 (-)	2655 (82)	93 (+)	2664 (65)
111 (+)	<i>p47</i> (transcription regulator)	100662→101861 (1200)	109 (+)	1200 (100)	40 (-)	1200 (100)	101 (+)	1200 (99)	33 (-)	1200 (68)	32 (-)	1200 (68)	40 (-)	1206 (68)	45 (-)	1200 (81)	105 (+)	1209 (64)
131 (-)	<i>pif-2</i> (per os infection factor-2)	115123→116271 (1149)	129 (-)	1149 (100)	20 (+)	1149 (100)	120 (-)	1149 (99)	14 (+)	1149 (77)	14 (+)	1149 (77)	22 (+)	1149 (79)	20 (+)	1149 (87)	123 (-)	1146 (73)
137 (+)	<i>lef-1</i> (late expression factor 1)	120460→121191 (732)	136 (+)	732 (100)	13 (-)	732 (100)	127 (+)	732 (99)	6 (-)	813 (51)	6 (-)	813 (51)	14 (-)	801 (51)	13 (-)	732 (66)	130 (+)	705 (51)
146 (+)	<i>lef-2</i> (late expression factor)	125679→126419 (741)	144 (+)	741 (100)	4 (-)	609 (82.11)	137 (+)	741 (99)	143 (+)	633 (55)	141 (+)	633 (55)	6 (+)	633 (54)	6 (-)	615 (74)	134 (+)	663 (46)

ORFs (Anpr53, Anpr44 and Anpr145) have no homology with the ORFs of AnpeNPV-L2 while two ORFs (Anpr53 and Anpr96) were absent in AnpeNPV-Z.

A total of 9,313 bp comprising 7.33 % of the genome were distributed in 109 intergenic regions ranging from 1 to 454 base pair (Table 2). Intergenic regions together with other functionary elements, enhancers, and promoters, play key roles in the gene transcription (Xu *et al.*, 2010; Nakagawa *et al.*, 2014). Most of the intergenic regions correspond to those of the AnpeNPV-L2 and AnpeNPV-Z. The largest intergenic region of AnprNPV (454 bp) was found between *pe-38* (Anpr5) and Anpr6. In AnpeNPV-L2 and AnpeNPV-Z, the largest intergenic region was also associated with Anpe6 (Anpr6) and Anpe7 (Anpr6), respectively. Variation in the position and size of intergenic regions of closely related species was also observed in BomaNPV and BmNPV (Xu *et al.*, 2010).

Microsatellites are the most variable type of DNA sequences present within the viral genome. These sequences play various roles in diverse activities in the eukaryotic, prokaryotic and viral genomes (Atia *et al.*, 2016). Microsatellites/Simple sequence repeats (SSRs) motif analysis within the intergenic regions of AnprNPV

found 239 SSR motifs, comprising mono-, di-, tri-, tetra-, penta- and hexa-nucleotide repeats, corresponding to 1.88 motif/kb. In *Spodoptera littoralis* NPV (SpliMNPV-AN1956), only 55 different SSR motifs having mono-, di-, tri- and hexa-nucleotide repeats were identified within the entire genome, corresponding to 0.39 motif/kb. Higher abundance of SSR motifs ranging in size between 2.32 and 5.05 motif/kb was observed in an earlier summary of 30 alphabaculoviruses (Alam *et al.*, 2014). Variation in the relative abundance of SSR motifs in different viral genomes may be attributed to the variation in the genome sizes. In the AnprNPV intergenic regions, trinucleotide repeats occurred at the maximum frequency of 47.89 % followed by dinucleotide (19.32 %) and tetranucleotide (18.48 %). This distribution pattern of repeat motifs agreed with the SpliMNPV motif frequencies (Atia *et al.*, 2016) where trinucleotides are the most abundant repeat (80 %) followed by dinucleotides (13 %). Analysis of 30 different alphavirus genomes revealed that mononucleotide repeats were the most abundant motifs, followed by dinucleotides and trinucleotides (Alam *et al.*, 2014).

Gene descriptions

One hundred forty seven ORFs of the AnprNPV were

Table 2: Distribution of intergenic spaces within the AnprNPV genome

Sl. No.	Position	length (bp)	GC (%)	Microsatellite repeats	No. of micro-satellites
1	3523-3560	38	13	AATAAT	1
2	4470-4640	171	50	GCGCGC, TATATA, CGCGCGCG	3
3	4812-5265	454	37	TTTTTT, GTGTGT, CACACACACACACA, TATATA	4
4	5704-5819	116	29	GGTGGT, AATAAATA, TTATTA	3
5	6892-7181	290	41	AGTAAAGTAA, AGTAAAGTAA, TTTTTT, TTTTTT, TACTAC, TTATTA, ATTAATTA	7
6	8307-8383	77	16	AAAAAA, ATTTATTT	2
7	9829-10169	341	47	CGTCGT, CTCCTT, CAACAA, TCTTCT, GCGGCG, GGTGGT, GCGCGCGC	7
8	10740-10787	48	21	TTTATTTA, TTATTA	2
9	14665-14905	241	52	AACAAC, GCGCGC, TTATTTAT, CGGCGG, ACAACA, CGGCGG, TATATA, GCGCGCGC, GGCGGC	9
10	16340-16374	35	17	TATATA, TATGTATG	2
11	17385-17535	151	39	TGCTGC, GTGTGT	2
12	18280-18372	93	34	TTTTTT	1
13	20551-20592	42	14	ATAATAATA	1
14	21316-21382	67	51	GTGTGT, TATATA	2
15	22874-23029	156	42	AATAAAAATAAA, ACATACAT, GCGCGC	3
16	23660-23685	26	8	TAATAA	1
17	27446-27865	420	43	TTTATTTA, GTTAGGTTAG, CGCCGC, ATGATG, TGCCTGCGC, TATATA, GCGCGC, TATATA	8
18	29396-29458	63	10	TTTTTTTT, TATATATA, AAAATAAAAT, TGTTGT	4
19	30434-30474	41	12	ATAAATAA	1
20	32134-32284	151	40	AAAAAA, CATTCATT	2
21	32918-32950	33	64	CGCGCG	1
22	33644-33728	85	11	TATATA, AATAAT, TTTATTTA, TTAAATTTAAT, TAATAA	5
23	34783-34954	172	36	TTATTA, AACAAC, TTATTA, GCCGGCCG	4
24	35207-35238	32	59	-	-
25	36967-37007	41	24	ATAAATAA, AACAAC	2
26	37989-38017	29	66	GGCGGC	1
27	40455-40559	105	48	CGTCGT, CAGCAG	2
28	41530-41550	21	57	CGCCGC	1
29	44063-44150	88	30	AATAAT, ATTTATTT,	3
30	46194-46387	194	43	TTATTA	4
31	48276-48356	81	10	CGTTCGTT, TATATA, GCGCGC, TGCTGC	3

32	56065-56113	49	49	TTAATTAA, GCGGGCGG	2
33	60261-60299	39	18	AATAAT	1
34	62228-62240	13	15	TTTATTTA	3
35	65925-66326	402	37	ACACAC, TTATTA, TTGTTG, AATAAT, AAATAAAT, TTTATTTA, CGCCGC, CCAACCAA, AAAAAA, ATTTATTT, TAATAA, TAATAA	4
36	66531-66892	362	33	TAATAA, GCAGCA, TTTTIT, TTTATTTA, AAATAAAT, ATGATG, AATAAT, AATAAAAAATAAA, GTGTGT	3
37	80247-80364	118	47	GAGGAG, CGCGCCGCGC, TACTAC	7
38	83127-83185	59	25	AAAAAA, TTATTA	2
39	84281-84307	27	19	AATAAT	7
40	85796-85843	48	50	CGTCCGTC, CTACTA, TTGTTG	2
41	86474-86660	187	36	TAAATAAA, ATTAATTA	9
42	87885-88256	372	31	TATATA, CAAACAAA, CAACAA, TAAATAAA, GTAGTA, ATAATA, TCATCA, ATTATT, AAAGTAAAGT, AAATATAAATAT	2
43	88491-88726	236	36	TATATA, ATTATT, ACAACAAA, TTAATTAA, AAAAAAAA, TTGCTTGC, ACGACG, GCGCGC	2
44	88937-88997	61	49	ATAATA, ACAACA	1
45	90632-90894	263	42	AGCAGC, TAATAA, ACAACA, GCGCGC, CGAACGAA, TTATTA, ATAATA, TTCTTC, TATTAT, GCGGCG	1
46	91855-91877	23	78	GCGCGC	2
47	94500-94534	35	57	GCGCGC	3
48	95610-95637	28	82	GCGGCCGCGC, GCGCGC	1
49	95908-95997	90	24	ATTATT, TAAATAAA, TTATTA, AGTCAGTC, TTGTTG	8
50	98038-98111	74	23	ACGACG, TTATTA	4
51	103292-103343	52	50	GCTGCT, GCGCGC, TATATATA	1
52	107657-107665	9	56	TGCTGC	2
53	108371-108478	108	47	TGTGTG, ATATAT	1
54	109438-109522	85	15	ATTATT, ACAACA	5
55	110081-110255	175	57	GCGCGC, GCGCGC, GCGGCG	4
56	111096-111178	83	17	AATAAT, TATATA, AAAAAA, TAAAAATAAAAA, TTATTA	-
57	112838-112971	134	48	TTGTTG, GCGCGC, ACTACT, AATAAT, TTTATTTA	2
58	114937-115122	186	44	AACAAC, TATATA, GCTGCT, ACAACA	1
59	116272-116291	20	40	TAGTAG	2
60	117267-117478	212	20	ATTATT, ATTATT, TTATTA, TATTAT, TGTTTGTI, TTGTTTGT, TAATTAAT, TAATAA, AAATAAAAAATAA	1
61	118854-118889	36	19	TTATTA	3
62	120073-120459	387	43	AATAAT, CGGCGG, AAAAAA, CGCCGC, CGCCGC, GGTGGT, CCACCA, AGAAGA, TTTTTT	4
63	122073-122129	57	32	TATATA	3
64	122391-122472	82	21	AATTAATT, ACAACA	2

65	123484-123603	120	49	TGCTGC, GCGCGC, TATATA	3
66	124280-124428	149	25	AAATAAAT, TAATAA, ATTATT, TTAATA, TATTTTATTT, GTGTGT, TTTTTT	7
67	124912-125090	179	42	AATAAT, TTTATTTTAT, CAACAA, AATAAATA, TTTTTT, GGCGGC, CAACAA	7
68	125541-125584	44	20	ATATAT	1
69	126830-126930	101	22	AAAAAA, TTTATTTA, ATAATA, GTAGTA	4

broadly divided into seven categories based upon the conserved and variable genes within the alpha-, beta-, gamma- and delta baculoviruses groups (Table 3). AnprNPV contained 37 baculovirus core genes, 25 genes conserved in all lepidopteran baculoviruses, 11 genes present in all lepidopteran NPVs, 41 genes present in all Group I NPVs, 27 genes present in some Group I NPVs, 5 genes specific to Saturniidae silkworm NPVs and 1 gene unique to AnprNPV. A similar type of gene distribution was noted in AnpeNPV-L2 (Fan *et al.*, 2007), AnpeNPV-Z (Nie *et al.*, 2007), PhcyNVP (Qian *et al.*, 2013), BmNPV (Xu *et al.*, 2010) and AcMNPV (Ayres *et al.*, 1994). Based on the host species and orders (Lepidoptera, Diptera or Hymenoptera), the distribution and number of encoded ORFs vary among baculoviruses, but the core genes are conserved among all baculoviruses (Miele *et al.*, 2011).

DNA replication genes

AnprNPV shares 17 genes responsible for DNA replication with other baculoviruses (Table 4). *Helicase* (Anpr63), *dnapol* (Anpr89), *lef-1* (Anpr136) and *lef-2* (Anpr146) are essential, conserved core genes that are required for transient DNA replication, ATPase and proofreading activities. Deletion of, or insertion of mutations in, *Helicase* and *dnapol* destroys replication capacity, whereas, a mutant *lef-2* results in an inability to produce infectious virus particles in AcMNPV (McDougal and Guarino, 2000; Haas-Stapleton *et al.*, 2005) and BmNPV (Gomi *et al.*, 1997). The conserved *ie-1* (Anpr122) gene is a transcription factor responsible for DNA replication (Olson *et al.*, 2003). The *pcna* (Anpr103) gene is non-essential though it is required for DNA synthesis, cell cycle progression and DNA repair. Its deletion does not affect DNA replication of AcMNPV

(Iwahori *et al.*, 2002), and it is absent in PhcyNPV, BmNPV, BomaNPV and LoobNPV. The *pe38* (Anpr5) gene is present in both group I NPV and GV genomes and is involved in DNA replication and as a transcriptional trans activator for early promoters (Kool *et al.*, 1994; Milks *et al.*, 2003).

Transcription genes

Fourteen main genes are involved in the transcription of AnprNPV (Table 4). Six ORFs of AnprNPV are identified as core transcription genes, namely Anpr59, Anpr68, Anpr78, Anpr91, Anpr102 and Anpr111. In the AnpeNPV-Z genome, 12 transcription genes were described (Nie *et al.*, 2007). Anpr85 has 60 % similarity with the AcMNPV (*ac69*) *met* gene (Rohmann, 2013) which was confirmed to stimulate late gene transcription in a transient assay (Li *et al.*, 1999). The Anpr112 ORF is similar to the PKIP gene of AcMNPV, which is involved in very late transcription (McLachlin *et al.*, 1998).

Structural protein genes

A total of 47 AnprNPV ORFs were identified as structural proteins involved in packaging, assembly, released of the virion particles and in the envelope (Table 4). Nineteen of the structural core genes were identified as: *alk-exo* (Anpr25), *odv-ec43* (Anpr48), *p48/p45* (Anpr55), *p40* (Anpr57), *p6.9* (Anpr58), *38k* (Anpr60), *odv-e25* (Anpr64), *p18* (Anpr65), *p33* (Anpr66), *vp39* (Anpr69), *vp91/p95* (Anpr72), *gp41* (Anpr75), *ac78* (Anpr77), *odv-nc42* (Anpr86), *desmoplakin* (Anpr88), *vp1054* (Anpr98), *odv-e56* (Anpr10), *odv-e18* (Anpr15) and *p49* (Anpr16). The *desmoplakin* and *odv-e18* genes were previously categorized as unknown genes of conserved lepidopteran

Table 3: Gene classification of *A. proylei* nucleopolyhedrovirus (AnprNPV)

Group	Sub-group	Gene name	Total number of genes
Core genes of baculovirus genomes		Anpr20 (<i>p74</i>), Anpr25 (<i>alk-exo</i>), Anpr38 (<i>pif-1</i>), Anpr42 (<i>pif-3</i>), Anpr48 (<i>odv-ec43</i>), Anpr55 (<i>p48</i>), Anpr57 (<i>p40</i>), Anpr58 (<i>p6.9</i>), Anpr59 (<i>lef-5</i>), Anpr60 (<i>38k</i>), Anpr62 (<i>pif-4/19k/odv-e28</i>), Anpr63 (<i>helicase</i>), Anpr64 (<i>odv-e25</i>), Anpr65 (<i>p18</i>), Anpr66 (<i>p33</i>), Anpr68 (<i>lef-4</i>), Anpr69 (<i>vp39</i>), Anpr72 (<i>vp91</i>), Anpr74 (<i>ac81</i>), Anpr75 (<i>gp41</i>), Anpr77/Ac78 (hypothetical protein), Anpr78 (<i>vlf-1</i>), Anpr86 (<i>ac68</i>), Anpr88 (<i>desmoplakin</i>), Anpr89 (<i>DNA polymerase</i>), Anpr91 (<i>lef-9</i>), Anpr98 (<i>vp1054</i>), Anpr100/Ac53 (hypothetical protein), Anpr102 (<i>lef-8</i>), Anpr111 (<i>p47</i>), Anpr131 (<i>pif-2</i>), Anpr137 (<i>lef-1</i>), Anpr146 (<i>lef-2</i>), Anpr10 (<i>odv-e56</i>), Anpr14 (<i>odv-e27</i>), Anpr15 (<i>odv-e18</i>), Anpr16 (<i>p49</i>).	37
Genes conserved in all lepidopteran baculoviruses		Anpr46 (<i>ac110</i>), Anpr50 (<i>ac107</i>), Anpr56 (<i>p12</i>), Anpr73 (<i>telokin</i>), Anpr79 (hypothetical protein), Anpr80 (hypothetical protein), Anpr87 (<i>lef-3</i>), Anpr92 (<i>fp25k</i>), Anpr106 (<i>odv-e66</i>), Anpr113 (<i>ssdbp</i>), Anpr116 (<i>lef-6</i>), Anpr117 (<i>ac29</i>), Anpr124 (<i>fgf</i>), Anpr126 (<i>ubiquitin</i>), Anpr127 (<i>p31</i>), Anpr128 (<i>lef-11</i>), Anpr129 (hypothetical protein), Anpr130 (<i>Fl envelope protein</i>), Anpr138 (<i>38.7k</i>), Anpr1 (<i>polyhed</i>), Anpr3 (<i>pk-1</i>), Anpr11 (<i>ie-1</i>), Anpr13 (<i>chtB 1</i>), Anpr12 (<i>ac146</i>), Anpr18 (<i>me53</i>).	25
Genes present in all lepidopteran NPVs	Exclusive to lepidopteran NPVs	Anpr27 (<i>calyx</i>), Anpr49 (<i>ac108</i>), Anpr54 (<i>p87</i>), Anpr94 (<i>ChaB</i>), Anpr97 (hypothetical protein), Anpr125 (<i>ac34</i>).	6
	Exist in one or more GV	Anpr29 (<i>p24</i>), Anpr31 (<i>cath</i>), Anpr93 (<i>fp</i>), Anpr91 (<i>lef-10</i>), Anpr17 (<i>ie-0</i>).	5
Genes present in all Group I NPVs	Exclusive to all Group I NPVs	Anpr26 (<i>ac132</i>), Anpr30 (<i>gp64</i>), Anpr35 (<i>ac124</i>), Anpr43 (<i>ac114</i>), Anpr82 (<i>ac73</i>), Anpr83 (<i>ac72</i>), Anpr109 (<i>gta</i>), Anpr118 (<i>ac30</i>), Anpr136 (<i>odv-e26</i>), Anpr141 (<i>ptp-1</i>), Anpr145 (<i>ac5</i>), Anpr8 (<i>ie-2</i>).	12
	Exist in one or more Group II NPVs (or GV)	Anpr21 (<i>p10</i>), Anpr22 (<i>p26</i>), Anpr28 (<i>gp16</i>), Anpr32 (<i>chitinase</i>), Anpr33 (<i>lef-7</i>), Anpr37 (<i>ac120</i>), Anpr40 (<i>ac117</i>), Anpr45 (<i>ac111</i>), Anpr67 (<i>ac91</i>), Anpr70 (<i>cg30</i>), Anpr76 (<i>ac79</i>), Anpr81 (<i>ac74</i>), Anpr84 (<i>iap-2</i>), Anpr90 (<i>gp37</i>), Anpr95 (<i>ac57</i>), Anpr101 (<i>bjdp</i>), Anpr108 (<i>ac43</i>), Anpr110 (<i>lef-12</i>), Anpr112 (<i>pkip</i>), Anpr114 (<i>ac26</i>), Anpr115 (<i>iap-1</i>), Anpr132 (<i>arif-1</i>), Anpr133 (<i>ac19</i>), Anpr134 (<i>ac18</i>), Anpr135 (<i>ac17</i>), Anpr140 (<i>ac11</i>), Anpr144 (<i>ac4</i>), Anpr2 (<i>1629-capsid</i>), Anpr5 (<i>pe38</i>).	29
Genes present in some Group I NPVs		Anpr19 (<i>op135</i>), Anpr23 (<i>94k</i>), Anpr24 (<i>p22.2</i>), Anpr34 (<i>v-trex</i>), Anpr36 (<i>ac122</i>), Anpr39 (<i>op118</i>), Anpr41 (<i>bro-a</i>), Anpr44 (<i>hycu43</i>), Anpr47 (<i>op110</i>), Anpr51 (<i>pnk/pnl</i>), Anpr52 (<i>he65</i>), Anpr61 (<i>bro-b</i>), Anpr85 (<i>met</i>), Anpr103 (<i>pcna</i>), Anpr104 (<i>eim</i>), Anpr105 (<i>ets</i>), Anpr107 (<i>ac44</i>), Anpr119 (<i>op37</i>), Anpr121 (<i>op36</i>), Anpr122 (<i>sod</i>), Anpr123 (<i>ctl-2</i>), Anpr139 (<i>cf11</i>), Anpr142 (<i>ctl-1</i>), Anpr143 (<i>ptp-2</i>), Anpr147 (<i>cf2</i>), Anpr4 (<i>cfdef147</i>), Anpr6 (<i>cfdef142</i>).	27
Genes specific to one or more saturniid silkmoths		Anpr71 (<i>phcy067</i>), Anpr96 (<i>Anpe-L₉₄</i>), Anpr120 (<i>phcy110</i>), Anpr7 (<i>phcy07</i>), Anpr9 (<i>Anpe-L₉</i>).	5
Unique to AnprNPV		Anpr53 (<i>he65-like</i>)	1
Total			147

baculoviruses (Nie *et al.*, 2007), but more recently they have been identified as core proteins (Miele *et al.*, 2011). *p48/p45* (Anpr55), *p40* (Anpr57), *p12* (Anpr56) and *p18*

(Anpr65) are capsid-associated proteins of both BV and ODV of OpMNPV, AcMNPV and other baculovirus genomes (Muller *et al.*, 1990; Braunagel *et al.*, 2003). The

Table 4: Categorization of conserved and non-conserved genes of AnprNPV with their functions

Category of gene functions	Conserved baculoviruses genes	Lepidopteran conserved baculoviruses genes	Genes present in other baculoviruses groups
Replication (17)	<i>helicase</i> (Anpr63), <i>dnapol</i> (Anpr89), <i>lef-1</i> (Anpr137), <i>lef-2</i> (Anpr146).	<i>lef-3</i> (Anpr87), <i>ssdbp</i> (Anpr113), <i>ie-1</i> (Anpr11) ^b , <i>me53</i> (Anpr18).	<i>lef-7</i> (Anpr15), <i>bro-a</i> (Anpr41), <i>bro-b</i> (Anpr61), <i>ChaBfp</i> (Anpr93), <i>ChaB</i> (Anpr94), <i>pcna</i> (Anpr103) ^d , <i>ac43</i> (Anpr108), <i>pe38</i> (Anpr5) ^b , <i>ie-2</i> (Anpr8).
Transcription (14)	<i>lef-5</i> (Anpr59), <i>lef-4</i> (Anpr68), <i>vlf-1</i> (Anpr78), <i>lef-9</i> (Anpr91), <i>lef-8</i> (Anpr102), <i>p47</i> (Anpr111).	<i>lef-6</i> (Anpr116), <i>pp31/39k</i> (Anpr127), <i>lef-11</i> (Anpr128).	<i>met</i> (Anpr85), <i>lef-10</i> (Anpr99), <i>lef-12</i> (Anpr110), <i>pkip-1</i> (Anpr112) ^c , <i>ie-0</i> (Anpr17).
Structural proteins (packaging, assembly and release) (47)	<i>alk-exo</i> (Anpr25), <i>odv-ec43</i> (Anpr48), <i>p48/p45</i> (Anpr55), <i>p40</i> (Anpr57), <i>p6.9</i> (Anpr58), <i>38k</i> (Anpr60), <i>odv-25</i> (Anpr64), <i>p18</i> (Anpr65), <i>p33</i> (Anpr66), <i>vp39</i> (Anpr69), <i>vp91/p95</i> (Anpr72), <i>ac81</i> (Anpr74) ^b , <i>gp41</i> (Anpr75), <i>ac78</i> (Anpr77), <i>odv-nc42/pif-6/Ac68</i> (Anpr86), <i>DSP</i> (Anpr88), <i>vp1054</i> (Anpr98), <i>odv-e56</i> (Anpr10), <i>odv-e18</i> (Anpr15), <i>odv-e27</i> (Anpr14) ^b , <i>p49</i> (Anpr16).	<i>p12</i> (Anpr56) ^a , <i>telokin-like protein</i> (Anpr73) ^f , <i>ac76</i> (Anpr79), <i>ac75</i> (Anpr80), <i>odv-e66</i> (Anpr106), <i>fp25k</i> (Anpr92), <i>polh</i> (Anpr1), <i>Nudix</i> (Anpr129), <i>Fprotein</i> (Anpr130), <i>pk1</i> (Anpr3) ^c , <i>ac146</i> (Anpr12).	<i>p10</i> (Anpr21) ^e , <i>ac132</i> (Anpr26), <i>calyx</i> (Anpr27), <i>p24</i> (Anpr29), <i>gp64</i> (Anpr30), <i>ac124</i> (Anpr35), <i>ac114</i> (Anpr43), <i>p87</i> (Anpr54), <i>cg30</i> (Anpr70), <i>ac74</i> (Anpr81) ^f , <i>ac73</i> (Anpr82), <i>gp37</i> (Anpr90) ^a , <i>ac17</i> (Anpr135) ^f , <i>odv-e26</i> (Anpr136), <i>1629-capsid</i> (Anpr2).
Auxiliary (12)	<i>ac53</i> (Anpr100) ^c	<i>fgf</i> (Anpr124), <i>ubiquitin</i> (Anpr126) ^c .	<i>gp16</i> (Anpr28), <i>cathepsin</i> (Anpr31), <i>chitinase</i> (Anpr32), <i>sod</i> (Anpr122), <i>ctl-2</i> (Anpr123), <i>arjif-1</i> (Anpr132), <i>ptp-1</i> (Anpr141) ^c , <i>ctl-1</i> (Anpr142), <i>ptp-2</i> (Anpr143) ^c .
Per os infectivity factors & other infection genes (14)	<i>p74</i> (Anpr20) ^a , <i>pif-1</i> (Anpr38) ^a , <i>pif-3</i> (Anpr42), <i>pif-4/19k/odv-e28</i> (Anpr62), <i>pif-2</i> (Anpr131) ^a .		<i>chtB2</i> (Anpr47), <i>ac108</i> (Anpr49) ^a , <i>ac107/106</i> (Anpr50), <i>bjdp</i> (Anpr101) ^a , <i>gta</i> (Anpr109) ^c , <i>ac26</i> (Anpr114), <i>ac34</i> (Anpr125) ^a , <i>ac5</i> (Anpr145), <i>chtB1/p11</i> (Anpr13) ^a .
Apoptosis inhibiting proteins (3)			<i>iap-2</i> (Anpr84), <i>ac44</i> (Anpr107), <i>iap-1</i> (Anpr115).
DNA/RNA repair (3)			<i>exo III v-trex</i> (Anpr34), <i>pnk/pnl</i> (Anpr51) endonuclease (Anpr76).
Other accessory functions (22)		<i>ac110</i> (Anpr46), <i>ac29</i> (Anpr117), <i>38.7k</i> (Anpr138).	<i>p26</i> (Anpr22), <i>94k</i> (Anpr23), <i>p22.2</i> (Anpr24), <i>ac122</i> (Anpr36), <i>ac120</i> (Anpr37), <i>ac117</i> (Anpr40), <i>ac111</i> (Anpr45), <i>he65</i> (Anpr52), <i>ac91</i> (Anpr67), <i>ac72</i> (Anpr83), <i>ac57</i> (Anpr95), <i>ac55</i> (Anpr97), <i>elm</i> (Anpr104), <i>ets</i> (Anpr105), <i>ac30</i> (Anpr118), <i>ac19</i> (Anpr133), <i>ac18</i> (Anpr134), <i>ac11</i> (Anpr140), <i>ac4</i> (Anpr144).

Unknown (15)		<i>he65-like</i> (Anpr53), <i>phcy110</i> (Anpr120), <i>phcy7</i> (Anpr7), <i>phcy140</i> (Anpr9), <i>Phcy67</i> (Anpr71), <i>Anpe94</i> (Anpr96).	<i>op135</i> (Anpr19), <i>op118</i> (Anpr39), <i>hycu43</i> (Anpr44), <i>op37</i> (Anpr119), <i>op36</i> (Anpr121), <i>cdef19</i> (Anpr139), <i>op5</i> (Anpr147), <i>cdef147</i> (Anpr4), <i>cfdef142</i> (Anpr6).
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Underlined text represents non-essential genes.

^a*Per os* infectivity factors with structural protein function

^bGene for DNA-replication and transcription

^cGene for transcription, DNA repair, *per os* infectivity and auxiliary function

^dGene for DNA-replication, DNA repair and auxiliary function

^eGene with structural- and auxiliary function

^fGene for structural function and replication

^gGene for cell cycle arrest and structural function

^hGene for replication, transcriptional and structural function

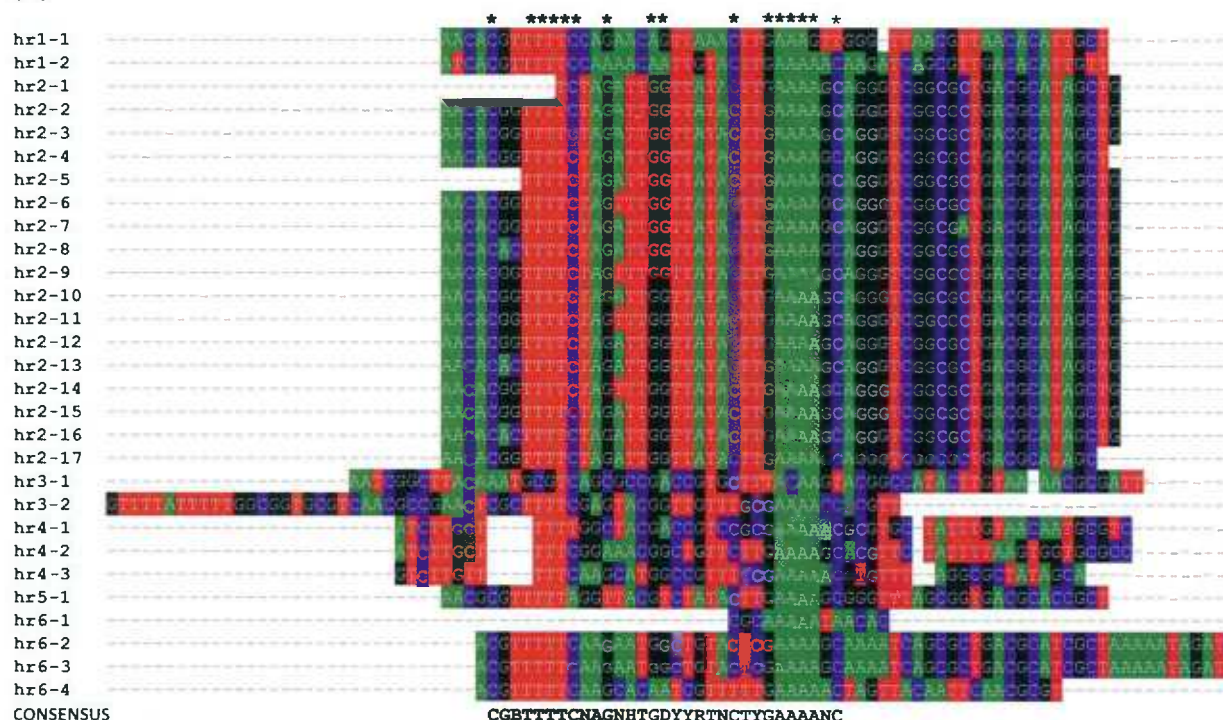
Table 5: Predicted AnprNPV ORFs with signal peptide

Sl. No.	AnprNPV ORF	Similarity score (%)	Signal peptide, putative cleavage sites (After AA)	Reference name
1	AnprNPV-6	46	54	UL121_HCMVA
2	AnprNPV-19	35	13	GC_PSHV1
3	AnprNPV-30	63	17	FUS_NPVOP
4	AnprNPV-31	100	16	CATV_NPVAP
5	AnprNPV-32	71	16	CHIT_NPVOP
6	AnprNPV-36	31	14	UDPE_GVLO
7	AnprNPV-38	65	20	Y119_NPVOP
8	AnprNPV-71	55	59	Y085_NPVOP
9	AnprNPV-72	88	53	VP91_NPVOP
10	AnprNPV-90	66	19	SPLR_NPVAC
11	AnprNPV-123	73	30	CXOL2_NPVOP
12	AnprNPV-124	52	17	FGFH_NPVO
13	AnprNPV-130	53	16	Y023_NPVOP
14	AnprNPV-142	81	18	CXOL_NPVAC
15	AnprNPV-7 ^a	-	20	

^aNot in database

telokir-like protein (Anpr73) is associated with the envelope or tegument of budded virions (Gauthier *et al.*, 2012), but appears to be a non-essential gene, as its deletion in BmNPV did not affect virus infectious capacity, although BV production and DNA replication were delayed (Ono *et al.*, 2012). Anpr129 is homologous

(a)



(b)



Figure 2: Multiple alignment of six homologous regions of the *A. proylei* NPV genome depicting the presence of perfect or imperfect palindromes associated with the direct repeat. The perfect or imperfect palindrome of 30 bp present in hr regions was represented by the sequence CGBTTTTCNAGNHTGDYYRTNCTYGAAAANC and is associated with the direct repeat TCRGCGCTGA (a). Conserved consensus sequences observed in Alpha group 1 NPVs of AnpeNPV-Z, AnpeNPV-L2, BmNPV, AcMNPV and OpMNPV with slight changes in nucleotides (b).

with *ac38* and exhibits a conserved Nudix motif (Mildvan *et al.*, 2005) that associates with the envelopes of budded virions in *Helicoverpa armigera* NPV (Wang *et al.*, 2005). *p24* (*ac129*, Anpr29), *ac124* (Anpr35) and *ac114* (Anpr43) are non-essential structural genes, as deletion or mutation of these genes in other baculoviruses results in normal virions (Slavicek and Hayes-Plazolles, 2003; Ono *et al.*, 2012).

Auxiliary genes

Auxiliary genes are those genes that enable the virus to carry out some selective advantageous functions such as

to reduce the dependency of the virus on its host cells. The core gene, *ac53* (Anpr100) has a U-box/ring-like domain that is common to the E3 ubiquitin ligase family and is likely to be involved in nucleocapsid assembly (Cuartas *et al.*, 2015). Twelve genes were categorized as auxiliary genes in AnprNPV, of which three genes, *ac53* (Anpr100), *fgf* (Anpr124) and *ubiquitin* (Anpr126) are conserved among all lepidopteran baculoviruses. In AcMNPV, fibroblast growth factor (*fgf*) is a secreted protein that mediates cell motility in insects (Detvisitsakun *et al.*, 2005). Mutation in *ubiquitin*, *ac35* (Anpr126) reduced BV production 5-10 fold (Reilly and Guarino, 1996). The *gp16* (Anpr28) gene corresponds to the *Op128* gene,

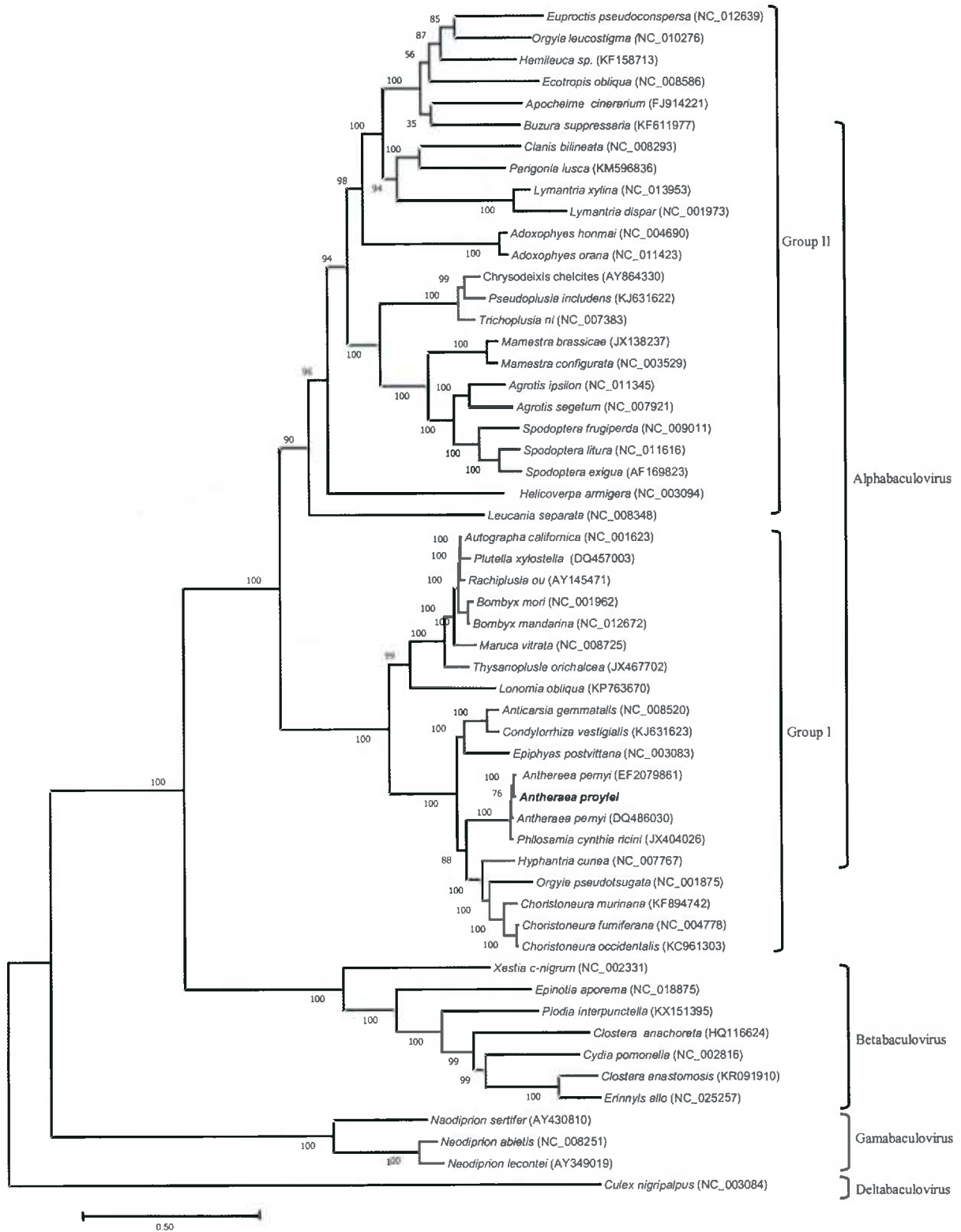


Figure 3: Evolutionary phylogenetic tree drawn from 55 baculoviruses based on the concatenated nucleotide sequence of 37 core genes. The phylogeny was inferred by the maximum likelihood (ML) method.

which is involved in envelopment of nucleocapsids in the cytoplasm, but has been defined as a non-essential gene in BmNPV (Ono *et al.*, 2012). A partial *egt* gene was encoded in the 5' end of AnprNPV, but its length is less than 50 amino acids, so it was not considered as a complete gene. In AnpeNPV, also a partial *egt* gene was encoded (Nie *et al.*, 2007), but it has been found intact in other baculoviruses such as OpMNPV, AcMNPV, BmNPV, BomaNPV and LoobNPV (Ayres *et al.*, 1994; Ahrens *et al.*, 1997; Xu *et al.*, 2010; Aragão-Silva *et al.*, 2016). This gene blocks moulting and pupation in infected larvae to produce higher virus yield during the prolonged larval period (Haas *et al.*, 1996), but it is a non-essential gene for virus production in either insects or cell culture (O'Reilly and Miller, 1990).

Per os infectivity factors (PIF) and other infection genes

Altogether, 14 PIF genes were detected in the AnprNPV genome-sequence that are responsible for infection and spread of the virus to *A. proylei* larvae. Conserved core genes of PIFs are *pif-74* (Anpr20), *pif-1* (Anpr38), *pif-3* (Anpr42), *pif-4/19k/odv-e28* (Anpr62) and *pif-2* (Anpr95). *chtB-2* (Anpr47) and *chtB 1/p11* (Anpr13) are chitin-binding proteins homologous to *Op110* (57 %) and *ac145* (72 %). Loss of these genes results in a 39-fold reduction in infectivity in *Heliothis armigera* during oral infection. *ac107/106* (Anpr50) and *bjdp* (Anpr101) are essential genes that when deleted in BmNPV affects viral spreading (Ono *et al.*, 2012). The global transactivator gene, *Gta* corresponds to Anpr109 and is involved in various functions, such as regulation of transcription, DNA recombination, repair and unwinding of chromatin as well as infection (Katsuma *et al.*, 2008).

Genes of other accessory functions

Twenty-two different genes are categorized as accessory function proteins. A large number of these genes are non-essential and deprivation of each individual gene in different baculoviruses showed little effect on virus infectivity aside from slight variations in lethal time

(Rohrmann, 2013). There were three apoptosis inhibiting proteins identified in the AnprNPV sequence, *iap-2* (Anpr84), *ac44* (Anpr107) and *iap-1* (Anpr115). In AcMNPV, *P35* gene is also involved in suppressing apoptosis, triggering the translation arrest (Thiem and Chejanovsky, 2004). The *pnk/pnl* (Anpr51) gene is involved in the DNA/RNA repair pathway, but is a non-essential one (Table 4). This gene is absent in other baculoviruses, such as BmNPV, OpMNPV and LoobNPV (Durantel *et al.*, 1998; Aragão-Silva *et al.*, 2016).

Unknown and specific genes of AnprNPV

Five ORFs of unassigned function that are specific to Saturniid silkworm NPVs were encoded in the AnprNPV genome sequence: Anpr71 (*Phcy067*), Anpr96 (*Anpe-L₂94*), Anpr120 (*Phcy110*), Anpr7 (*Phcy07*) and Anpr9 (*Anpe-L₂9*) (Fan *et al.*, 2007; Nie *et al.*, 2007; Qian *et al.*, 2013) (Tables 3, 4). Additionally, one *he65-like* gene (Anpr53) specific to the AnprNPV genome was also identified and assigned based upon the (~100 %) similarity of a 223 bp nucleotide sequence downstream of the *he65* (Anpr52) gene. Even though, the *he65* gene is involved in nuclear localization of G-actin (Ohkawa *et al.*, 2002), it is a non-essential gene in AcMNPV and BmNPV (Ono *et al.*, 2012) and absent in OpMNPV (Krejmer *et al.*, 2015). The presence of the partial encoded *he65-like* gene in AnprNPV might enhance the cellular mechanism of actin regulation for better infection of the host. Variable genes of unknown function that are present in other group I alphabaculoviruses (OpMNPV, HycuNPV and CfdefMNPV) are also observed in the AnprNPV sequence.

Signal-peptide detected AnprNPV ORFs

Signal peptides are essential for efficient protein secretion in the baculovirus-silkworm expression and are widely employed in recombinant secretory proteins for medical and industrial applications (Soejima *et al.*, 2013; Zhang *et al.*, 2015). In cultured lepidopteran cells, recombinant baculoviruses are employed for the expression of foreign gene products due to high levels of

foreign gene expression and the fact that these cells provide most of the protein processing pathways present in higher eukaryotes (Jarvis *et al.*, 1993). Out of the 147 ORFs of AnprNPV-TS, 15 ORFs were detected possessing potential signal peptides (Table 5). Among them, AnprNPV-31 showed 100 % similarity with the reference signal peptide, CATV_NPVAP, whereas, AnprNPV-7 exhibits an unreported signal peptide that does not share similarity with any signal peptide available in the database Uniprot 2014.05 (Viruses). The signal peptide bombyxin from *B. mori* integrated with the *UreB* gene was used to generate the Bacmid/BmNPV/(signal peptide)-*UreB* baculovirus expression system for usage as an edible vaccine against *Helicobacter pylori* infection in humans (Zhang *et al.*, 2015). Similarly, the signal peptide encoding ORFs of AnprNPV might also have significant applications in the future.

Homologous regions

Six homologous regions (hr1 to hr6) were observed with a total length of 1,803 bp, contributing 1.42 % to the AnprNPV genome (Figure 2 a). The presence of hr regions is a characteristic feature of most baculoviruses. Homologous regions are usually composed of a perfect or imperfect palindrome associated with a direct repeat (Fan *et al.*, 2007; Nie *et al.*, 2007). They contribute as origins of DNA replication and enhancers of RNA polymerase II facilitated transcription (Ahrens and Rohrmann, 1995; Kool *et al.*, 1995). In the AnprNPV sequence, there were 30 perfect or imperfect palindromes (CGBTTTTTCNAGNHTGDYYRTNCTYGAAAANC) ranging from 1-17 in each hr region, and these were usually accompanied by a direct repeat (TCRGCGCTGA). The core palindromic consensus is conserved in various Alpha group I NPVs; (AnpeNPV-Z) (Fan *et al.*, 2007), AnpeNPV-L2 (Nie *et al.*, 2007), PhcyNPV (Qian *et al.*, 2013), BmNPV (Xu *et al.*, 2010), AcMNPV (Ayres *et al.*, 1994) and OpMNPV (Ahrens *et al.*, 1997) with slight nucleotide changes (Figure 2 b). It was suggested that the hrs of group I alphabaculoviruses are descended from a common ancestor. Hrs of the AnprNPV and AnpeNPV-L2 showed 100 % similarity

except for the hr1 and hr5.

Some genes associated with hrs were conserved in group I NPVs. AnprNPV hr1 was flanked by genes *Op135* (Anpr19) and *me53* (Anpr18), similar to the hrs positioned in AnpeNPV-Zhr3, AnpeNPV-L2hr2 (Nie *et al.*, 2007), CfDEFNPVhr11 (Theilmann and Stewart, 1992), EppoNPVhr4 (de Jong *et al.*, 2005), HycuNPVhr5 (Hyink *et al.*, 2002) and OpMNPVhr5 (Ahrens *et al.*, 1997). Hrs associated with the *sod* gene include AnprNPVhr5, AnpeNPV-Zhr1, AnpeNPV-L2hr6, BmNPVhr2, AcMNPVhr2 (Ayres *et al.*, 1994) and RoMNPVhr2 (Ikeda *et al.*, 2006), while the *pif-1* gene is associated with AnprNPVhr3, AnpeNPV-Zhr2, AnpeNPV-L2hr3, HypuNPVhr4 and EppoNPVhr3. Hr sites are also considered an important spot for inter- or intra molecular homologous recombination as reflected by the high degree of genome variability around the hr regions among these viruses (Harrison and Bonning, 2003).

Phylogenetic analysis

A phylogenetic tree was drawn based on the nucleotides of 37 conserved core genes from 55 baculoviruses sequences belonging to alpha-, beta-, gamma- and delta groups (Figure 3). From the phylogenetic analysis, it was apparent that AnprNPV belongs to the Group I alphabaculovirus and is within the same clade as AnpeNPV and PhcyNPV indicating a close relationship among the baculoviruses infesting saturniid silkworm species. Additionally, the nucleotide identity between *A. pernyi* NPV and *A. proylei* NPV is 98 %, which suggests that AnprNPV is a local strain of AnpeNPV. There was also a close relationship among HycuNPV, OpMNPV, ChmuMNPV, CfMNPV and ChocMNPV and to a lesser extent with the AgMNPV, CoveMNPV and EppoNPV. Several studies suggest that the close relationship of HycuNPV, OpMNPV, ChmuMNPV and CfMNPV may be attributed to geographical overlapping of their hosts (Afonso *et al.*, 2001; Hyink *et al.*, 2002; Nie *et al.*, 2007). The alpha group viruses, AcMNPV, PlxyNPV, RoMNPV, MaviMNPV, ThorMNPV, LoobMNPV, BmNPV and BomaNPV showed a distant

relationship to AnprNPV. A similar observation was also recorded for the baculovirus of the saturniid lepidopteran species, *L. obliqua*, LoobMNPV (Aragão-Silva *et al.*, 2016).

Conclusion

This study identified a group I alphabaculovirus (AnprNPV) as the causal agent of the *A. proylei* tiger band disease. AnprNPV shows close similarity with the ORFs of both *A. pernyi* NPV isolates AnpeNPV-L2 and AnpeNPV-Z and is in the same clade in the phylogenetic tree. AnprNPV and AnpeNPV isolates had a nucleotide sequence identity above 95 %, which leads to the conclusion that AnprNPV is a variant of AnpeNPV. AnprNPV contains five genes that are only found in the saturniid silkworm alpha group I NPVs (AnprNPV, AnpeMNPV-L2, AnpeMNPV-Z and PhcyNPV). In addition to the *he65* gene, which is only present in AnpeMNPV, the unique gene *he65-like* is found in AnprNPV. During the course of infestation and adaptation to the new host (*A. proylei*), changes in the genome content, losses and acquisition of new genes might have occurred as a result of selection pressure for successful AnpeNPV infection. This additional information on saturniid baculovirus will be useful in diversity analysis and in developing baculovirus-resistance strains specific to saturniid silkworm species.

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Article scientifique

CARACTERISATION GENETIQUE D'UN BACULOVIRUS RESPONSIBLE DE LA MALADIE DE LA BANDE TIGRE CHEZ LE VER A SOIE TASAR DU CHENE, *ANTHERAEA PROYLEI* J (LEPIDOPTERE: SATURNIDE)

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RESUME

Dans la récente décennie une maladie redoutable connue sous le nom de bande du tigre qui affecte le ver à soie du chêne *Antheraea proylei* a entraîné des pertes sévères dans la production de soie. Le virus a été isolé et la séquence complète de son génome déterminée, révélant qu'un alphabaculovirus du groupe I, le virus de la polyèdrose nucléaire de *Antheraea proylei* (AnprNPV) est la cause de cette maladie. La taille du génome est de 126 930 pb avec 53,46 % de G+C. Il code 147 phases de lecture ouvertes (ORFs) représentant 37 gènes principaux, 25 gènes conservés de baculovirus de lépidoptères, 11 gènes de NPV de lépidoptères, 41 gènes présents dans tous les baculovirus du groupe I, 27 gènes présents chez certains baculovirus du groupe I, 5 gènes spécifiques des baculovirus des vers à soie saturnidés et 1 gène additionnel unique *he65-like* (Anpr 35). Il existe 109 régions intergéniques de 1 à 454 pb contribuant à 7,33 % du génome total. Dans ces régions, il existe 239 motifs microsatellites (SSRs) présentant des motifs répétés de 1 à 6 nucléotides distribués où l'abondance de trinuécléotides est maximale (47,89 %). Six régions homologues (HrI à Hr6) ont des palindromes parfaits ou imparfaits de 30 pb CGBTTTTTCNAGNHTGDYYRTNCTYGAAAANC associés avec une répétition directe TCRGCGCTGA. L'analyse phylogénétique montre que le AnprNPV appartient au même clade que les alphabaculovirus du groupe I des vers à soie saturnidés *A. pernyi* et *Philosamia cynthia ricini* ce qui indique une relation étroite avec ces virus. De plus, AnprNPV et AnpeNPV présentent une séquence nucléotidique identique à plus de 95 % ce qui suggère que AnprNPV est un variant régional de AnpeNPV.

Mots-clés: *Antheraea proylei*, virus de la polyèdrose nucléaire, ver à soie saturnide, génome viral



INVENTORY AND POPULATION ASSESSMENT OF NATIVE PREDATORS ATTACKING MEALYBUGS INFESTING MULBERRY IN WEST BENGAL

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ABSTRACT

The pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) and papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink have attained the status of major sucking pests of mulberry (*Morus alba* L.), the sole host plant of silkworm; *Bombyx mori* (L.), which provides the livelihood option for millions of sericulture farmers in India. The Indo-gangetic plains of West Bengal harbour rich fauna of coccinellid predators, which can be effectively utilized for the management of the major pests of mulberry. Information on native arthropod predators and their impact on mealybug complex are useful for implementing biological control. During 2014-15, a study was conducted to explore and characterize foliar-dwelling, arthropod native predators in four locations (Murshidabad, Nadia, Birbhum and Malda) in West Bengal. Surveillance studies revealed sixteen predator species, consistently associated with mealybugs infesting mulberry. They are *Brumoides suturalis* (F), *Coccinella septempunctata* L., *Jauravia pallidula* Mots, *Menochilus sexmaculatus* (F), *Nephus regularis* Sicard, *Nephus* sp. nr. *roepkei* de Fluiter., *Nephus bipunctatus* Kugelann, *Scymnus bourdilloni* Kapur, *Scymnus coccivora* Ayyar, *Scymnus nubilus* Mulsant, *Scymnus pallidicollis* Mulsant, *Scymnus pyrocheilus* Mulsant, *Spalgis epius* (Westwood), *Mallada desjardinsi* (Navas) and one anthocorid bug (unidentified). Apart from these arthropods, one new species of *Scymnus* Kugelann, *Scymnus* (*Pullus*) *latifolius* Poorani sp. nov, was collected and reported for the first time. The present study highlights the predator species composition, abundance and their seasonal phenology.

Key words: *Maconellicoccus hirsutus*, mulberry, native predators, *Paracoccus marginatus*.

INTRODUCTION

In eastern and north-eastern parts of India, the major pests of mulberry are thrips, mealybug, whitefly, root mealybug and leaf webber. The mealybug menace in mulberry poses a serious threat to sericulture industry by reducing the availability of quality leaves for silkworm rearing. The pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) manifests symptoms of curling and crinkling of mulberry leaves at the growing tips, thickening and twisting of the apical portion of the shoots, shortening of the inter nodal distance with bushy top appearance, bearing dark green leaves initially, which

turn pale yellow and fall off prematurely. These symptoms are collectively referred to as 'Tukra'. An avoidable leaf yield loss of 69.87 kg/ha/crop was incurred due to *M. hirsutus* infestation (Lalitha *et al.*, 2015 a). This leads to considerable economic loss by depriving the farmers of 100 DFL silkworm rearing.

Recently, the invasive exotic pest, papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink got entry into West Bengal infesting papaya (Seni and Sahoo, 2013) and mulberry (Lalitha *et al.*, 2015 b). Infestation of *P. marginatus* appears as clusters of cotton-like masses on the above-ground portion of mulberry

plants with terminal shoots becoming bunched and distorted. Immature and adult stages suck the sap of the plant and weaken it. The leaves become crinkled, yellowish and wither (Mahalingam *et al.*, 2010). The honey dew excreted by the bug and the associated black sooty mould formation impairs photosynthetic efficiency of the affected plants. The qualitative and quantitative yield of mulberry plants was affected due to the infestation of this pest. Further, feeding the silkworms with the mealy bug infested leaves proved detrimental as it affected the growth and development of the silkworms and also the quality parameters of the cocoon (Selvaraju and Sakthivel, 2011; Sakthivel *et al.*, 2012)

Some entomologists attempted to collect the natural enemies associated with the mealybug pests of mulberry. The species that were reported earlier include *Scymnus nubilus* (Santhakumar and Chakraborty, 1997), *S. (Pullus) bourdilloni*, *S. brunnescens* Mots., *S. fuscatus* Bohem, *S. apiciflavus* Mots., *S. pyrocheilus*, *S. ceylonicus* (Mots.), *S. quadrillus* (Mots.), *Pharoscygnus* sp., *Brumoides suturalis* (Fabricius), (Santhakumar *et al.*, 1995), *S. coccivora* (Santhakumar and Chakraborty, 1997), *Nephus* sp. *near roepkei*, *N. sp.* (Chakraborty *et al.*, 1999), *N. tagiapatus* (Kamiya), *Stethorus indira* Kapur and *S. tetranychii* Kapur (Poorani, 2002).

Biological control is regarded as the most effective long-term solution to mealybug infestation. The biocontrol agents are self-perpetuating and persist even when the mealybug is at low population densities. The present study envisages that the native generalist predator group can contribute additively to any effective bio-control model over the time scale. The objective of this study was to survey the native predators of mealybug pest of mulberry and ascertain their population in four districts of West Bengal. The species composition, abundance and their seasonal phenology are discussed.

MATERIALS AND METHODS

The population dynamics, phenology and species diversity of the arthropod predators of mealybugs

infesting mulberry were studied. The study was carried out in the mulberry fields of four sericulture districts, namely Murshidabad (24.1°N latitude, 88.25°E longitude and 18 m above MSL), Nadia (23.97° N latitude, 88.62° E longitude and 15 m above MSL), Birbhum (24° 35' 0" N latitude, 87° 5' 25" and 88° 1' 40" E longitude and 42 m above MSL) and Malda (24°40'20" N latitude, 87°45'50" E longitude and 31 m above MSL) during 2014 and 2015.

The surveillance studies were based on a fixed plot survey in each of the above location at monthly intervals. From each location, three mulberry gardens of 0.5 ac. were selected as one replication unit. The *S₁* cultivar mulberry plantation was considered for data collection. The mulberry crops were pruned five times in a year for rearing 4-5 scheduled commercial silkworm crops. The selected sample plots of mulberry plantation were maintained as per standard agronomic package of practices (Krishnaswami, 1986; Choudhury and Giridhar, 1987) without insecticidal application either on foliage or in soil (Patnaik *et al.*, 2011). The standard pruning schedules were adopted in all the experimental plots as per the sericulture crop recommendations of West Bengal (Choudhury *et al.*, 1991).

Sampling of mealybugs and its associated arthropod predators was conducted at monthly intervals. For monitoring the population trends of the mealybug infestation and associated predators, 25 plants from each plot / replication (N=3) were selected and examined at random by taking five plants each from the four corners and one from the middle of the plot in every location (Mukhopadhyay, 2006).

Percentage of plants infested by mealybugs (pink hibiscus mealybug and papaya mealybug) was assessed by observing twenty five randomly selected plants / plot. The per cent infestation of mealybugs in mulberry was calculated by the following formula (Mastoi *et al.*, 2014)

$$\text{Mealybug infestation per cent} = \frac{\text{Number of infested plants}}{\text{Total number of observed plants}} \times 100$$

Five infested mulberry shoots (one sample unit) were collected from each field (N=3) from four locations. The mealybug associated arthropod predators were evaluated monthly by the method of destructive sampling (Geiger and Daane, 2001). This sampling involves the removal of the sample unit (mealybug infested terminal shoots of 15 cm length) for later assessment. This is more accurate as it provided scope for rearing of immature insects that cannot be identified until adult emergence. The predator insects that are likely to escape during the counting process will also be accounted in this method. The mealybug infested shoot portions of mulberry were cut along with the host population and all predator stages, intact, were reared in the laboratory until the adult predators emerged. The absolute counts of all arthropod predators associated with mealybugs in each sampling unit were recorded.

The predators were sorted out and duly identified. Individuals which could not be identified to species level were initially grouped at genus level and sent to NBAIR, ICAR, Bengaluru for further species level identification. Month-wise and species-wise data were tabulated to study the species composition, abundance and phenology.

RESULTS AND DISCUSSION

Pink hibiscus mealybug, *M. hirsutus* was prevalent from 2nd fortnight of March to 2nd fortnight of August months while *P. marginatus* was recorded during June-July months of the year in West Bengal. The mealybugs were found to breed abundantly in summer months in the region, resulting in increased infestation of mulberry plants. During the study (2014-2015), per cent mealybug infestation ranged between 10 and 25 in the sampled mulberry gardens (Tables 1, 2).

More natural enemies were noticed in 2014 (Table 1) than in 2015 (Table 2), corresponding to higher mealybug infestations in respective locations. But the total number of species recorded was 15 and 16 during 2014 and 2015, respectively. Active feeding on mealybugs by all of these natural enemies was observed in the field *in situ*. Lady beetles were the most frequent natural enemies sampled

each year. Various stages of lady beetles were represented in samples over a period of time. Lacewing abundance was marked by the numbers of their eggs found throughout the season. Among *Scymnus* spp., it was impractical to identify larvae to species and hence their rearing was completed in the laboratory. However, coccinellids that are distinct such as *B. suturalis*, *Coccinella septempunctata*, *Menochilus sexmaculatus* were accounted with ease.

Based on the species abundance and monthly distribution, phenology graphs were prepared with inset graph showing frequency distribution of predators (Figure 1) for the two years studied. The graphical representation of species abundance reflected the mean number of arthropod predators collected from destructive counts from five sampled mealybug infested mulberry shoots from four locations during 2014 and 2015.

The data revealed that two species viz., *S. latifolius* sp. nov. and *S. coccivora* were collected in 10-15 nos. per sampling unit during the period of both the years (Figure 1). During 2015, seven species were recorded in 5-10 nos. per sampling unit. Seven predator species viz., *B. suturalis*, *J. pallidula*, *S. bourdilloni*, *S. nubilus*, *S. pallidicollis*, *S. pyrocheilus* and *M. sexmaculatus* recorded 5-10 nos. per sample unit and another seven predators, *N. regularis*, *N. sp. nr. roepkei*, *N. bipunctatus*, *Spalgis epius*, *Mallada desjardinsi*, *C. septempunctata* and one anthocorid bug recorded 0-5 nos. per sample unit during the study.

Species composition

A diverse assemblage of arthropod predators was associated with mealybugs infesting mulberry in West Bengal (Figure 2). Coccinellid species belonging to six genera, spread across three tribes of the family Coccinellidae were collected and identified in this study. Besides coccinellids, one lepidopteran, one hemipteran and one neuropteran predator were collected.

The predator species that were collected during the study period (Figure 4) were *J. pallidula*, (9%) *B. suturalis*

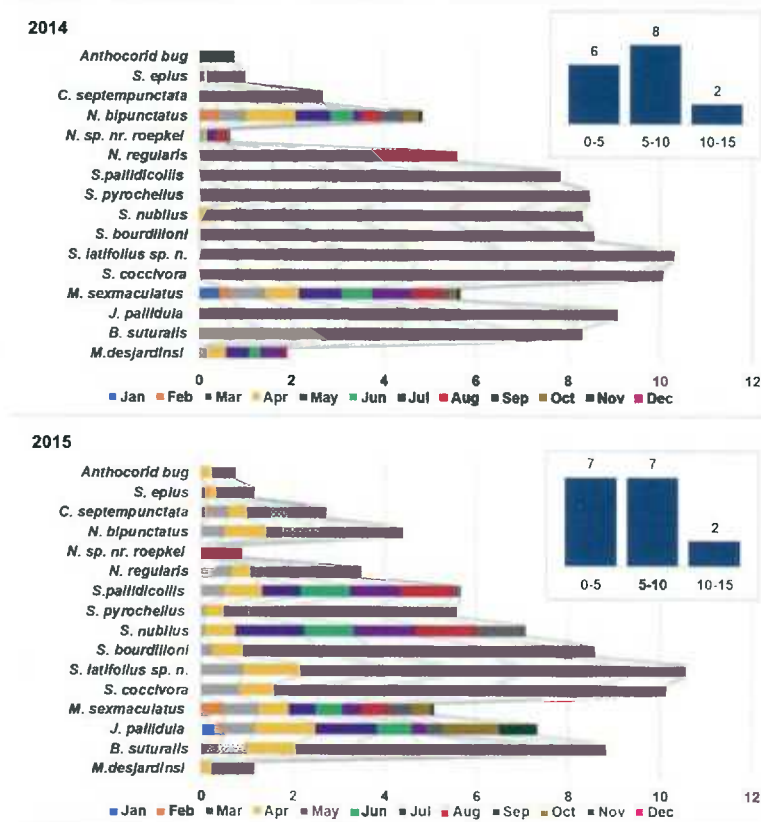


Figure 1: Year-wise phenology graphs with inset showing frequency distribution of predators

Table 1: Native arthropod predators feeding on mealybugs infesting mulberry at different districts of West Bengal during 2014

Month	Murshidabad			Nadia			Birbhum			Malda			Total species	Pooled No. of individuals*	Proportional abundance (%)
	Mealy bug infestation (%)	Total species	No. of individuals*	Mealy bug infestation (%)	Total species	No. of individuals*	Mealy bug infestation (%)	Total species	No. of individuals*	Mealy bug infestation (%)	Total species	No. of individuals*			
January	0.00	3	8	0.00	3	7	0.00	3	3	0.67	4	7	4	25	2.21
February	0.67	6	12	0.67	7	9	0.67	1	3	1.33	8	12	8	36	3.18
March	4.67	12	29	6.67	13	25	6.67	8	10	4.67	11	23	13	87	7.69
April	12.67	13	45	12.67	14	39	12.67	11	15	14.00	14	32	14	131	11.57
May	15.33	13	46	23.33	15	44	23.33	12	24	24.00	13	36	15	150	13.25
June	14.00	11	49	12.00	14	37	12.00	12	33	24.00	14	41	14	160	14.13
July	23.33	10	53	18.67	15	49	18.67	13	38	27.33	13	46	15	186	16.43
August	24.00	10	51	22.00	14	38	22.00	13	40	20.67	10	44	14	173	15.28
September	14.00	10	31	4.00	13	21	4.00	13	36	14.00	10	30	13	118	10.42
October	12.00	6	11	2.00	5	7	2.00	11	20	12.00	3	8	11	46	4.06
November	2.00	2	4	0.67	1	2	0.67	6	8	2.00	2	4	6	18	1.59
December	0.67	0	0	0.00	0	0	0.00	2	2	0.67	0	0	2	2	0.18
Total		13	339		15	278		13	232		14	283	15	1132	100

*Predators recorded on five mealybug infested mulberry shoots

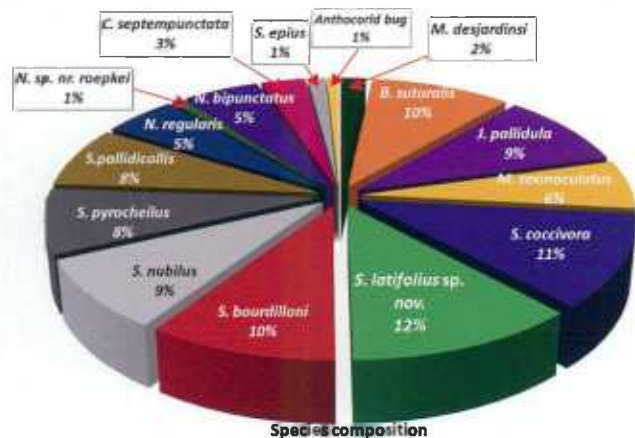


Figure 2: Species composition of mealybug predators in mulberry

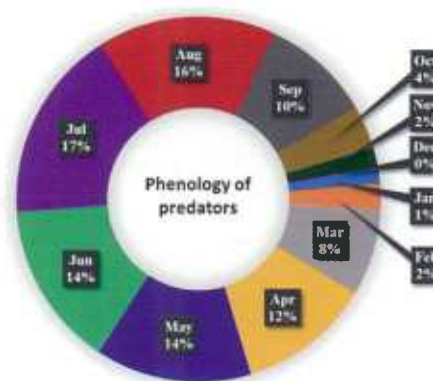


Figure 3: Phenology of the mealybug predators in mulberry

(10%), *C. septempunctata* (3%), *M. sexmaculatus* (6%), *S. coccivora* (11%), *S. nubilus* (9%), *S. bourdilloni* (10%), *S. pyrocheilus* (8%), *S. pallidicollis* (8%), *N. regularis* (5%), *N. bipunctatus* (5%), *N. sp. nr. roepkei* (1%), *S. epius* (1%), *M. desjardinsi* (2%) and one anthocorid bug (1%). Besides these predator species, one new species of *Scymnus* Kugelann, *Scymnus* (*Pullus*)

latifolius Poorani sp. nov (12 %) (Figure 4 E) was collected and reported for the first time (Poorani and Lalitha, 2018). This was earlier misidentified and accounted as *S. pallidicollis* or *S. apiciflavus*. Owing to the small size and difficulty in examination of male genitalia, classification of accurate species and identification of Scymnini tribe is often difficult (Wilson 1927; Pang and

Table 2: Native arthropod predators feeding on mealybugs infesting mulberry at different districts of West Bengal during 2015

Month	Murshidabad			Nadia			Birbhum			Malda			Total species	Pooled No. of individuals*	Proportional abundance (%)
	Mealy bug infestation (%)	Total species	No. of individuals*	Mealy bug infestation (%)	Total species	No. of individuals*	Mealy bug infestation (%)	Total species	No. of individuals*	Mealy bug infestation (%)	Total species	No. of individuals*			
January	0.67	1	1	0.00	1	1	0.00	2	2	0.67	2	2	2	6	0.60
February	1.33	2	2	0.67	4	6	0.67	3	3	1.33	3	4	4	15	1.50
March	4.00	9	23	2.00	11	16	2.00	9	11	4.00	10	25	11	75	7.48
April	15.33	14	34	9.33	15	31	9.33	12	20	15.33	13	36	15	121	12.06
May	18.00	13	40	22.00	16	43	22.00	13	23	16.00	13	40	16	146	14.56
June	10.00	13	43	14.00	14	45	14.00	11	22	17.33	14	41	14	151	15.05
July	16.00	13	48	16.67	16	52	16.67	14	38	23.33	13	43	16	181	18.05
August	21.33	10	38	20.00	13	51	20.00	12	35	16.00	11	39	13	163	16.25
September	10.00	10	19	6.00	13	31	6.00	12	20	10.00	11	22	13	92	9.17
October	7.33	3	8	2.00	6	11	2.00	5	7	7.33	4	9	6	35	3.49
November	2.00	2	4	0.00	2	4	0.00	2	3	2.00	4	6	4	17	1.69
December	0.00	0	0	0.00	0	0	0.00	0	0	0.00	1	1	1	1	0.10
Total		14	260		16	291		14	184		14	268	16	1003	100

*Predators recorded on five mealybug infested mulberry shoots

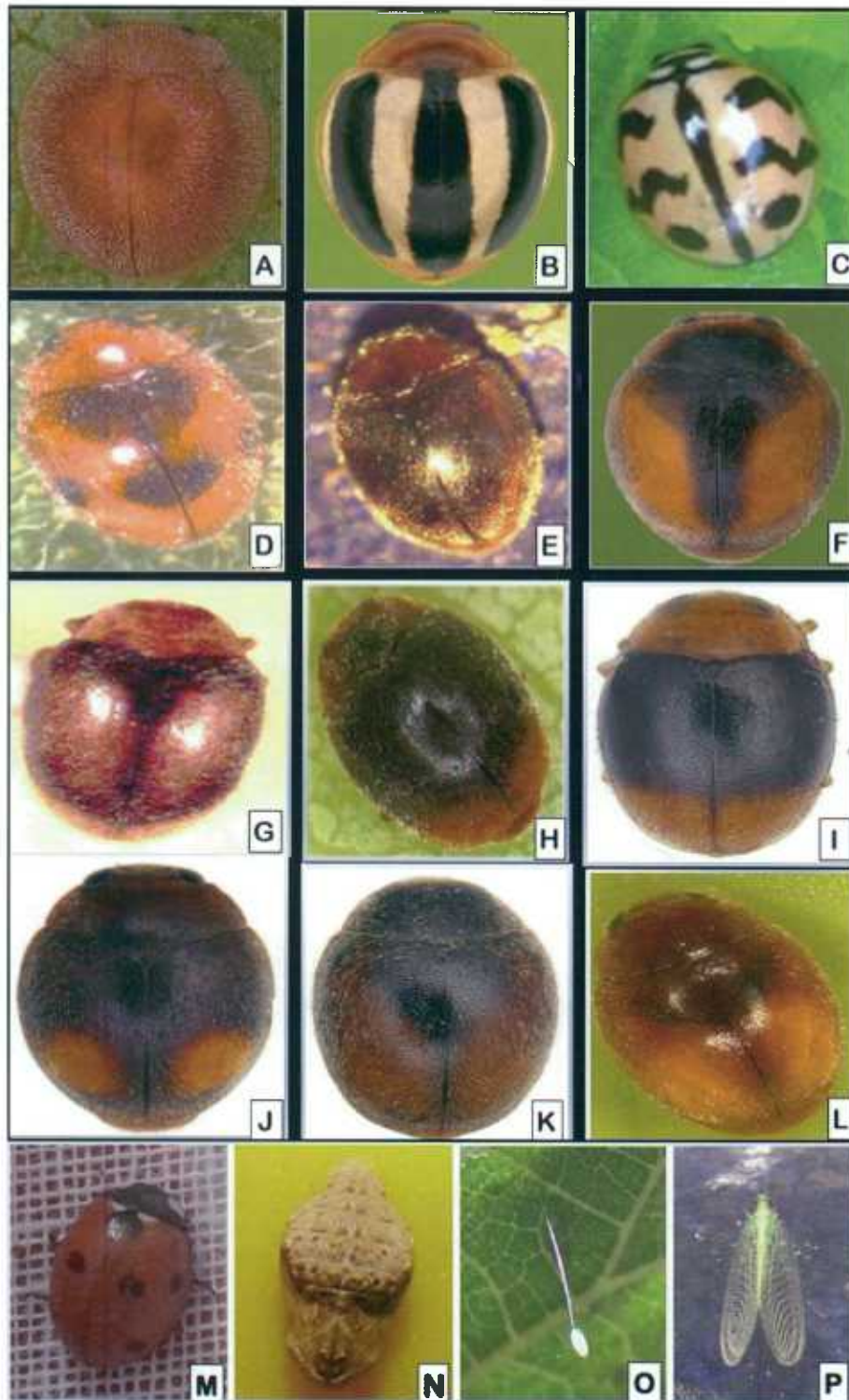


Figure 4: Predators collected from mealybug infested mulberry gardens of West Bengal. A) *Jauravia pallidula*, B) *Brumoides suturalis*, C) *Menochilus sexmaculatus*, D) *Scymnus coccivora*, E) *S. latifolius* sp. nov., F) *S. nubilus* G) *S. bourdilloni*, H) *S. pyrocheilus*, I) *S. pallidicollis*, J) *Nephus regularis*, K) *N. sp. nr. roepkei* de Fluiter, L) *N. bipunctatus*, M) *Coccinella septempunctata*, N) Pupa of *Spalgis epius*, O) egg of *Mallada desjardinsi* (Navas) and P) adult of *M. aejardinsi* (Navas).

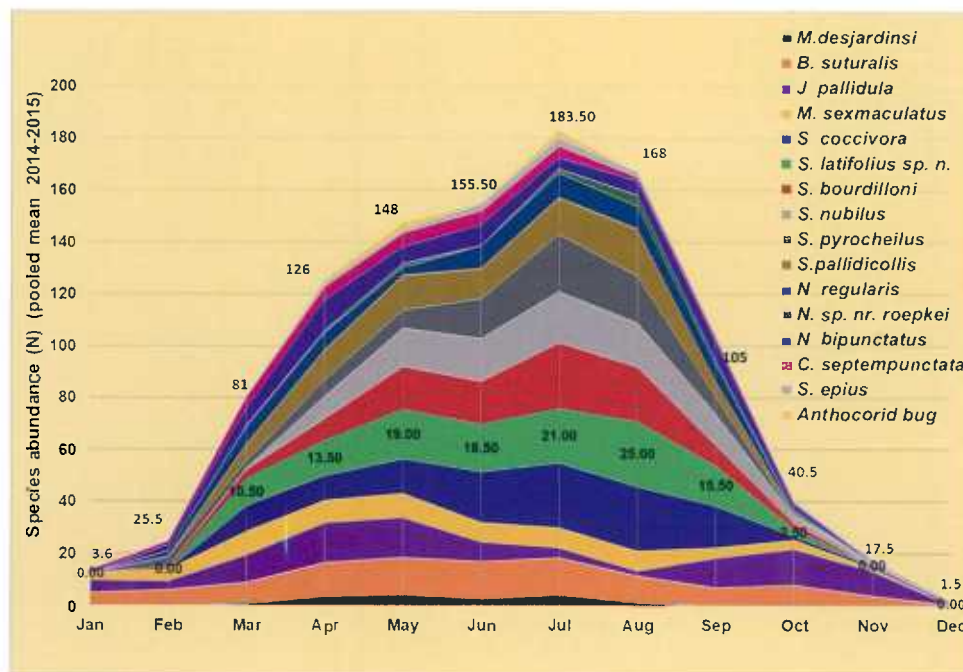


Figure 5: Species abundance (pooled) and monthly distribution of mealybug predators

Gordon, 1986).

Seasonal phenology

The predator population was recorded >100 between the months of April and September in 2014 and April to August in 2015 with proportional abundance ranging from 10 to 18 %. Low predator population (<50) with proportional abundance, 1 to 4 % was recorded during the winter months (October to December and January to February). The seasonal phenology of the predators (pooled for two years) is represented in Figure 3. The highest record of predators was during July and distributed between April and September months of the year coinciding with the peak incidence of mealybugs in mulberry with optimum climatic conditions, which is in concurrence with the previous findings (Mahimasanthi *et al.*, 2015).

Species abundance

The species-wise assembled comprehensive inventory of predators associated with mealybugs infesting

mulberry from four locations, pooled for the period 2014 and 2015 are presented in Table 3. The pooled, species abundance with month-wise distribution is presented in Figure 5. Among the different predatory arthropods captured, *S. latifolius* sp. nov., *S. coccivora* and *S. bourdilloni*, recorded more than 20 numbers per sample unit of five mulberry shoots, during July and August months of the year (Figure 5). *S. pallidicollis* was also recorded more than 20 numbers per sample unit during July month. During May, June and September months, the population of *Scymnus* spp. ranged from 15.1 to 20 nos. per sample unit.

All the six *Scymnus* spp. collected in the study (*S. bourdilloni*, *S. coccivora*, *S. latifolius*, *S. nubilus*, *S. pallidicollis* and *S. pyrocheilus*) were relatively abundant and were collected at different stages (egg, grub, pupa or adult) along with the mealybug life stages during April to September. This suggests that all the *Scymnus* spp. were dominant coccidophagous taxa that successfully multiplied and reproduced amidst mealybug hosts. The population of *Scymnus* spp. increased and decreased with respect to the mealybug population indicating their close

Table 3: Species-wise mealybug predator population across the months, recorded in mulberry from four districts of West Bengal

Predator species	Larva /				JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	Total	Species ranking
	Egg	nymph	Pupa	Adult														
<i>S. latifolius</i> sp. nov.	✓	✓	✓	✓	0.0	0.0	10.5	13.5	19.0	18.5	21.0	25.0	15.5	2.5	0.0	0.0	125.5	1
<i>S. coccivora</i>	✓	✓	✓	✓	0.0	0.0	10.0	9.5	13.5	19.5	25.0	25.0	16.0	1.5	1.0	0.5	121.5	2
<i>S. bourdilloni</i>	✓	✓	✓	✓	0	1.0	3.5	8.5	16.5	16.5	25.5	21	8	2.5	0	0	103.0	3
<i>B. suturalis</i>	✗	✗	✓	✓	5.0	6.0	8.0	12.5	14.0	14.0	14.0	10.0	7.0	8.0	4.0	0.5	103.0	3
<i>J. pallidula</i>	✗	✗	✗	✓	5.0	3.5	10.5	15.5	15.5	7.5	4.0	2.0	11.5	14.0	9.5	0.0	98.5	5
<i>S. nubilus</i>	✓	✓	✓	✓	0.0	0.0	0.5	8.5	15.0	16.5	19.5	17.0	12.0	2.5	1.0	0.0	92.5	6
<i>S. pallidicollis</i>	✓	✓	✓	✓	0.0	4.0	3.0	6.5	7.0	15.5	22.5	18.5	7.0	0.5	0.0	0.0	84.5	7
<i>S. pyrocheilus</i>	✓	✓	✓	✓	0.0	0.0	6.0	11.5	13.5	11.5	14.0	18.0	5.5	1.0	0.0	0.0	81.0	8
<i>M. sexmaculatus</i>	✗	✗	✗	✓	2.5	4.5	9.0	8.5	9.0	7.5	7.5	7.5	4.0	3.5	1.0	0.0	64.5	9
<i>N. bipunctatus</i>	✗	✗	✗	✓	0.0	2.5	6.5	12.0	6.5	8.0	4.5	5.5	7.0	2.5	0.5	0.0	55.5	10
<i>N. regularis</i>	✗	✗	✗	✓	0.0	2.0	7.0	7.0	3.0	9.0	9.5	9.0	7.0	1.0	0.0	0.0	54.5	11
<i>C. septempunctata</i>	✗	✗	✗	✓	1.5	1.5	4.5	5.0	5.5	5.5	4.5	1.5	1.5	0.5	0.5	0.5	32.5	12
<i>M. desjardinsi</i>	✓	✓	✗	✗	0.0	0.0	1.0	4.0	4.5	3.0	4.5	1.5	0.0	0.0	0.0	0.0	18.5	13
<i>S. epius</i>	✓	✓	✓	✓	0.5	0.5	0.5	1.5	1.0	2.5	3.0	2.0	1.0	0.5	0.0	0.0	13.0	14
<i>N. sp. nr. roepkei</i>	✗	✗	✗	✓	0.0	0.0	0.5	0.5	1.5	0.0	1.5	4.0	1.5	0.0	0.0	0.0	9.5	15
Anthocorid bug	✗	✗	✗	✓	0.0	0.0	0.0	1.5	3.0	0.5	3.0	0.5	0.5	0.0	0.0	0.0	9.0	16
Total*					14.5	25.5	81.0	126.0	148.0	155.5	183.5	168.0	105.0	40.5	17.5	1.5	1066.5	N=16

Predator numbers per sample unit (five mulberry shoots) pooled for two years, 2014 and 2015

Legend  <5.0 5.1-10.0 10.1-15.0 15.1-20.0 >20.0

association with the host.

Coccinellids such as *B. suturalis*, *J. pallidula*, *M. sexmaculatus* and *C. septempunctata* were collected generally as adults from the infested mulberry shoots. Apart from feeding mealybugs, *B. suturalis* and *J. pallidula* were often found predating the egg and nymphal stages of the whiteflies infesting mulberry. *M. sexmaculatus* and *C. septempunctata* were also found to check the population build-up of thrips in mulberry (Lalitha *et al.*, 2018). Hence, these generalist predators were found distributed all through the year (Figure 5).

N. regularis, *N. sp. nr. roepkei* and *N. bipunctatus* (Coleoptera : Coccinellidae) were collected mostly as adults in very few numbers from the infested mulberry shoots.

The chrysopid, *M. desjardinsi* (Navas) (Neuroptera: Chrysopidae) was recorded as eggs or nymphs in very few samples. Adults are not predatory in habit. Eggs were not proportionately represented as nymphs in the collected samples. Chrysopids are generally subject to high intraguild predation in agroecosystem (Rosenheim *et al.*, 1999) and this may have decreased their relative larval abundance in the samples.

The carnivorous lycaenid butterfly, *S. epius* (Lepidoptera: Lycaenidae) was recorded, generally as larva, pupa, and rarely as adult. The effective predation of *S. epius* on *M. hirsutus* (Pushpaveni *et al.*, 1973) and *P. marginatus* (Thangamalar *et al.*, 2010) were reported earlier.

Anthocorid bugs (Hemiptera: Anthocoridae) were

sparsely recorded as nymphs and adults in a few locations. Ballal *et al.*, 2012 reported the predation of *P. marginatus* by anthocorid bug in Karnataka.

Although, earlier workers (Santhakumar *et al.*, 1995; Santhakumar and Chakraborty, 1999; Chakraborty *et al.*, 1999) reported several native predators of mulberry pests in West Bengal, some of those predators were not traceable in the present study. Instead, a few new ones were added to the predator inventory. In mulberry crop grown for sericulture, predators are forced to emigrate upon pruning, crop senescence or leaf harvesting. Conservation of species diversity is strongly recommended for effective utilisation of native predators in bio control. Enhancing plant diversity in the mulberry ecosystems, either through the use of non-crops in undergrowth or field margins can ensure non-prey food resources for the native predator species. Avoiding insecticide use and habitat management will determine the sustenance and survivability of these listed native predators in the future.

The present study reveals the distribution of the new promising mealybug predator *S. latifolius* sp. nov. Being native to the Eastern zone of India, the predator survives the adverse climatic conditions especially high temperature and high humid conditions. Hence, there is enough scope to develop mass multiplication protocol and utilise this predator in biocontrol programmes.

CONCLUSION

A diverse assemblage of arthropod natural enemies is associated with mealybugs infesting mulberry in West Bengal. The two year surveillance study evidently suggests that the native predators are important in suppressing growing mealybug population. Nevertheless, no parasitoids and entomopathogens were found on mealybug colonies during the study. The seasonal phenology of the predators provides information on the native predators that are density dependent with respect to host mealybugs. Beyond these basic information, a “field guide” with photographs was

provided for general public. The newly reported promising predator, *S. latifolius* Poorani sp. nov could be attempted for mass multiplication and released to enhance field population and boost biological control.

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Article scientifique

INVENTAIRE ET EVALUATION DES POPULATIONS DES PREDATEURS ATTAQUANT LES PUNAISES DE LA FARINE QUI INFESTENT LE MURIER AU BENGALE DE L'OUEST

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RESUME

La punaise de l'hibiscus rose, *Maconellicoccus hirsutus* (Green), et de la papaye *Paracoccus marginatus* Williams and Granara de Willink sont les pestes suceuses majeures du mûrier (*Morus alba* L.), la seule plante hôte du ver à soie *Bombyx mori* (L.), qui est l'option de survie de millions de fermiers en Inde. Les plaines indo-gangétiques du Bengale de l'ouest présentent une faune riche de prédateurs coccinélidés qui peuvent être utilisés pour la gestion des pestes majeures du mûrier. Une information sur les arthropodes prédateurs et leur impact sur les punaises de la farine et utile pour développer une lutte biologique. En 2014-2015 une étude a été menée pour explorer et caractériser l'habitat foliaire, des arthropodes prédateurs dans quatre localités (Murshidabad, Nadia, Birbhum et Malda). Ce sont *Brumoides suturalis* (F), *Coccinella septempunctata* L., *Jauravia pallidula* Mots, *Menochilus sexmaculatus* (F), *Nephus regularis* Sicard, *Nephus* sp. nr. *roepkei* de Fluiter., *Nephus bipunctatus* Kugelann, *Scymnus bourdilloni* Kapur, *Scymnus coccivora* Ayyar, *Scymnus nubilus* Mulsant, *Scymnus pallidicollis* Mulsant, *Scymnus pyrocheilus* Mulsant, *Spalgis epius* (Westwood), *Mallada desjardinsi* (Navas) et un anthocoridé (non identifié). En dehors de ces arthropodes, une nouvelle espèce de *Scymnus* Kugelann, *Scymnus (Pullus) latifolius* Poorani sp. nov, a été collectée et décrite pour la première fois. Notre étude éclaire la composition en espèces de prédateurs, leur abondance et leur cycle saisonnier.

Mots-clés: *Maconellicoccus hirsutus*, mûrier, prédateurs natifs, *Paracoccus marginatus*.



INFLUENCE OF VARIOUS FACTORS ON THE PREVALENCE OF SILKWORM DISEASES AND LOW COCOON PRODUCTIVITY IN THE SELECTED SERICULTURAL AREAS OF ANDHRA PRADESH, INDIA

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ABSTRACT

Prevalence of silkworm diseases causes significant damage to cocoon production. Information on influential factors on the prevalence of silkworm diseases and the resultant low cocoon productivity at farmers' conditions is essential for formulating silkworm disease management strategies. Periodic survey on silkworm diseases, in the affected areas will help to find out the pattern of prevalence of diseases in the field at farmers' conditions and the factors responsible for low cocoon productivity as well. Hence, in the present study, a survey was conducted for two years (October, 2012 to September, 2014) during silkworm rearing (V instar) to find out the point prevalence of silkworm diseases in the selected areas of Andhra Pradesh, India. After cocoon harvest, all the farmers' rearing houses were re-visited to collect the information on cocoon yield and also factors responsible for low cocoon yield (<60 kg/100 DFLs) if any. The average data on the point prevalence of diseases in two years indicated the prevalence of 3.25 % of total diseases with 1.96, 0.92 and 0.22 % of grasserie, flacherie and muscardine diseases, respectively during survey day. There was no incidence of pebrine disease in any of the crops surveyed during the two years period. Prevalence of grasserie was observed comparatively more during summer (2.76 %), followed by rainy (1.67 %) and winter (1.45 %) seasons. In the case of flacherie, the incidence was more during rainy (0.94 %) and winter (0.93 %) seasons followed by summer (0.89 %). However, muscardine disease was more prevalent during winter (0.58 %) and less in rainy and winter seasons. Among 875 farmers' crops surveyed during the period, 697 farmers (79.66 %) harvested >60 kg cocoons / 100 DFLs of rearing and 178 farmers (20.34 %) obtained <60 kg cocoons / 100 DFLs of rearing. The influence of major factors on the prevalence of diseases and low cocoon productivity (<60 kg cocoons / 100 DFLs) were analyzed and discussed.

Key words: Andhra Pradesh, cocoon yield, diseases, point prevalence, silkworm.

INTRODUCTION

In silk production, one of the major constraints is the disease occurrence during silkworm rearing. The domestication and continuous rearing of mulberry silkworm, *Bombyx mori* L. for over 4000 years have made them sensitive to environmental, nutritional and microbial factors thereby enhancing susceptibility to

infectious and non-infectious diseases and cocoon crop losses. Among the infectious diseases of silkworm, grasserie, flacherie, muscardine and pebrine are the major ones. In India, 30-40 % crop loss was reported in nineteen sixties (Vaidya, 1960; Janakiraman, 1961) and 15-20 % in seventies (Chitra *et al.*, 1975). The silkworm crop loss due to muscardine varies from 5 to 50 % in sericultural countries (Jayaramaiah and Kuberappa, 1987) and it is

common during winter and rainy seasons in all sericultural areas of Karnataka. Samson *et al.* (1990) reported the prevalence of 57 % of flacherie among the total diseases. Later, Nataraju *et al.* (1998) reported that nuclear polyhedrosis virus (BmNPV) was prevalent throughout the year in Karnataka to an extent of 33 – 53 % among the diseases. Pebrine disease was rampant during 1990-1992 (Baig, 1994; Nataraju and Dandin, 2006) and drastically declined later.

In India, bivoltine silkworm breeds / hybrids (CSR) developed by Central Sericultural Research and Training Institute, Mysuru, under BSTD (Bivoltine Sericulture Technology Development) project of JICA (Japan International Collaboration Agency), Japan during 1990-96 are having the potential to yield 60 - 75 kg of cocoons/100 DFLs of rearing provided the bivoltine silkworm rearing technology [new mulberry variety, V1 with suitable package of practices for its cultivation, concept of chawki (young age) rearing under Chawki Rearing Centre, chawki certification, shoot rearing method for late age silkworms, and effective silkworm disease management] is followed meticulously. During 1997-2002, this bivoltine technology was demonstrated in the field under PPPBST (Promotion of Popularization of Practical Bivoltine Sericulture Technology) project of JICA, Japan in limited areas of Karnataka, Tamil Nadu and Andhra Pradesh. During the period, Nataraju *et al.* (2002) formulated and demonstrated a practical technology for diagnosis and management of diseases in silkworm rearing to avoid crop losses due to silkworm diseases, which boosted the confidence of farmers to go for bivoltine crops. Again, during 2002-2007, during the 3rd phase of JICA PEBS (Strengthening of Extension System for Bivoltine Sericulture in India) project, large scale demonstrations were conducted in southern states of India and the Extension system was strengthened for bivoltine sericulture. However, due to some hurdles in adopting the bivoltine technology fully by all the farmers, the potential of the breeds / hybrids is not exploited fully resulting in low cocoon productivity. Recently, Balavenkatasubbaiah *et al.* (2014, 2015) reported the point prevalence of silkworm diseases and the factors

influencing the low cocoon productivity in selected sericultural areas of Karnataka and Tamil Nadu. Similar way, in the present study also, a survey was conducted for two years (October, 2012 to September, 2014) in four sericultural areas of Andhra Pradesh, India during silkworm rearing (V instar) to estimate the point prevalence of silkworm diseases and cocoon productivity apart from collecting information on the factors influencing the prevalence of diseases and low cocoon productivity.

MATERIALS AND METHODS

A survey was conducted at fortnightly interval in the following selected areas/districts of Andhra Pradesh from October, 2012 to September, 2014, to ascertain the point prevalence of silkworm diseases covering all the months/seasons viz., winter, summer and rainy. In total, 875 farmers' crops were covered during the period of two years.

Selected area	District
Madakasira	Anantapur
Eluru	West Godavari
Vikarabad	Ranga Reddy (at present, Telangana state)
V. Kota	Chittoor

- In Andhra Pradesh, the three seasons in a year are differentiated as the following periods.

Season	Duration
Winter	November ~ February
Summer	March ~ June
Rainy	July ~ October

- In every fortnight, five farmers having V instar larvae (4th to 6th day) were selected randomly for the survey in each selected area.
- The programme covered two fortnight surveys in each month and 6 in every quarter and 24 in a year and 48 in two years (in V. Kota area, survey was conducted from June, 2012 to September, 2014).
- Total larval rearing bed area, number of larvae /sq ft

bed area and total number of larvae in the rearing bed were the parameters recorded / estimated.

- 10 % of the bed area was observed for assessing the point prevalence of silkworm diseases viz., grasserie, flacherie, muscardine and pebrine. Based on the visual symptoms expressed by larvae and microscopic examination of haemolymph / midgut as reported by Balavenkatasubbaiah *et al.* (2014), the incidence of diseases was recorded and the per cent point prevalence of each disease was calculated.
- Later, after completion of crop, the information on the quantity of cocoons harvested, number of DFLs brushed *etc.* were collected from the farmers to arrive at the figure of cocoon yield / 100 DFLs of rearing.
- The important factors which affect the cocoon crop harvest were noted along with the excerpts of discussion / opinion of the farmers after the cocoon crop harvest. Apart from this, the following crucial aspects that play a key role in successful silkworm rearing were noted.
 - Mulberry leaf quality - mulberry variety, type of soil, irrigation schedule, fertilizer dose and application and mulberry crop protection methods followed.
 - Disinfection of silkworm rearing environment – Method of disinfection practiced for rearing house, appliance and rearing house surroundings, type of disinfectant / quantity of disinfectant solution used, disinfection schedule followed and type of bed disinfectant used and its quantity.
 - Maintenance of hygiene – Personal and rearing hygienic measures followed during rearing.
 - Rearing management – Silkworm rearing management practices followed during rearing.
 - Environmental conditions – Temperature and humidity levels of rearing house during rearing

period.

- Other factors – Heavy rainfall during rearing period and mortality due to toxicity of chemicals / pesticides *etc.*

The above factors were correlated with the prevalence of silkworm diseases / low cocoon productivity (<60 kg cocoon yield/100 DFLs of rearing). The monthly data on the point prevalence of diseases and cocoon yield harvested by farmers were tabulated season-wise / area-wise with their averages. The impact of the above mentioned factors on the prevalence of diseases and cocoon yield is also discussed in the present study.

RESULTS

The seasonal average data on the point prevalence of silkworm diseases and cocoon yield in the selected sericultural areas of Andhra Pradesh during 2012 - 2014 are presented in Table 1. On an average, the total incidence of diseases during survey day was 3.25 % consisting 1.96, 0.92 and 0.22 % of grasserie, flacherie and muscardine, respectively with zero incidence of pebrine. When comparison was made between seasons, grasserie was observed more prevalent during summer (2.76 %) followed by rainy (1.67 %) and winter (1.45 %) seasons. Flacherie incidence was recorded more in rainy (0.94 %) followed by winter and summer seasons. Muscardine was noticed more prevalent during winter (0.58 %) than rainy and summer seasons. The cocoon crop harvested / 100 DFLs of rearing amounted to 66.77, 62.63 and 64.20 kg during winter, summer and rainy seasons, respectively.

The seasonal data on the point prevalence of silkworm diseases and cocoon yield in Madakasira, Eluru, Vikarabad and V. Kota areas during 2012 - 14 are presented in Table 2. On an average, Madakasira area recorded 0.90 % grasserie, 1.29 % flacherie and 0.23 % of muscardine. In the case of Eluru, high incidence (4.68 %) of grasserie and 1.11 % of flacherie and 0.25 % of muscardine was recorded. In Vikarabad area, the total incidence of diseases was 2.83 % out of which 1.73, 0.87

Table 1: Point prevalence of silkworm diseases and cocoon yield in different seasons in Andhra Pradesh during 2012 - 14

Season	Grasserie (%)	Flacherie (%)	Muscardine (%)	Pebrine (%)	Total (%)	Cocoon yield / 100 DFLs (kg)
Winter	1.45	0.93	0.58	0.00	2.96	66.77
Summer	2.76	0.89	0.03	0.00	3.67	62.63
Rainy	1.67	0.94	0.06	0.00	3.13	64.20
Average	1.96	0.92	0.22	0.00	3.25	64.53

Table 2: Point prevalence of silkworm diseases (%) and cocoon yield (kg) in different sericultural areas of Andhra Pradesh during 2012 - 14

Year / Season	Madakasira					Eluru					Vikarabad					V. Kota				
	Gra.	Fla.	Mus.	Total	Yield / 100 DFLs	Gra.	Fla.	Mus.	Total	Yield / 100 DFLs	Gra.	Fla.	Mus.	Total	Yield / 100 DFLs	Gra.	Fla.	Mus.	Total	Yield / 100 DFLs
2012-13																				
Winter	0.85	0.98	1.02	2.85	67.58	4.70	1.35	1.43	7.48	60.59	2.07	0.31	1.32	3.70	69.94	0.22	0.98	0.00	1.20	67.68
Summer	1.16	1.19	0.13	2.48	66.75	10.43	0.65	0.00	11.08	49.35	1.99	1.70	0.00	3.69	64.34	0.40	1.02	0.00	1.42	68.48
Rainy	1.00	1.30	0.00	2.30	66.95	5.25	2.30	0.00	7.55	56.48	2.28	0.95	0.00	3.23	63.26	1.07	2.16	0.44	3.67	69.27
2013-14																				
Winter	0.99	0.98	0.24	2.21	65.56	1.19	1.55	0.00	2.74	63.60	1.43	0.49	0.60	2.52	65.09	0.16	0.76	0.00	0.92	74.06
Summer	0.91	0.93	0.00	1.84	66.50	4.20	0.20	0.05	4.45	48.41	1.75	0.78	0.00	2.53	62.46	0.07	0.60	0.00	0.67	74.76
Rainy	0.46	2.33	0.00	2.79	67.39	2.35	0.58	0.00	2.93	55.58	0.84	0.99	0.05	1.88	67.50	0.09	0.59	0.00	0.68	72.18
Average	0.90	1.29	0.23	2.42	66.79	4.68	1.11	0.25	6.04	55.67	1.73	0.87	0.33	2.93	65.43	0.34	0.87	0.07	1.43	71.07

Gra. - Grasserie; Fla. - Flacherie; Mus. - Muscardine.

and 0.33 % were accounted towards the incidence of grasserie, flacherie and muscardine, respectively. The total point prevalence of diseases in V. Kota area was only 1.43 % with grasserie incidence of 0.34 %, flacherie of 0.87 % and muscardine of 0.07 %. Incidence of pebrine disease was not reported in any of the areas during October 2012 to September 2014. The cocoon crop harvested / 100 DFLs of rearing was 66.79, 55.67, 65.43 and 71.07 kg in Madakasira, Eluru, Vikarabad and V. Kota areas, respectively. When the seasonal impact was compared, summer season displayed higher incidence of grasserie and flacherie. However, in the case of muscardine, as expected, it was more prevalent during winter followed by rainy and summer seasons. The cocoon yield was comparatively higher in winter. In the case of V. Kota area, the prevalence of diseases was low in all the seasons and hence, the range of cocoon yield was narrow with the average yield harvested being around 71.07 kg / 100 DFLs.

In total, 875 farmers harvested an average cocoon yield of 64.53 kg / 100 DFLs. Out of 875 farmers' crops surveyed during the period, 697 farmers (79.66 %) harvested >60 kg cocoon yield / 100 DFLs of rearing and 178 of them (20.34 %) obtained <60 kg. Between the areas, Madakasira registered an average of 66.70 kg cocoons / 100 DFLs of rearing in respect of 240 farmers surveyed. Out of this, 215 farmers (89.58 %) recorded >60 and 25 farmers (10.42 %), <60 kg of cocoon yield / 100 DFLs of rearing. In Eluru area, the total sample of 240 farmers harvested an average of 55.67 kg cocoons / 100 DFLs of rearing with 99 (41.25 %) at a level of >60 and 141 (58.75 %) at <60 kg / 100 DFLs. In the case of Vikarabad, it was 65.43 kg cocoons / 100 DFLs of rearing for 240 farmers wherein 228 (95 %) belonged to the higher yield category of >60 kg cocoon yield / 100 DFLs and only 12 farmers (5 %) harvested <60 kg / 100 DFLs. V. Kota area presented a different picture where, all the 155

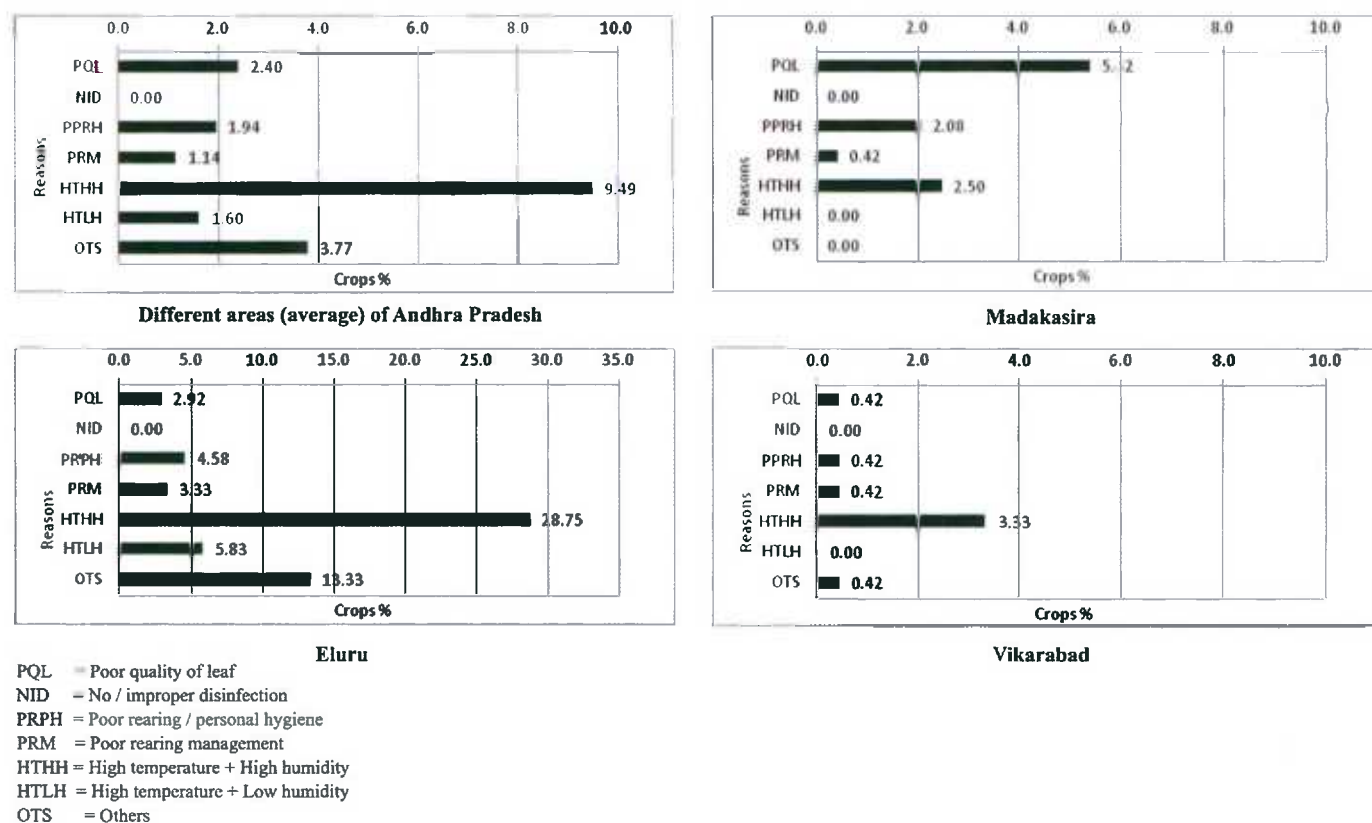


Figure 1: Reasons for harvesting low cocoon yield (< 60 kg / 100 DFLs)

Table 3: Cocoon yield /100 DFLs of rearing in different sericultural areas of Andhra Pradesh during 2012 - 14

Yield / 100 DFLs	Madakasira		Eluru		Vikarabad		V. Kota		Total	
	No. of crops	%	No. of crops	%	No. of crops	%	No. of crops	%	No. of crops	%
< 50 kg	2	0.83	73	30.42	1	0.42	0	0	76	8.69
50- 55 kg	7	2.92	28	11.67	0	0.00	0	0	35	4.00
56 - 60 kg	16	6.67	40	16.66	11	4.58	0	0	67	7.66
Total < 60 kg	25	10.42	141	58.75	12	5.00	0	0	178	20.34
61 - 65 kg	65	27.08	47	19.58	111	46.25	17	10.97	240	27.43
66 - 70 kg	75	31.25	38	15.83	64	26.67	60	38.71	237	27.09
71 - 75 kg	53	22.08	7	2.92	38	15.83	37	23.87	135	15.43
>75 kg	22	9.17	7	2.92	15	6.25	41	26.45	85	9.71
Total >60 kg	215	89.58	99	41.25	228	95.00	155	100.00	697	79.66
Total crops	240	100	240	100	240	100	155	100.00	875	100

farmers harvested >60 kg with an average yield of 71.07kg/100 DFLs (Table 3).

The information collected from all the farmers of Andhra Pradesh on reasons for harvesting low cocoon

yield (<60 kg cocoons / 100 DFLs) is presented in Figure 1. In general, high temperature with high humidity (9.49%), other factors (3.77 %) such as heavy rains in coastal area (West Godavari) and poor quality leaf (2.40%) were the major factors that influenced the cocoon

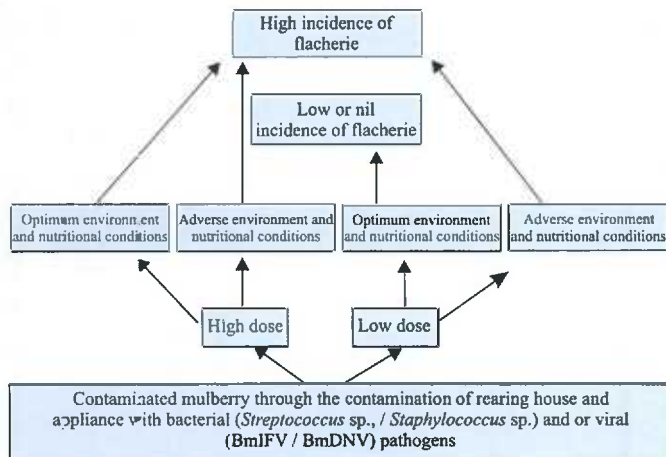


Figure 2 a: Influence of factors on causation of flacherie in the presence of pathogen load

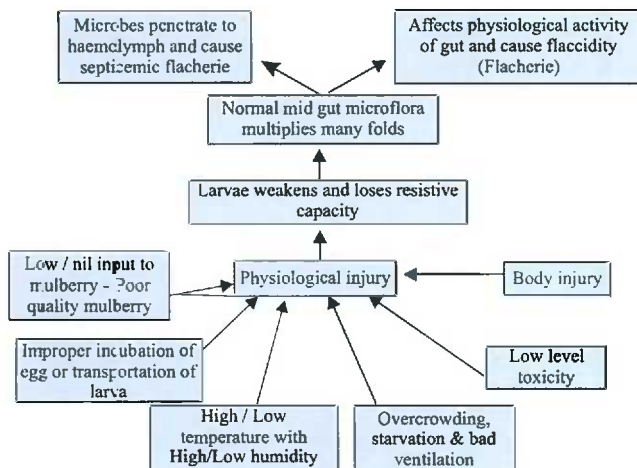


Figure 2 b: Influence of factors on causation of flacherie in the absence of pathogen load

productivity. However, different set of reasons were attributed to different areas for low cocoon harvest. In Madakasira, poor nutritional quality of leaf (5.42 %) was ascertained as the major factor responsible for harvesting <60 kg cocoons / 100 DFLs of rearing. High temperature with high humidity (28.75 %) and other factors viz., heavy rains (13.33 %) acted as crucial factors responsible for low cocoon productivity (< 60 kg cocoons / 100 DFLs of rearing) in Eluru, a coastal area. The adverse effects of high temperature (3.33 %) was revealed in Vikarabad in terms of low cocoon yield. V. Kota, where all the farmers could harvest potential yield of the hybrids stood as a testimony of successful and stable bivoltine rearing as the

farmers followed all the recommended technologies meticulously.

DISCUSSION

The results of the present study, clearly indicates that throughout the survey period, there was no incidence of pebrine disease in the field. This is a clear indication that the eggs produced and supplied by the Seed Production Centers to the different Chawki Rearing Centres of Andhra Pradesh were of high quality. The point prevalence of silkworm diseases in the field during rearing period was low. The total prevalence of grasserie, flacherie and muscardine was only 3.25 % and majority of the farmers (79.66 %) harvested >60 kg cocoons / 100 DFLs of rearing in which, 9.71 % farmers harvested > 75 kg / 100 DFLs, and 15.43 % farmers harvested 71-75 kg / 100 DFLs and 27.09 % farmers harvested 65-70 kg / 100 DFLs and 27.43 % farmers harvested 61-65 kg / 100 DFLs of rearing. Remaining 20.35 % farmers harvested < 60 kg / 100 DFLs. The potential of the bivoltine hybrid was fully exploited by 25.14 % farmers who obtained maximum cocoon yield of > 70 kg / 100 DFLs of rearing by following all the recommended packages / technologies at the field level. However, Eluru (West Godavari) area recorded only 55.67 kg cocoons / 100 DFLs of rearing. Out of 240 farmers' crops surveyed, 141 farmers (58.75 %) harvested <60 kg cocoons / 100 DFLs. As West Godavari (Eluru) area is a coastal area, the reasons attributed for low yield were high temperature and high humid (28.75 %) conditions prevailed along with frequent heavy rains (13.33 %).

Earlier Selvakumar *et al.*, 2002 conducted a survey over a period of 4 years in Karnataka, involving 2,821 farmers' crops in multivoltine, bivoltine, multi × bivoltine and bivoltine hybrid areas and estimated the point prevalence of silkworm diseases and cocoon crop loss due to common silkworm diseases. The point prevalence of nuclear polyhedrosis ranged from 1.16 (winter) to 2.34 % (summer) and flacherie from 0.92 (winter) to 3.43 % (summer) while muscardine ranged from 0.79 (winter) to 1.08 % (rainy). The estimated total loss due to diseases at the point prevalence level was 11.486, 14.864 and

14.500 kg/ 100 DFLs during winter, summer and rainy seasons, respectively. Recently, Balavenkatasubbaiah *et al.* (2014) conducted a survey in Karnataka covering 3 sericultural areas viz., Bangalore Rural, Kolar and Mandya for two years (October, 2012 to September, 2014). The average data on the point prevalence of diseases in two years was 3.28 % with 1.03, 2.15 and 0.19% of grasserie, flacherie and muscardine, respectively with no incidence of pebrine. Among 720 farmers' crops surveyed, 86.81 % farmers harvested > 60 and 13.10 % farmers harvested <60 kg cocoons / 100 DFLs of rearing. Balavenkatasubbaiah *et al.* (2015) also conducted a similar study in Tamil Nadu covering Salem, Erode and Tiruppur areas. The average data on the point prevalence of diseases in two years indicated the prevalence of 1.65 % of diseases in which grasserie was 0.46 %, flacherie was 1.19 % with no incidence of muscardine and pebrine throughout the period. Among 720 farmers' crops surveyed, 568 farmers (78.89 %) harvested >60 kg cocoons / 100 DFLs of rearing and 152 farmers (21.11 %) harvested <60 kg cocoons / 100 DFLs of rearing. The major reasons for harvesting <60 kg cocoons / 100 DFLs (less than potential of the hybrid) were poor quality of leaf (16.11 %) and high temperature with low humidity (1.25 %). Earlier, Vijaya Kumari *et al.* (2000, 2001) studied the influence of larval density and mulberry leaf maturity as stress factors for rearing of bivoltine hybrid silkworms. Sudipta Mukherjee *et al.* (2005) reported BmNPV as a biotic cause for post cocoon mortality in silkworm. Later, Nataraju *et al.* (2007) reported the cause and management of flacherie disease in silkworm, where the influence of factors viz., environment conditions prevailing in the rearing room, leaf quality and silkworm rearing practices in the presence / absence of pathogen load in the rearing bed was discussed (Figure 2 a, b).

In general, Andhra Pradesh farmers have well adopted the bivoltine sericulture technologies recommended by CSRTI, Mysuru and hence, majority of the farmers' cocoon crop yields were improved compared to earlier period. The survey data also revealed that majority of the farmers' (79.66 %) harvested > 60 kg / 100 DFLs of

rearing. In V. Kota, which is a leading bivoltine cocoon production area in Andhra Pradesh, majority of the farmers were already been trained on bivoltine sericulture technologies under various programmes of JICA / CSB / DoS of Andhra Pradesh. Farmers also attended many awareness programmes / group discussions / enlightenment programmes arranged in their areas. In general, this area, consists of many innovative and progressive farmers following all the recommended technologies of CSRTI, Mysuru very effectively for production of quality bivoltine silk. The subsidy schemes introduced by Department of Sericulture, Govt. of Andhra Pradesh also evoked positive response towards stabilizing cocoon productivity at farmers' level.

Madakasira area also witnessed a favourable response regarding bivoltine sericulture and earlier during JICA projects period, many programmes in this direction were conducted in this area and hence, farmers have adopted these technologies well. It is inspiring to note that in Vikarabad of Ranga Reddy district, the number of farmers taking up bivoltine rearing is increasing day by day. However, in these two areas, many bivoltine programmes are to be conducted to educate and sustain the farmers on bivoltine crop rearings as these are potential areas for bivoltine silk production. In the coastal area of Eluru (West Godavari), there is an urgent need to develop / identify suitable, tolerant breeds / hybrids that survive under high temperature and high humidity conditions and also development of suitable rearing houses / rearing packages to reduce the impact of high temperature and high humidity in the rearing environment. In this direction, CSRTI, Mysuru has already initiated a few programmes. Overall, there is a need for introducing disease monitoring system in the sericultural areas of Andhra Pradesh to alert the farmers immediately in case of disease outbreaks and also to suggest suitable remedial measures for the management of silkworm diseases and thereby reduce the crop losses at the farmers' level.

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Article scientifique

INFLUENCE DE DIVERS FACTEURS SUR L'APPARITION DES MALADIES DU VER A SOIE ET LA PRODUCTION DE COCONS DANS DES ZONES SELECTIONNEES DE L'ANDHRA PRADESH EN INDE

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RESUME

L'apparition de maladies chez le ver à soie entraîne de sérieux dommages sur la production de cocons. Une connaissance des facteurs influençant l'apparition des maladies et la productivité en cocons dans les conditions des fermiers est essentielle pour définir des stratégies de gestion des maladies du ver à soie. Des surveillances périodiques dans les zones concernées doit aider à trouver le profil d'apparition des maladies et les facteurs responsables de la faible production de cocons. Aussi, dans cette étude, nous avons effectué une surveillance pendant deux ans (Octobre 2012 à Septembre 2014) pendant les élevages de vers à soie (stade V) dans des zones sélectionnées de l'Andhra Pradesh en Inde. Après la récolte des cocons, toutes les magnaneries ont été revisitées pour collecter des informations sur le rendement en cocons et sur les facteurs responsables d'un faible rendement (<60 kg/100 pontes saines). Les données moyennes indiquent une incidence totale des maladies de 3,25 % avec 1,96 %, 0,92 et 0,22 % de grasserie, flacherie et muscardine respectivement. On ne constate pas de pébrine dans tous les élevages au cours des deux années. La grasserie est observée surtout pendant l'été (2,76 %) puis la saison des pluies (1,67 %) et l'hiver (1,45 %). Pour la flacherie, l'incidence est plus importante pendant la saison des pluies (0,94 %) puis en hiver (0,93 %) et en été (0,89 %). La muscardine est maximale pendant l'hiver et faible pendant la saison des pluies et l'hiver. Parmi 875 récoltes de fermiers analysées pendant la période, 697 fermiers (79,66 %) ont récolté plus de 60 kg de cocons/100 pontes saines et 178 (20,34 %) moins de 60 kg/100 pontes saines. L'influence des facteurs principaux sur l'apparition des maladies et la faible productivité en cocons sont analysés et discutés ici.

Mots-clés: Andhra Pradesh, rendement en cocons, maladies, ver à soie.



COMPARATIVE ACCOUNT ON THE TECHNO-ECONOMICS OF THE AUTOMATIC AND MULTIEND SILK REELING FILATURES IN THE PRESENT INDIAN CONTEXT

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ABSTRACT

The technological advantages of automatic silk reeling machinery package vis-à-vis multiend silk reeling machinery package in terms of cocoon processing, economic parameters of silk reeling units, including break even point (BEP), return on investment (ROI), net present value, (NPV) and internal rate of return (IRR); technical aspects of silk reeling viz., consumption of cocoons, productivity, labour requirement, cost of production, quality of raw silk *etc.*, were studied. The automatic silk reeling machinery package was noticeably superior in terms of bulk quantity of superior grade raw silk production (3A ~ 4A grade) at less cost, using comparatively less labour. However, multiend silk reeling technology package also helps the marginal reelers for the production of 2A ~ 3A grade raw silk and consumes large quantities of cocoons presently. Further, the automatic silk reeling technology package ensures higher returns for the investment and net profit is almost doubled compared to multiend reeling technology. The reelers are of the opinion that with the current set up and availability of quality bivoltine cocoons, it is more advantageous to invest on automatic silk reeling technology package in view of better returns. Thus, the Indian silk reeling industry is presently equipped to produce international grade raw silk as import substitute to enable the weavers to produce quality fabrics of both domestic and export requirements.

Key words: Automatic silk reeling, break even point (BEP), multiend silk reeling, net present value (NPV), return on investment (ROI).

INTRODUCTION

Indian silk industry is aiming high for the development of bivoltine sericulture so that import substitute raw silk can be produced indigenously in adequate quantities for both domestic consumption as well as for exports. Though the bivoltine sericulture was initiated in India even during 1970's, the commercial exploitation was restricted to the level of use as male component for cross breed (multivoltine x bivoltine) cocoon production, whereas, for reeling purpose, it was seldom used. Hence in 1990's, bivoltine sericulture was reintroduced with the help of Japan International cooperation agency, Japan which enabled development of improved CSR race hybrids and double hybrids by Central Sericultural Research and Training Institute (CSRTI), Mysuru as well as

popularization of bivoltine sericulture package and simultaneous establishment of many number of multiend reeling units and automatic reeling units by Central Silk Technological Research Institute (CSTRI), Bengaluru in association with Departments of Sericulture of states, thus facilitating the production of superior grade bivoltine raw silk in the country. The rate of development of bivoltine sericulture in terms of raw silk production over a period of time is depicted in Figure 1.

It is quite evident that bivoltine cocoon as well as silk production have been increasing significantly, thus making it possible, the introduction of improved reeling contrivances in silk industry (Somashekar and Kawakami, 2003). Hence, Central Silk Board in association with

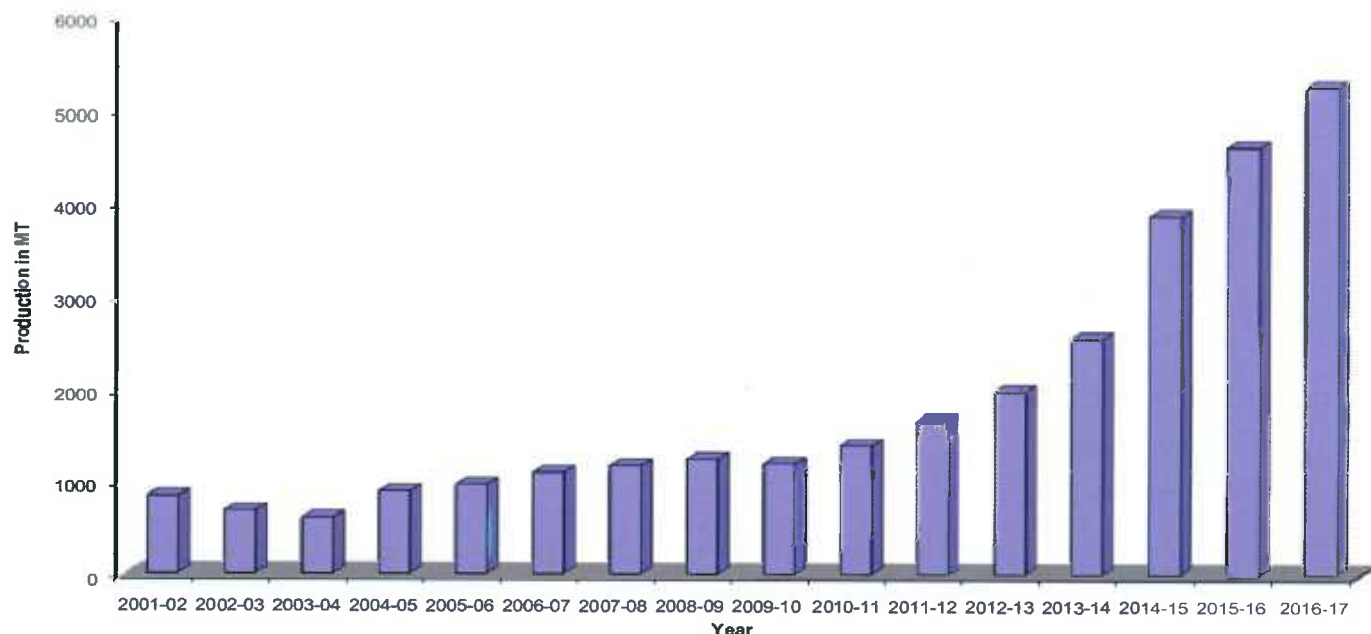


Figure 1: Production of bivoltine raw silk (MT) during the period 2001-02 to 2016-17

Departments of sericulture of respective states has started establishing automatic silk reeling filatures in India under plan schemes. So far, 37 automatic silk reeling filatures are running successfully in India and 38 units are under various stages of establishment. In this context, it was thought that a study could be conducted to analyze technological advantages of automatic silk reeling filatures working successfully in southern states of India along with multiend silk reeling units to derive effective comparisons.

MATERIALS AND METHODS

CSR race bivoltine hybrid cocoons procured from the farmers in the Govt. cocoon markets were reeled on automatic silk reeling filatures as well as multiend silk reeling filatures using standard practices. In automatic silk reeling filature, the following sequence of machineries viz., conveyor cocoon drying machine – deflossing machine – cocoon sorting machine – cocoon vacuum permeation chamber – conveyor cocoon cooking machine – mechanical brushing and automatic silk reeling machine with denier detection and auto casting mechanism – reel permeation chamber – re-reeling machine – lacing – skeinling – bundling and packing were used to convert the cocoons into raw silk. Whereas,

in multiend silk reeling filature, the sequence of machineries used was, batch type hot air drier, manual sorting of cocoons, 2 pan / pressurized cooking machine, manual brushing in 2 pan cooking unit, multiend silk reeling machine with manual casting and mixed reeling of cocoons, small reel permeation, closed type re-reeling machine, lacing, skeinling and silk finishing. The standard process parameters were adopted for processing the bivoltine cocoons in automatic as well as multiend silk reeling filatures. The data pertaining to technological advantages of cocoon processing, economic parameters of both silk reeling units, including break even point (BEP), return on investment (ROI), net present value, (NPV), internal rate of return (IRR); technical analysis of silk reeling viz., consumption of cocoons, productivity, labour requirement, cost of production *etc.*, were studied. Further, the raw silk thus reeled was tested as per ISA standards in the case of automatic silk reeling filatures whereas, BIS standards were adopted for multiend silk reeling filatures.

RESULTS AND DISCUSSION

The economics of the whole sequence of cocoon processing and silk reeling activities worked out for both

Table 1: Techno-economics of automatic and multiend silk reeling units

#	Particulars	Multiend silk reeling unit	Automatic silk reeling unit
I	Installed capacity		
1	Machinery	10 basin / 100 ends	400 ends
2	Production (kg/annum)	4200	36000
II	Average performance		
1	No. of shifts / day	1	2
2	No. of hours / shift	10	8
3	Purchase price of cocoons (₹/kg)	350	400
4	Selling price of raw silk (₹/kg)	3200	3500
III	Project cost (building, machinery, miscellaneous and fixed assets (MFA), pre and post operative expenses (P & POE) & working capital margin (WCM)) (lakh ₹)	31.90	319.90
IV	Working capital		
1	Cycle (days)	26	40
2	Working capital requirement (lakh ₹)	10.40	132.10
V	Sales / Turnover (lakh ₹)	119.70	1109.20
VI	Net profit / Sales (%)	3.90	7.20
VII	Break even point (operation capacity) (%)	56.25	49.16
VIII	Cash flows		
1	Return on investment (ROI) (%)	26.04	36.21
2	Net present value (NPV) (lakh ₹)	7.50	283.65
3	Internal rate of return (IRR) (%)	21.28	34.76

the silk reeling units are presented in Table 1. Further, a comparative picture of the status of parameters viz., consumption of cocoons, productivity, labour requirement and cost of production is provided in Table 2. Also, the average quality characteristics of 20/22 denier raw silk reeled on both automatic and multiend silk reeling machines were analyzed as presented in Table 3.

Technological advantages of automatic and multiend silk reeling machinery

I) Automatic silk reeling machinery package

The automatic silk reeling machinery packages being popularized by CSTRI, CSB in association with Department of sericulture of respective states have the

following machineries and processing technology (Subhas *et al.*, 2015).

Conveyor hot air cocoon drier with hot air generator:

The bivoltine cocoons procured from the farmers are hot air dried to the optimum level with 4-band conveyor type cocoon drying machine. The hot air required for drying the cocoons is generated using hot air generator associated with force draft (FD), induced draft (ID) and exhaust fans, wherein the atmospheric air is forced into hot air generator, heated by fire wood burning and induced into hot air drier. The moist air generated during the process of drying the cocoons are exhausted from the drier using exhaust fan, for effective drying of cocoons. The capacity of the drying machine is to handle 2 tonnes of cocoons per day. The following temperature profile was used to dry the cocoons.

Table 2: Technical analysis of automatic and multiend silk reeling units

#	Particulars	Multiend silk reeling unit	Automatic silk reeling unit
1	Reeling process	Manual	Semi automatic cum automatic
2	Raw silk production / shift (kg)	14	60
3	Labour requirement / kg raw silk production	1.3	0.37
4	Labour requirement / shift	18	22
5	Raw silk quality	2A - 3A grade	3A - 4A grade
6	Raw silk supply	Small quantity	Bulk quantity
7	By-product utilization	Not very effective	Very effective
		Disposed to agents for processing	Processed at the unit
		Pollution issues	Pollution issues addressed
		Moderate returns	Significantly higher returns
8	Working atmosphere	Moderate	Very hygienic

The cocoons are dried for 5 hours per batch.

I Band	- 110 ~ 115 °C
II Band	- 95 °C
III Band	- 90 °C
IV Band	- 80 ~ 70 °C

Cocoon deflossing: The hot air dried cocoons are deflossed using deflossing machine to remove the outer layers *i.e.*, floss. About 1 ton of cocoons can be deflossed per 12 hours shift using deflossing machine.

Cocoon sorting: Defective cocoons are sorted out from the cocoon lots using cocoon sorting machine. The output of the cocoon-sorting machine is one tonne per 12 hours.

Cocoon pre-treatment and cooking: The cocoons are pre-treated using vacuum permeation chamber followed by cooking in conveyor cocoon cooking machine. Cocoons are first treated in the vacuum permeation chamber at 0.09 MPa vacuum pressure to enable water to permeate inside the cocoons. The pre-treatment is carried out in about 5 – 7 minutes. The vacuum permeation chamber can permeate 1.5 tonnes of cocoons in a shift of 8 hours. The water-permeated cocoons are then cooked in conveyor cocoon cooking machine under the following temperature profile (Hariraj *et al.*, 2005). The cooked

cocoons are then collected and fed to automatic silk reeling machine.

Cooking zone	Temperature (°C)	Cooking zone	Temperature (°C)
1 st Zone (Water)	40 – 50	5 th Zone (Steam)	94 – 98
2 nd Zone (Water)	40 – 50	6 th Zone (Water)	60 – 70
3 rd Zone (Steam)	98 – 100	7 th Zone (Water)	45 – 55
4 th Zone (Water)	98 – 100	8 th Zone (Water)	40 – 45

Mechanical brushing of cocoons: The cooked cocoons are brushed mechanically using mechanical brushing unit, which is a part of automatic silk reeling machine. During the process of brushing the true ends of the cocoons are found and dropped into moving trays for supplying to reeling basins. The silk waste generated during brushing process is accumulated automatically in the brushing chamber.

Automatic silk reeling: The brushed cocoons are reeled on 400 ends automatic silk reeling machine. The automatic silk reeling machine mainly consists of units *viz.*, mechanical brushing, groping and end picking, cocoon carrier system, denier indicator and mechanical casting of cocoons and dropped cocoons / pelade carrier and separation system. The cocoons are reeled on

Table 3: Average quality characteristics of 20/22 denier raw silk samples produced from automatic and multiend silk reeling filatures

#	Particulars	Automatic silk reeling filatures		Multiend silk reeling filatures	
I	Average size (d)	20/22		20/22	
II	Major tests	Range	Grade	Range	Grade
1	Standard size deviation (d)	0.94 ~ 1.3	4A ~ 3A	1.34 ~ 1.59	3A ~ 2A
2	Evenness variation- I (Stripes)	25 ~ 123	4A ~ 4A	120 ~ 170	4A ~ 3A
3	Evenness variation- II (Stripes)	0 ~ 8	4A ~ 4A	10 ~ 15	4A ~ 3A
4	Cleanness (%)	96 ~ 98	3A ~ 4A	93 ~ 95	2A ~ 3A
5	Neatness (%)	94 ~ 97	4A ~ 4A	93 ~ 95	3A ~ 4A
6	Low neatness (%)	90 ~ 95	4A ~ 4A	88 ~ 90	3A ~ 4A
III	Auxiliary tests		Class		Class
1	Maximum deviation (d)	2.6 ~ 3.5	1 ~ 2	3.0 ~ 3.5	1 ~ 2
2	Evenness variation- III (Stripes)	0 ~ 1	1 ~ 1	0 ~ 1	1 ~ 2
3	Winding breaks / 40 skeins / hour	4 ~ 8	1 ~ 2	4 ~ 10	1 ~ 2
4	Tenacity (g/d)	3.8 ~ 4.0	1 ~ 1	3.7 ~ 3.8	1 ~ 1
5	Elongation (%)	18 ~ 20	1 ~ 1	18 ~ 19	1 ~ 1
6	Cohesion (Strokes)	80 ~ 120	1 ~ 1	70 ~ 85	1 ~ 1
	Overall grade of raw silk		3A ~ 4A		2A ~ 3A

automatic silk reeling machine with following process parameters.

Reeling speed: 120–130 m/min
Croissure length: 8–10 cm

Reel permeation: The raw silk reeled on small reels are treated in reel permeation chamber to wet the silk before re-reeling. Small reels are subjected to vacuum permeation pressure of 0.6 MPa and this process is repeated for 8 cycles.

Re-reeling: The permeated raw silk is re-reeled on closed type of re-reeling machine. Re-reeling machine consists of 40 double-sided windows and runs at 170–180 rpm. The raw silk is dried during the re-reeling process by maintaining 35–40 °C temperature inside re-reeling machine.

Long skein book making machine: The raw silk removed from re-reeling machine are made into 5 kg

books using motorized long skein book making machine.

II) Multiend silk reeling machinery package

The multiend silk reeling machinery packages being popularized by CSTRI, CSB for the past 2 decades in association with Department of sericulture of respective states have the following machineries (Subhas *et al.*, 2003).

Batch type hot air drier: The Multiend reelers also procure bivoltine cocoons from the farmers and hot air dry to the optimum level with batch type hot air drier following temperature sequence of 110-100-85-70-55 °C for a period of 5 hours. About 50 kg cocoons are dried per batch.

Cocoon sorting: The defective cocoons are sorted out manually from the cocoon lots using cocoon sorting table and collected separately.

Cocoon cooking: The cocoons are either cooked on two pan system of cooking or pressurized cooking system adopting standard cooking procedures. The cocoons are cooked in two pan using CSTR I cocoon cooking cage following low-high-low temperature principle for about 5-7 minutes and the ends are groped. The temperature and duration profile is as follows:

1 st pan	2 nd pan
i. Soaking at 55 °C for 45 seconds	ii. High temperature (90 °C) permeation for 60-90 seconds
iii. Low temperature (65-70 °C) permeation for 45-60 seconds	iv. Cooking at 95-96 °C for 60-90 seconds
	v. Stop heating and allow the cocoons to remain for 60 seconds
	vi. Sprinkle cold water to reduce temperature to 80 °C in 75-90 seconds
	vii. Brush the cocoons at 80 °C

Multiend silk reeling: The brushed bivoltine cocoons are reeled on CSTR I multiend reeling machine with following parameters to produce superior grade raw silk.

1. Reel speed : 100 - 120 meters/min.
2. Croissure length : 8 - 10 cm
3. Reeling basin water temperature : 40 °C
4. Good quality reeling button with appropriate hole size developed by CSTR I.

Small reel permeation: In the reel permeation chamber, small reels are soaked in water using low pressure (vacuum up to 300 to 400 mm Hg). Permeation is achieved by creating vacuum condition for 1-2 minutes and released. This cycle is repeated three times to achieve better reel permeation.

Re-reeling: The permeated raw silk is re-reeled on closed type of re-reeling machine. Re-reeling machine consists

of 10 double-sided windows and runs at 160 rpm. During the re-reeling process, 35–40 °C temperature and 45 % RH is maintained inside the re-reeling machine. The raw silk thus produced are skeinned and marketed.

Indeed, both the automatic and multiend silk reeling technologies are capable of processing bivoltine cocoons for the production of superior grade raw silk.

Techno-economics of automatic and multiend silk reeling filatures

The economics of 400 ends automatic silk reeling filatures and 10 basin multiend silk reeling filatures was worked out for comparative purpose based on the existing field conditions of important silk reeling clusters in Karnataka (Table 1). From the data, it could be observed that 10 basin multiend silk reeling filatures produce raw silk of about 4200 kg (20/22 denier) per annum, working for single shift of 10 hours with about 300 working days, whereas, 400 ends automatic silk reeling filatures produce about 36000 kg per annum, working for 2 shifts (8 h each). The total investment (project cost) is quite huge in the case of automatic silk reeling unit amounting to ₹320 lakhs, while for a multiend reeling unit, it is about 32 lakhs. This is due to the high end technology and huge production capacity of the former unit.

Further, the details of sales, various fixed & variable costs and profitability were worked out at 85 % capacity utilization in both the cases. The working capital requirement for a 400 ends automatic silk reeling unit (₹1109 lakhs) is significantly higher as compared to 10 basin multiend silk reeling unit (₹12 lakhs) due to the high production capacity and larger working capital cycle. The automatic silk reeling unit needs large inventories, especially of raw material, i.e., cocoons for smooth and uninterrupted production process. The unit has to buy large quantities of cocoons of uniform quality, hot air dry and store them for longer periods in order to utilize the installed capacity optimally. The annual turnover of the automatic silk reeling unit is significantly higher due to larger production capacity and importantly, higher sales

realization due to better quality of raw silk produced in bulk quantities. Hence, the net profit / sales % were found to be higher (7.20 %) in the case of automatic silk reeling unit as compared to multiend silk reeling unit (3.90 %). The BEP works out to only 49.16 % at operating capacity in automatic silk reeling unit as compared to 56.25 % in multiend silk reeling units indicating that the risk involved is less in automatic silk reeling units.

It could also be observed that, the cash flows (both absolute and discounted) in terms of ROI, NPV and IRR are found to be favourable in both the cases and significantly higher in the case of automatic silk reeling unit (Table 1). The positive NPV and high IRR indicate that the investment in silk reeling is profitable in both the cases and more so in automatic silk reeling units.

Technical aspects of automatic and multiend silk reeling units including Labour requirements

The details of labour requirements and technical aspects in both automatic and multiend silk reeling filatures are shown in Table 2. It is stated that about 22 labourers work in automatic silk reeling filatures to produce 60 kg raw silk per 8 hours shift compared to 18 labourers working in multiend silk reeling filatures to generate 14 kg raw silk / 10 hours. Further, the labour requirement in the case of automatic silk reeling filatures is 0.37 for the production of one kilogram of raw silk, which is significantly lower than that of multiend silk reeling filatures (1.3 labourers), thus revealing the appreciably higher efficiency of automatic silk reeling filatures.

The automatic silk reeling filatures and multiend silk reeling filatures were analyzed for the quality characteristics of raw silk produced from both the filatures (Table 3). It is obvious that majority of the lots produced on automatic silk reeling filatures were of 3A to 4A international grade, which may be attributed to the advanced technology adopted in automatic silk reeling filatures including denier detector and auto casting mechanism whereas, multiend silk reeling filatures could also deliver the nearest international grade of 2A to 3A.

Further, the study revealed that by-product utilization in automatic silk reeling filatures has not only improved the hygiene of the filature, but also addressed the pollution problems faced by multiend reeling filatures, apart from increasing the returns from by-products.

Conclusion

Based on the above analysis, it is evident that in view of increased production of bivoltine cocoons in the country, adoption of automatic silk reeling units would result in augmenting production of bulk quantity of superior grade raw silk. Multiend silk reeling technology package also helps the marginal reelers for the production of 2A ~ 3A grade raw silk by consuming large quantities of cocoons. Both of these technologies have been motivating more number of farmers to adopt bivoltine sericulture in the country as their produce is sold at appropriate price and assured of forward linkages. The establishment of more number of silk reeling filatures is expected to support the horizontal expansion of sericulture due to assured market and better price for the produce. Moreover, the automatic silk reeling filatures assist in production of import substitute raw material to the weavers, which would increase their productivity and quality to meet the global requirements. The positive NPV and high IRR indicate that the investment in silk reeling is profitable in both the cases and more so, in automatic silk reeling units. The better environment of working and hygienic conditions in automatic silk reeling units would provide opportunity to the young generation to work in these units and reduce the labour shortage problems. The reelers are of the opinion that with the current scenario and availability of quality bivoltine cocoons, it is more advantageous to invest on automatic silk reeling technology package in view of better returns.

Thus it could be concluded that the economic viability of automatic reeling is significantly higher than that of multiend reeling, along with the added advantages of labour reduction, quality improvement, better marketability and congenial as well as pollution free working atmosphere.

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Rapport technique

COMPARATIF TECHNICO-ECONOMIQUE DES FILATURES DE SOIE AUTOMATIQUE ET MULTI-BOUTS DANS LE CONTEXTE ACTUEL DE L'INDE

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RESUME

Dans notre étude, nous comparons les avantages technologiques des filatures automatiques par rapport aux filatures multi-bouts en ce qui concerne le traitement des cocons, les paramètres économiques incluant les points de rupture (BEP), le retour sur investissements (ROI) la valeur nette actuelle (NPV) et le taux interne de retour (IRR) ; les aspects techniques, la consommation de cocons, productivité, la main d'œuvre nécessaire, le coût de production, la qualité de la soie grège sont également étudiés. La filature automatique est supérieure en termes de quantités de production de soie grège de qualité (grade 3A-4A) à un coût de production moindre en utilisant moins de main d'œuvre. Cependant, la filature multi-bouts aide des filateurs marginaux à produire de la soie grège 2A-3A et consomme de larges quantités de cocons. De plus, la filature automatique assure des retours plus élevés pour les investissements et le profit net est environ le double de celui obtenu avec la technologie multi-bouts. Les filateurs estiment qu'avec la disponibilité actuelle des cocons bivoltins de qualité, il est économique d'investir dans la technologie de filature automatique. L'industrie de la filature de soie indienne est équipée aujourd'hui pour produire de la soie grège de grade international qui peut se substituer à la soie importée et permettre aux tisseurs de produire des tissus de qualité pour le marché domestique et pour l'exportation.

Mots-clés: Filature de soie automatique, point de rupture (BEP), filature de soie multibouts, valeur nette (NPV), retour sur investissements (ROI).



DEVELOPMENT OF A SENSITIVE REAL-TIME PCR ASSAY FOR THE DETECTION OF MICROSPORIDIA IN SILKWORMS

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ABSTRACT

Microsporidia are obligate intracellular parasites that infect a broad range of invertebrates, and vertebrates including humans. Pebrine disease caused by microsporidia has been a constant threat to sericulture industry. A real-time PCR - based method was developed for the specific and sensitive diagnosis of microsporidia from silkworms. A pair of primers from the conserved regions coding for the 16S small subunit ribosomal RNA gene across microsporidia gave discrete PCR product. As few as 10 spores could be detected from DNA extracted from both gut and eggs of silkworm, *Bombyx mori*. Further, the detection method was extended to the wild tasar silkworm, *Antheraea mylitta*. It is validated that this method is highly reliable for early and sensitive detection of microsporidia and thus could be a valuable addition in quarantine and surveillance activities in sericulture.

Key words: *Antheraea mylitta*, *Bombyx mori*, real-time PCR.

INTRODUCTION

Microsporidia are obligate intracellular parasites causing infection to almost all animal phyla. Apart from causing human infections in immuno-compromised patients, microsporidia also cause extensive damage to aquaculture and apiculture industry (Williams, 2009). Microsporidiosis has been a focus of research for over 150 years and was even the subject of study of Louis Pasteur who investigated the cause of the pebrine disease of silkworms that threatened to devastate the silk industry in the 19th century (Pasteur, 1870). Pebrine caused by *Nosema bombycis* and other species of microsporidia is still a threat to silk industry because of its unique and recurrent occurrence. The disease spreads quickly and takes a heavy toll of silkworm resulting in considerably high economic loss in sericulture. The current method used for diagnosing the microsporidiosis is based on light microscopy which has limited sensitivity. PCR diagnosis has greatly enhanced the sensitivity and specificity for microsporidia in silkworms (Kawakami *et al.*, 1995; Hatakeyama and Hayasaka, 2003; Kawarabata, 2003;

Ravikumar *et al.*, 2011; Roy *et al.*, 2017). Mostly, the 16S small subunit ribosomal RNA (SSU-rRNA) is used as the target gene, but intergenic spacer region and RNA polymerase as target sequences were also employed (Ghosh and Weiss, 2009; Roy *et al.*, 2017). Real-time PCR is a highly sensitive and faster technique than conventional PCR and has frequently been applied for the detection of pathogens. In silkworm, *Bombyx mori*, real time PCR-based detection of microsporidia were reported of late (Fu *et al.*, 2016; Wu *et al.*, 2017) but limiting the detection only for *N. bombycis*. Here, we demonstrate that conserved SSU-RNA primers could detect microsporidia from *B. mori* as well as tasar silkworm, *Antheraea mylitta*.

MATERIALS AND METHODS

The silkworms, *B. mori* (Pure Mysore) and *A. mylitta* (Daba) were used for the study. Microsporidian spores were isolated from infected silkworms by an isopycnic gradient of Percoll (Sigma). Spores were counted by haemocytometer and spore concentrations were adjusted to the desired concentrations in water by serial dilutions.

Different concentrations of spores ranging from <10 and 10^1 to 10^5 were mixed with 15 mg of gut tissue or eggs and subjected to DNA extraction. The extraction of DNA was carried out using 0.5 μ m glass beads (Sigma-Aldrich) and DNeasy kit (Qiagen) as per manufacturer's instructions. The integrity and quantification of DNA were analysed by agarose gel electrophoresis and NanoDrop 2000C spectrophotometer (Thermo Scientific). Primers were designed from the conserved regions of 16S SSU RNA across microsporidia as reported by us earlier (Ravikumar, 2011). A pair of primers, Forward: 5'-ACCATTGTAGTTCTAGCAGTAA-3' and Reverse: 5'-TGAGTCAAATTAAGCCGCACA-3' was employed in Real-Time PCR using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), according to the manufacturer's instructions. The PCR program consisted of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 30s, and 72 °C for 30 s in Step One Plus Real-Time PCR Machine (Applied Biosystems). Each sample was assayed in triplicate. Negative controls were DNA extracted from normal silkworms. Results were expressed as threshold cycle (Ct) values corresponding to the cycle at which PCR enters the exponential phase. Conventional PCR was carried out using DNA extracted from sample of 10^4 spores combined with 15 mg of eggs and PCR products were confirmed by sequencing.

RESULTS AND DISCUSSION

Real-time and conventional PCR with pan-microsporidia primers produced positive amplification in all microsporidia spiked samples (Figure 1, Table 1). The limit of detection was 10 spores using DNA extracted from serial dilutions of microsporidia spores prepared from gut and eggs from both silkworm species (Table 1). The Ct values were 29.32 ± 0.13 and 30.71 ± 0.07 for gut and egg samples, respectively, for *B. mori* and 29.86 ± 0.42 and 31.20 ± 0.11 , respectively for the same tissues for *A. mylitta*. As expected, there was direct correlation between the concentration of spores and corresponding Ct values. The Ct values obtained ranged from 17.10 to 31.20 (Table 1) wherein the former detected 10^5 spores. No



Figure 1: Agarose gel electrophoresis (2.5 %) showing amplification of microsporidia-specific DNA bands (182 bp) with SSU-RNA primers. Lane M: DNA size marker, lanes 1 & 2: Microsporidia of *B. mori* and *A. mylitta*, respectively.

Table 1: Real-time PCR analysis of DNA extracted from gut and eggs of silkworms spiked with different concentrations of microsporidia spores

Spore concentration	Ct Value			
	<i>B. mori</i>		<i>A. mylitta</i>	
	Gut	Egg	Gut	Egg
10^5	17.10 ± 0.72	18.22 ± 0.27	17.63 ± 0.19	17.91 ± 0.42
10^4	18.36 ± 0.14	19.70 ± 0.33	18.31 ± 0.70	19.00 ± 0.12
10^3	21.58 ± 0.59	21.65 ± 0.18	21.20 ± 0.06	22.67 ± 0.29
10^2	24.20 ± 0.18	25.00 ± 0.63	25.17 ± 0.39	27.32 ± 0.51
10	29.32 ± 0.13	30.71 ± 0.07	29.86 ± 0.42	31.20 ± 0.11
<10	-	-	-	-

-: Not detected.

amplification was observed for samples obtained from negative controls (data not shown).

Our results clearly indicated the importance of real time PCR-based assay for the specific and sensitive detection of microsporidia from silkworms. In our earlier report, the limit of detection using conventional PCR was stated as 1500 spores/20 mg tissue (Ravikumar *et al.*, 2011). In this study, we have improved the detection limit to 10 spores/15 mg tissue using real time quantitative PCR, enhancing the sensitivity to nearly 113 times. Apart from real-time PCR efficiency, the increased sensitivity of detection was also due to the glass bead disruption of

spores to maximise the DNA concentration. Real-time PCR method for the detection of microsporidia in *B. mori* reported to be 10 spores/whole sample and even 0.1 spore DNA in a reaction (Fu *et al.*, 2016). However, how DNA can effectively be extracted from a single *B. mori* egg is not yet clear. Multiplex real-time PCR showed the detection limit of 8.5×10^3 copies for *N. bombycis* and *B. mori* NPV (BmNPV) and 8.5×10^4 copies for *B. mori* DNV (BmDNV) (Wu *et al.*, 2017). These reports for the detection of microsporidia were aimed at the presence of *N. bombycis* and hence, limited to silkworm *B. mori*. Nevertheless, our results clearly show that the pan-microsporidian SSU primers could detect microsporidia from *B. mori* and *A. mylitta*; even though the latter is infected by different microsporidia (Hassan *et al.*, 2016). Comparing the results of microsporidia detection using conventional PCR reported by us (Ravikumar *et al.*, 2011), the real-time PCR method described here can be used for the early detection of microsporidia from silkworms. Moreover, the present real-time PCR method could be a valuable tool employed for the quarantine inspection and field monitoring of microsporidia across different silkworm species.

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Rapport technique

DEVELOPPEMENT D'UN ESSAI PCR EN TEMPS REEL POUR LA DETECTION DE MICROSPORIDIES CHEZ LE VER A SOIE

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RESUME

Les microsporidies sont des parasites intracellulaires obligatoires qui infectent de nombreux vertébrés et invertébrés y compris l'homme. La pébrine causée par une microsporidie est un phénomène permanent en sériciculture. Une méthode basée sur la PCR en temps réel a été développée pour un diagnostic spécifique et sensible chez les vers à soie. Une paire d'amorces des régions conservées parmi les microsporidies codant pour le gène de l'ARN ribosomique de la sous-unité ribosomique 16 S donne des bandes discrètes de produits PCR. On peut détecter 10 spores à partir de l'ADN extrait à la fois de l'intestin et des œufs du ver à soie *Bombyx mori*. La méthode de détection a été étendue au ver à soie tasarsauvage., *Antheraea mylitta*. Cette méthode est hautement utile pour la détection précoce et sensible de microsporidies et peut être un plus dans les activités de quarantaine et de surveillance en sériciculture.

Mots-clés: *Antheraea mylitta*, *Bombyx mori*, temps réel, essai PCR.

International Training Programme on Mulberry Silkworm Seed Production Technology conducted at Bangalore, India during 17-26 September 2018



In order to strengthen the Silkworm Seed Production Networks in Member Countries, ISC has organized a 10 days intensive training on **Mulberry Silkworm Seed Production Technology** at Bangalore, India during 17-26 September 2018. The training was conducted in the Silkworm Seed Production Centre of the National Silkworm Seed Organization, Central Silk Board, Bangalore, India. 22 trainees from 11 Member Countries have attended the training. The participating countries were; Afghanistan, Bangladesh, DPR Korea, Egypt, Ghana, Iran, Kenya, Madagascar, Romania, Thailand and Uzbekistan. The entire expenses related to the training like; international travel, accommodation, food, training cost, *etc.* were met by International Sericultural Commission.



The training programme was inaugurated by Mr. Takayuki KITAGAWA, Hon'ble Consul General of Japan in Bangalore on 17 September 2018 in a brief function held in the campus of Central Silk Board, Madiwala, Bangalore.

The trainees were exposed to the various intricate aspects of silkworm seed production through theoretical and practical classes. Besides, the trainees were also shown the sericulture areas and facilities in and around Bangalore and Mysore to understand the successful model of commercial sericulture being practised in India.





6th Asia-Pacific Congress of Sericulture and Insect Biotechnology (APSERI) to be held at Mysore, India during 2nd to 4th March, 2019.

The International Sericultural Commission (ISC), Bangalore, India would be hosting the 6th Asia-Pacific Congress of Sericulture and Insect Biotechnology (APSERI) at Hotel Southern Star, Mysore, India, during 2nd to 4th March, 2019. The Congress is an international forum committed exclusively for the

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Section	Theme
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|---|----------------------------------|
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Global Silk Production (in Metric Tonnes)

#	Country	2013	2014	2015	2016	2017
1	Bangladesh	43	44.5	44	44	41
2	Brazil	550	560	600	650	600
3	Bulgaria	8.5	8	8	9	10
4	China	1,30,000	1,46,000	1,70,000	1,58,400	1,42,000
5	Colombia	0.6	0.5	0.5	-	-
6	Egypt	0.7	0.8	0.8	1.2	1.1
7	India	26,480	28,708	28,523	30,348	31,906
8	Indonesia	16	10	8	4	2.5
9	Iran	123	110	120	125	120
10	Japan	30	30	30	32	20
11	North Korea	300	320	350	365	365
12	South Korea	1.6	1.2	1	1	1
13	Philippines	1	1.1	1.2	1.82	1.5
14	Syria	0.7	0.5	0.3	0.25	0.25
15	Thailand	680	692	698	712	680
16	Tunisia	4	4	3	2	2
17	Turkey	25	32	30	32	30
18	Uzbekistan	980	1,100	1,200	1,256	1,200
19	Vietnam	475	420	450	523	520
20	Madagascar	18	15	5	6	7
	Total	159737.10	178057.62	202072.83	192512.27	177507.35

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