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Epidemiology of the Thai Sacbrood Virus Disease Attacking Indian Honey Bee *Apis cerana indica* F and Morphological Characterization of the Virus Particle using Transmission Electron Microscope

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Honey bees are affected by various diseases caused by viruses, bacteria, fungi and mites. Of these, the Thai Sac Brood virus (TSBV) disease attacking Indian honey bee has been posing threat to hives of *Apis cerana indica* F. in different parts of Southern India. The virus is caused by picorna-like virus characterized under *Iflaviridae* family. The symptoms of TSBV are perforated brood scattered among capped brood, the presence of prepupae with typical raised and pointed heads in the comb cells, the dead larvae turn into sac-like structure filled with milky white fluid which when lifted up ruptured easily. Infective broods are odourless and show no ropiness. These symptoms were clearly visible in the present study. Studies conducted on the seasonal incidence of the disease revealed the prevalence of the disease during winter (October to January) season which prolonged to spring (Late January to March) season and was influenced by brood rearing. The TSBV disease incidence decreased in the month of April (2 / 30 colonies infected, 80 cells infected / colony) when the mean maximum temperature (34.3°C) was high while the relative humidity (RH 65%) and rainfall (62.7 mm) were low. In the succeeding four months namely May, June, July and August, the disease incidence was nil. The disease incidence started again in the month of September and reached a peak in the month of November (9 / 30 colonies were infected, 342 cells infected / colony) at which time the mean maximum temperature was low (28.6°C) while the RH (82%) and rainfall (191.3mm) were high. Thus the high disease incidence was found to be significantly correlated with low temperature and non significantly correlated with high RH and rainfall. The disease incidence also coincided with the active brood rearing period (November to March). Electron microscopic study of purified virus particles revealed that the virus is 32.3 ± 0.7 nm in diameter and icosahedral in shape. The morphology was found to be similar to that of sacbrood virus attacking Italian honey bee, *Apis mellifera*.

Key words: Thai sacbrood virus (TSBV), Epidemiology, Honey bee, TEM, Picorna virus.

Honey bees are truly eusocial insects, which play a vital role in the environment by pollinating both wild flowers and many agricultural crops as they forage for nectar and pollen, in addition to producing honey and beeswax. The essential and valuable activities of bees depend upon beekeepers maintaining a healthy population of honey bees, because like other insects and livestock, honeybees are subject to many diseases and pests. Virus-induced population decrease among honeybees thus affects not only the bee-farming economy but also other aspects of agriculture and plant ecology (Desphande and Chaphalkar, 2013).

TSBV is one of many insect viruses generally referred to as picornavirus-like. This presumed similarity has been based largely on biophysical properties and the presence of an RNA genome (Moore *et al.,* 1985). The sacbrood viruses fulfil many of the criteria of picornaviruses including having the correct size of single-stranded RNA (Bailey *et al.,* 1982). The genomic organization of SBV clearly resembles that of typical members of the Picornaviridae, with structural genes at the 5' end and nonstructural genes at the 3' end arranged in a similar order. Sequence comparison suggested that SBV is distantly related to infectious flacherie virus of the silkworm, a virus that possesses a genome of similar size and gene order (Ghosh *et al.,* 1999). Detection of honey bee viruses has been done through electron microscopy (Bailey *et al.,* 1964 and Rana et al., 1986). Recently, research is being conducted on the characterization of honey bee viruses on molecular basis through RT-PCR technique in different parts of the world. TSBV is considered to be a strain of SBV, but is more infections on Indian honey bee *A. cerana* and causes 5-30% colony loss annually.

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Materials and Methods

Sample collection

The infected honey bee prepupae collected from *Apis cerana indica* colonies, Department of Agricultural Entomology, Apiary located at TNAU, Coimbatore were used for the studies. The diseased samples were collected during August 2015 to February 2016. A part of the samples was used for purification and the remaining part was stored at -20° C for other studies. Healthy prepupae were also tested as control.

Symptomatology

Thirty colonies of *A. cerana indica* having almost same bee strength and queens of almost same age were selected out of the existing stock in the Department of Agricultural Entomology (Apiculture) TNAU, Coimbatore. Out of the selected colonies of *A.cerana indica*, those exhibiting typical symptoms of Thai sacbrood disease were studied regularly during the period of experimentation. The observations were recorded on alternate days with respect to state, change in colour, shape and texture of the brood in the diseased colonies. Finally the number colonies infected out of the thirty colonies were used in calculations.

Strength of colony

The adult bee population was recorded by calculating the total number of frames entirely covered by honey bees, percent area covered in partially covered frames and adding up to get an approximate figure. A fully covered brood frame with dense covering of bees on both sides was counted to be 1000 bees based on earlier estimation.

Brood area and number of brood cells

Honeybees seal their brood with wax once the larvae turn into pupae. Thus, the sealed brood in a colony is an indication of future adult population. The area of sealed brood was recorded using transparent OHP sheet with grid markings. The total brood area in the colony was calculated by finding out the total number of each 4 cm2 of area squares covering the brood area in all the brood frames and multiplying by four. The number of brood cells was obtained by multiplying the brood area in cm2 by 5.25 based on estimations made earlier. The number of brood cells infected in a colony was used directly in calculations or divided by total cells in a colony and multiplied by 1000 to get number infected cells / 1000 cells for the purpose of calculations.

Incidence of the disease

Studies on the incidence and prevalence of TSBV were conducted in TNAU apiary, Coimbatore during April 2015 to March 2016. Three colonies were selected at monthly intervals to assess the incidence of TSBV and adult population. These data were correlated with different weather parameters *viz.,* minimum and maximum temperatures, relative humidity and rainfall.

Purification of virus

Isolation and purification of Thai Sac Brood Virus of *A. cerana indica* was done as per the method given by Bailey *et al* (1981). Dead prepupae collected from different locations showing typical symptoms of the disease were used for the isolation of viral pathogen. The brood samples of *A.cerana indica* were ground in 0.5M potassium phosphate buffer (pH 8) containing 0.02 per cent ethylene diamine tetra acetate (EDTA) and 10 per cent diethyl ether followed by emulsification with 10 per cent (by volume) carbon tetrachloride (CCl4) in a pre cooled grinder for 2-4 min. Initially, grinding was done in a small volume of buffer with entire amount of CCl4 and later remaining buffer was added. The slurry was filtered through a small piece of cotton and the filtrate was clarified by a low speed centrifugation (500g, 10 min at 4^{o}C). The supernatant was collected and further clarified at 8000g for 10 min at 4^{o}C and then finally centrifuged at 100000g for 2h at 4^{o}C (HIMAC CP-80MX) to pellet/ sediment virus. After ultracentrifugation, only pellets were collected and resuspended in a small volume of potassium phosphate buffer (0.1, pH 8). Such partially purified virus suspension was purified further through sucrose density gradient ultracentrifugation to separate virus from remaining bee proteins. The partially purified virus preparations were stored overnight at 4^{o}C and then layered on 10, 20, 30 and 40 per cent (w/v) sucrose gradients for spinning at 75,000g for 2.5h at 4^{o}C in swinging bucket rotor (SW 25.1). Finally, the virus bands were collected with a glass syringe and the virus was pelleted at 100000g for 2h at 4^{o}C. The pelleted virus was resuspended in a small volume of 0.1M potassium phosphate buffer (pH 8.0) and stored at 4^{o}C or -20^{o}C for electron microscopy.

Electron Microscopy

Collodion coated copper grids were floated (film side down) on a drop of virus suspension for about 1 to 5 min. The grids were washed several times by dipping in distilled water to remove untrapped extraneous virus or host debris. These grids were drained by touching their edges with filter paper and immediately floating them on a freshly prepared 2 per cent aqueous uranyl acetate for 1-2 minutes. The grids were finally drained and air dried. These were screened under a transmission electron microscope (Model JEOL 100 CX-II) available at the Division of Virology, Indian Agricultural Research Institute, New Delhi.

Statistical Analysis

Correlation coffecient (r), coefficient of determination (R^2), mean and standard deviations were calculated wherever applicable using the analysis tool pack of MS EXCEL package.

Results and Discussion

Symptomatology

The typical symptoms of Thai sacbrood virus were observed and diseased colonies showed that the

mottled appearance and perforated brood cappings in combs (Fig. 1). The position of dead prepupae was straight in the comb cells lying lengthwise on

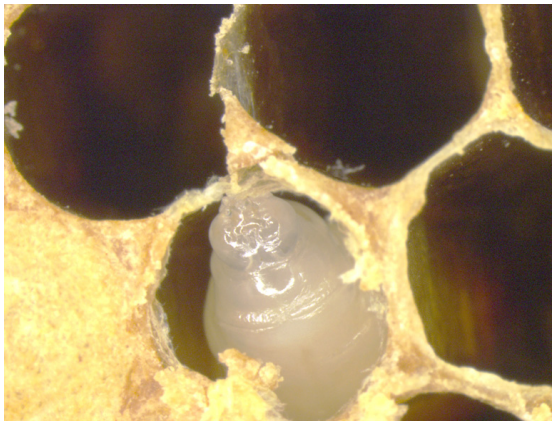

Fig. 1. Comb with typical symptom of TSBV

their backs with the formation of pointed tongue like projection at the tip of head pointing outwards. Death occurred on ninth day *i.e.* on second day after sealing of brood. The colour of the diseased prepupae changed from pearly white to pale yellow and finally dark brown. The prepupae had "sac-like" appearance which was typical symptom of the disease (Fig. 2). The larval cuticle was thin and loose which was filled with milky fluid. The dead prepupae became soft and boat-shaped scales. They were odourless and no ropiness was observed when matchstick was pierced through such dead brood and taken out slowly. The dead larvae dried up in 10 days and formed boat shaped scales lying at the floor of the comb.

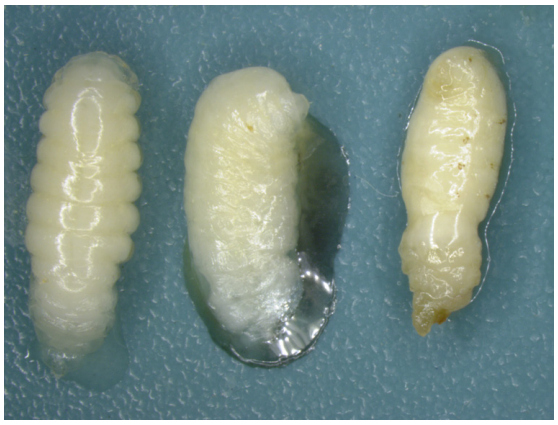

Fig. 2. TSBV infected prepupae showing sac-like appearance and colour changes from pale white to dark

These symptoms were almost identical to those described for Thai sacbrood virus of *A. cerana* by different researchers in India (Kshirsagar *et al.,* 1982; Joshi and Verma, 1985; Devanesan and Jacob, 2001). Infected larvae failed to pupate. In case of severe infection, the dead larvae were left in the cells as the nurse bees were unable to do their work properly. Later the hives had unpleasant odour which resulted in behavioural changes in worker and queen bees. Finally the bees quit of the colony. Besides the

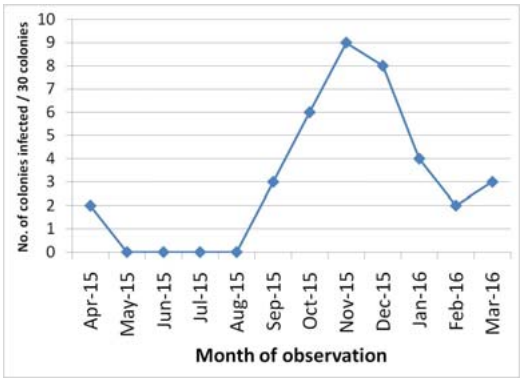

Fig. 3a. Seasonal occurrence of TSBV infected colonies in the apiary

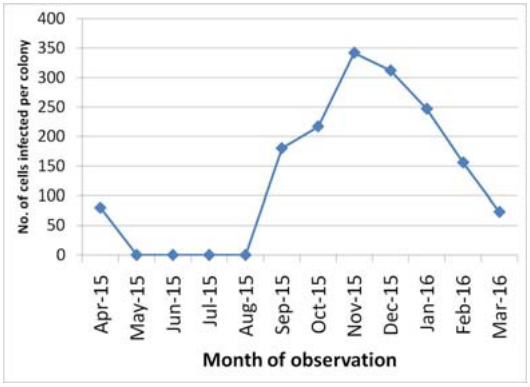

Fig. 3b. Seasonal variations in the number of TSBV infected cells per colony

behavioural changes in the worker bees due to TSBV infection, emergence of new brood gets inhibited resulting in a decrease of worker bee population, especially the house bees (Devanesan and Jacob, 2001). Mass absconding of *A. cerana indica* colonies infected with TSBV has been reported earlier by Phadke, 1983; Joshi and Verma, 1985 and Jacob *et al.,* 1992.

Incidence of TSBV

Studies were conducted on the incidence of TSBV during the period of April 2015 to March 2016 and the results revealed that *A. cerana indica* colonies

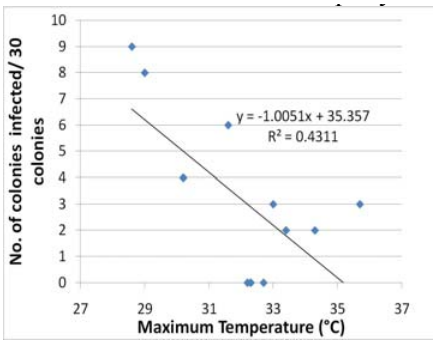

Fig. 4a. Effect of maximum temperature on number of infected colonies

were affected from September to April. The seasonal incidence of TSBV in relation to weather parameters at Coimbatore conditions showed a clear pattern.

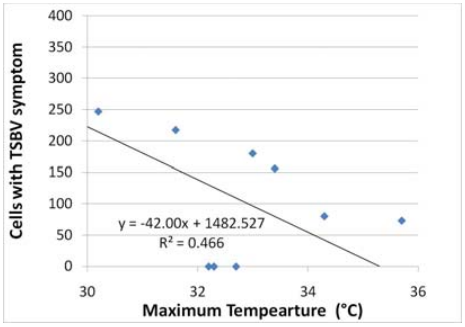

Fig. 4b. Effect of maximum temperature on number of prepupae showing TSBV symptoms / colony

The study showed that the disease was noticed in colonies during active brood rearing season *i.e.,* winter (October to January) and prolonged to spring

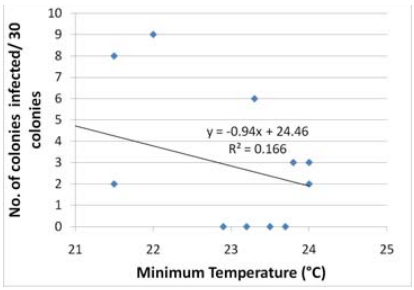

Fig. 5a. Effect of maximum temperature on number of infected colonies

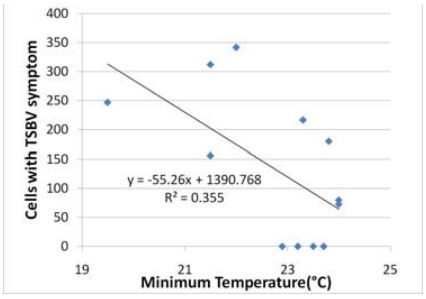

Fig. 5b. Effect of maximum temperature on number of prepupae showing TSBV symptoms / colony

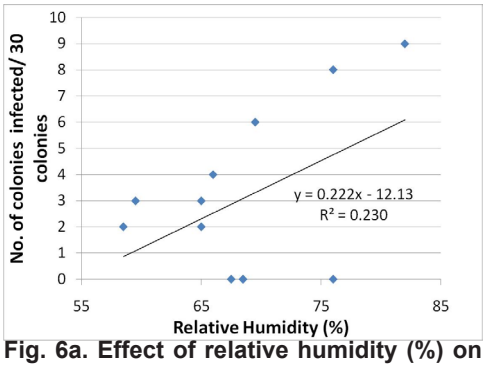

Fig. 6a. Effect of relative humidity (%) on number of infected colonies

(Late January to March) season. Kshirsagar et al (1982) recorded the incidence of TSBV during winter also in addition to early summer in Bihar and North-Eastern states of India. Freiberg *et al.* (2012) also stated that the virus was detected in larvae, foraging and nurse bees which had symptoms of SBV at the beginning of the winter season in June 2011 in Brazil.

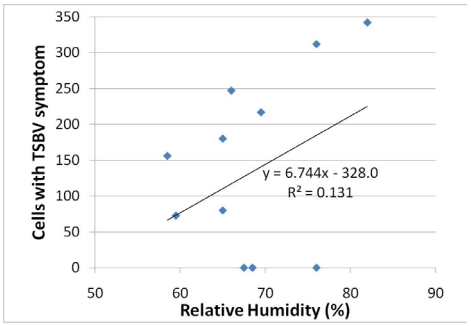

Fig. 6b. Effect of relative humidity on number of prepupae showing TSBV symptoms / colony

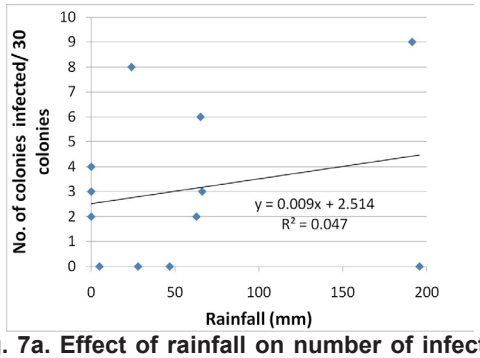

Fig. 7a. Effect of rainfall on number of infected colonies

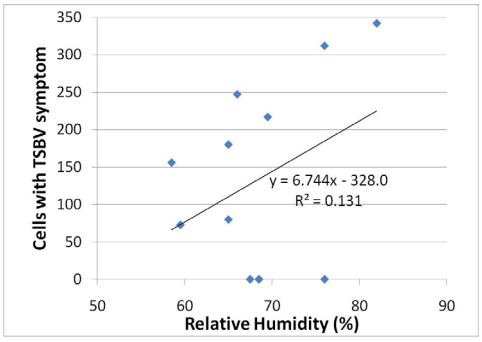

Fig. 7b. Effect of rainfall (mm) on number of prepupae showing TSBV symptoms / colony

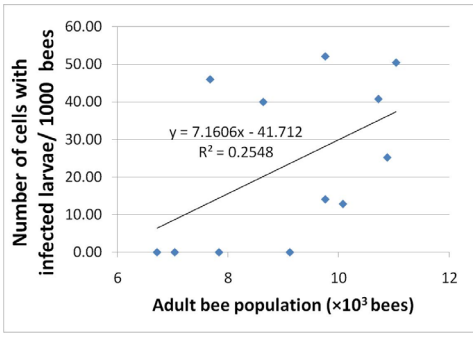

Fig. 8a. Effect of total adult bee population on number of infected prepupae for every 1000 adult bees

Seasonal incidence of TSBV incidence with weather parameters

During April 2015, only two colonies were infected out of 30 colonies and just 75 cells showed disease symptom, when the mean monthly maximum and

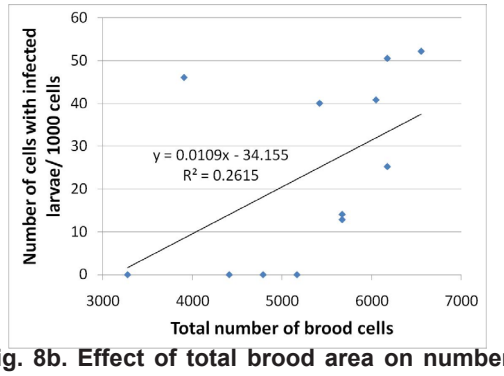

Fig. 8b. Effect of total brood area on number of infected prepupae for every 1000 prepupae

minimum temperatures were high (34.3°C and 24°C, respectively) and the RH and rainfall were low (65% and 62.7 mm, respectively). In the succeeding four months namely May, June, July and August, the disease incidence was nil. On the other hand, the TSBV incidence started again in the month of

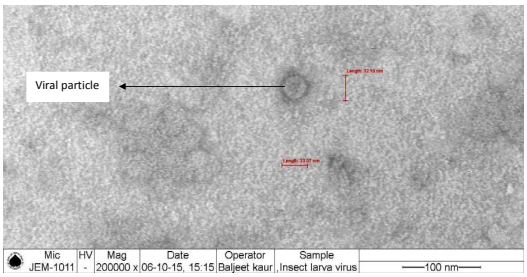

Fig. 9. Transmission Electron micrograph of Thai sacbrood virus (mean diameter of the viral particle = 32.3 ± 0.7 nm)

September and reached a peak in the month of November 2015 (nine colonies out of 30 colonies) at which time the maximum temperature and minimum temperature were low (28.6°C and 22°C, respectively) and RH and rainfall were high (82% and 191.3 mm, respectively, Fig 3a. and 3b.).

Table 1. Relationship of weather parameters with the number of Indian bee colonies infected by TSBV in the apiary

Weather parameters	Correlation with the number of Indian bee colonies infected by TSBV / 30 colonies	
	r	R^2
Maximum temperature (°C)	-0.66	0.43*
Minimum temperature (°C)	-0.41	0.17ns
Relative humidity (%)	0.48	0.23ns
Rainfall (mm)	0.22	0.05ns

*Significant at p=0.05, ns= non-significant

The overall mean incidence of TSBV observed during the study period indicated that the number of colonies infected fluctuated in a predictable manner. Seasonal incidence of TSBV incidence was significantly correlated with maximum temperature. The number of colonies infected had significant negative relationship (r = -0.66, R^2= 0.43, Fig. 4a, Table 1.) with maximum temperature. Simlarly, the number of cells with infected larvae also decreased with increase in maximum temperature (r= - 0.68, R^2 = 0.47, Fig. 4b. Table 2.).

Table 2. Relationship of weather parameters with the number of cells with TSBV infected prepupae per colony of Indian honey bee

Weather parameters	Correlation with the number of TSBV infected prepupae per colony	
	r	R^2
Maximum temperature (°C)	-0.68	0.47*
Minimum temperature (°C)	-0.60	0.36*
Relative humidity (%)	0.36	0.13ns
Rainfall (mm)	0.13	0.02ns

*Significant at p=0.05, ns= non-significant

A non-significant negative relationship was observed between number of colonies showing TSBV symptoms with minimum temperature (r = -0.41, R^2 =17, Fig. 5a). The number of cells with infected larvae per hive showed a significant negative relationship (r = -0.60, R^2 =36, Fig 5b.) with minimum temperature.

The prevailing relative humidity was non-significantly positively correlated with TSBV infected colonies (r = 0.48, R^2 = 0.23, Fig. 6a.) and with number of cells with TSBV infection / colony (r = 0.36, R^2 = 0.13, Fig 6b).

Table 3. Relationship of adult population or brood area with the TSBV infected prepupae of Indian honey bee

Adult population or brood area	Correlation with the number of TSBV infected prepupae	
	r	R2
Adult population / colony	0.503	0.254*
Total number of brood cells / colony	0.511	0.261*

* Significant at p=0.05, ns= non-significant

Rainfall was also non-significantly positively correlated with TSBV infected colonies (r = 0.22, R^2 = 0.05, Fig 7a) and with number of cells with TSBV infection / colony (r = 0.13, R^2 = 0.02, Fig 7b).

The results of our studies are in concurrence with Ball (1999) who observed that under natural environmental conditions sacbrood abates and usually disappears spontaneously during summer

even though larvae are easily infected by feeding them the virus at any time of the year. Further, they become more susceptible to chilling and loss in the field or from the bee cluster, especially in winter.

Correlation analysis of number of cells with infected larvae/ 1000 cells with total number of brood cells and adult bee population

The severity of TSBV disease in the colonies *A. cerana indica* was studied and found that the disease symptom was significantly positive correlated with total number of brood cells and adult bee population (Table 3). The number of cells with infected larvae/1000 cells was also significantly positively correlated with respect to brood cells (r = 0.5114) (Fig. 8a) and adult bee population (r = 0.5048) (Fig. 8b). Joshi and Verma (1985) also found that the infection of TSBV was recorded during brood rearing season in India and percent infestation increased with increase of brood rearing. SBV infection appears mainly in spring when the brood rearing begins (Ritter, 1996). This might be because when the adult population increases ultimately foraging activity from different places where the inoculation of the disease was carried over with pollen by other bees to the colonies.

Electron microscopy

Electron microscopy of the virus preparations from the samples were done to view the presence of virus particles along with shape and size. Electron microscopic study revealed the presence of large number of isometric virus particles of 32.3 ± 0.7 nm diameter in the processed samples of diseased prepupae of *A. cerana* (Fig. 9). Earlier studies have indicated that SBV particles are 28 nm in diameter, non enveloped, round and feathureless in appearance (Ghosh *et al.,* 1999; Bailey 1968 and Mingxiao *et al.,* 2011). Bailey *et al.,* (1982) and Zhang *et al.,* (2001) also studied the virus preparation from diseased prepupae of *A. cerana* using electron microscope and reported the presence of a very large number of isometric particles of TSBV with 30 nm in diameter. These results were also reported from Indian bee hive, *A. cerana* (Rana *et al.,* 1986 and Rana *et al.,* 1991) from India. Further studies at molecular level on the virulence of TSBV on *A. cerana indica* can help to devise management measures to save the bees and beekeeping industry from this serious disease.

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