Effect of feeding Silkworm (*Bombyx mori* L.) on Mulberry treated with Bio and Inorganic Fertilizer on Antennal Sensilla

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Abstract

The present work was directed to study the effect of bio and inorganic fertilizer treatments on the ultra structure antennae of female and male silkworm (*Bombyx mori* L.), as a trail to understand the effect of the type of fertilization on mulberry trees and silkworm antennal sensilla. The sensory organs on the antennae were visualized by scanning electron microscopy. The results showed that the surface of the antenna is covered with scales overlapped together and is provided with sense sensilla that found on the ventral and lateral surface of the antenna. On the silkmoth antennae, six different types of sensilla have been identified: Olfactory sensilla trichodea, s. basiconica, s. coeloconica, putative gustatory s. chaetica that are multimodal receptors, s. styloconica, that are thought to be thermo- and hygroreceptors and the mechano-sensory sensilla (squamiformia). Silkworm (*B. mori*) is a monophagous insect that drives almost all required nutrients for its growth and development from mulberry leaf. Mulberry trees were treated with bio fertilizes, chemical fertilizers and the mixture of them through the addition in soil. The results of chemical fertilizer, bio-fertilizer and its mixture increased the growth, development, health, feed consumption and larval weight, total larval duration and this reflected on the number and length of sensilla. Chemical fertilizer, bio-fertilizer and its mixture increased the size of sensilla than control. Moreover, application by bio-fertilizer resulted in better values larval weight, total larval duration and the size of sensilla. Both chemical and bio fertilizes exhibited better values for pupal weight which reflect on the size of sensilla.

Keywords: Silkworm, *Bombyx mori*, Antenna, Sense organs, SEM, Mulberry leaf, Bio-fertilizers, Inorganic fertilizers.

1 Introduction

Insects, like other invertebrate and vertebrate animals have evolved chemical sensing devices for detecting stimuli that have adaptive value, so that most of their receptors are sensitive to a specific range of available stimuli and this sense has become further differentiated into two categories ie. chemo and mechano receptors (Tousson and Gaaboub, 2004 and Gaaboub and Tousson, 2010). The antennal or tarsal receptors could influence the various modes of behaviour including avoidance (White & Chapman, 1990; Gaaboub, 2000; and Gaaboub *et al*., 2005), detection and selection of food sources (Blaney, 1974; Gaaboub and Hustert, 1998) and selection oviposition sites (Staedler *et al*., 1995; Kalogianni, 1996; Tousson *et al*., 1999; Newland *et al*., 2000).

Forty years of studying insect sensilla with the electron microscope have resulted in a general bauplan that-like a theme with variations-extends throughout all insect orders. Typical modality-specific features of the cuticular apparatus and of the receptor cell endings in many cases allow for correct prediction of the functional modality of a sensillum type before electrophysiological recording can yield final confirmation (Hunger and Steinbrecht, 1998).

The morphology and distribution of the sensilla present on some insect appendages as antennae and tarsi have been studied in different species of Lepidopterous insects, e.g., *Agrotis segetum* (Hallberg, 1981), *Yponomeuta vigintipunctatus* (Cuperus, 1983 and 1985a), *Manduca sexta* (Shields and Hildebrand, 2001).

The domesticated silkworm, *B. mori* L., is a monophagous insect that can be raised on fresh mulberry leaves (*Morus alba* L.). Good quality leaf production in mulberry is highly dependent on the supply of various inputs especially nitrogen and phosphorus fertilizers (Nasreen *et al.,* 1999 and El-Khayat *et al.,* 2013). The quality of mulberry leaf fed to silkworms is the most important factor that influences successful cocoon production (Singheal *et al.,* 1999). Usage of a combination of organic and inorganic fertilizers, increasing the carbohydrate and crude protein percentage of the mulberry leaves significantly increased the silkworm larval body weight, silk gland weight and ultimately the cocoon yield etc (Jadhav *et al.,* 2000).

The present studies aims to determine the impact of bio-fertilizers, inorganic fertilizers application and their mixture on the ultra structure antennae sensilla of female and male silkworm (*B. mori*), as a trail to understand the relation between different fertilizers on mulberry trees and antennal sensilla

2 Materials and Methods

Rearing technique

The mulberry silkworm (*B. mori*) eggs were obtained from the Sericulture Research Department of Plant Protection Research Institute, Agricultural Research Center, Ministry of Agriculture and Land Reclamation in Giza, Egypt. It was established in the Department of Plant Protection, Faculty of Agriculture Moshthour, Benha University and the department of applied Entomology, Faculty of Agriculture, Alexandria University during two successive spring seasons (2014 and 2015).

Nutrition methods and Food intake

The native mulberry (*Morus alba* var *rosa*) leaves were used to feed the mulberry silkworm larvae. Mulberry trees were treated with bio fertilizes, chemical fertilizers and the mixture of them through the addition in soil. Two bio-fertilizers were used; nitrogen containing *Azotobacter* spp. and *Azosprillum* spp. and phosphoren contains *Bacillus magatherium*. Also, chemical fertilizers were used as Nitrogen in the form of Urea (46 % N), phosphorus in the form of super phosphate (16 % P_2O_5) and potassium in the form of potassium sulphate (48 % K_2O). Treatments of bio-fertilizes, chemical fertilizers and their mixture were as shown in table (1).

Bioassay tests of Silkworm (*B. mori*)

The newly hatched silkworm larvae were divided into ten groups including control. Each treatment represented by three replicates. Each replicate have 50 larvae. For each group, chopped leaves were offered four times daily to young silkworms (first, second and third instars). While, whole leaves were offered for grown silkworm (fourth and fifth instars). The following characters were studied: fresh cocoon weight (FCW), cocoon shell weight (CSW), pupal weight (PW).

Scanning Electron Microscopy (SEM)

To identify the different sensory receptors on the antenna of the female and male silkworm (*B. mori*) moths were immersed in hexane and shaked for several minutes to remove the cuticular wax layer. Antennae rinsed in chloroform, dehydrated in ethanol and air-dried. After drying, they were sputtering coated with gold-palladium and examined using a scanning electron microscope (LEO –UIF: Leo 438VP) of Faculty of Science, Alexandria University.

Statistical Analysis

The statistical analysis was carried out using ANOVA with two factors under significance level of 0.05 for the whole results using SPSS (ver. 19) and complete randomized design were used according to (Steel *et al*., 1997). Multiple comparisons were carried out applying LSD.

3 Results and Discussion

In the silkmoth, the flagella are comb-shaped (Fig. 1) and the antennae of the male are somewhat larger than those of the female. Antenna 5 mm long and 1.2 mm width. Usually, the distal part, the flagellum is most prominent. The antennae have two rows of antennal branches (ribs), the shorter anterior and the longer posterior branches. Each rib ca. 68 µm wide. The shape can be thought of as an adaptation to optimise odorant catch. The males are described as having denser sensory hairs.

Scanning electron microscopy showed that flagellum is covered by many types of sensilla (Figure 3 B&C). Sensilla can usually be classified by their external appearance (Fig. 3). For example, olfactory sensilla generally have small pores or slits in their cuticular wall. These opening provide an interface between the inside and the outside to allow the exchange of odorant molecules. Six different types of sensilla have been identified: sensilla trichodea, sensilla basiconica, sensilla coeloconica sensilla chaetica , sensilla styloconica, and sensory sensilla squamiformia (Kaissling *et al*., 1978; Steinbrecht, 1988 and 1989; Pophof, 1997; Hunger and Steinbrecht, 1998).

- **Sensilla coeloconica**

On the silkmoth antenna, the s. coeloconica (pit pegs) stand on the outer side of the basketlike flagellum and the dorsal side of each branchs and stem. The most proximal segments of flagellum are devoid of them (Fig. 2 A-D). The coeloconic sensilla of *B. mori* double-walled, wall-pore sensilla are 10 µm long, located in pits, and longitudinally grooved in their distal half (grooved surface 30 μm^2).
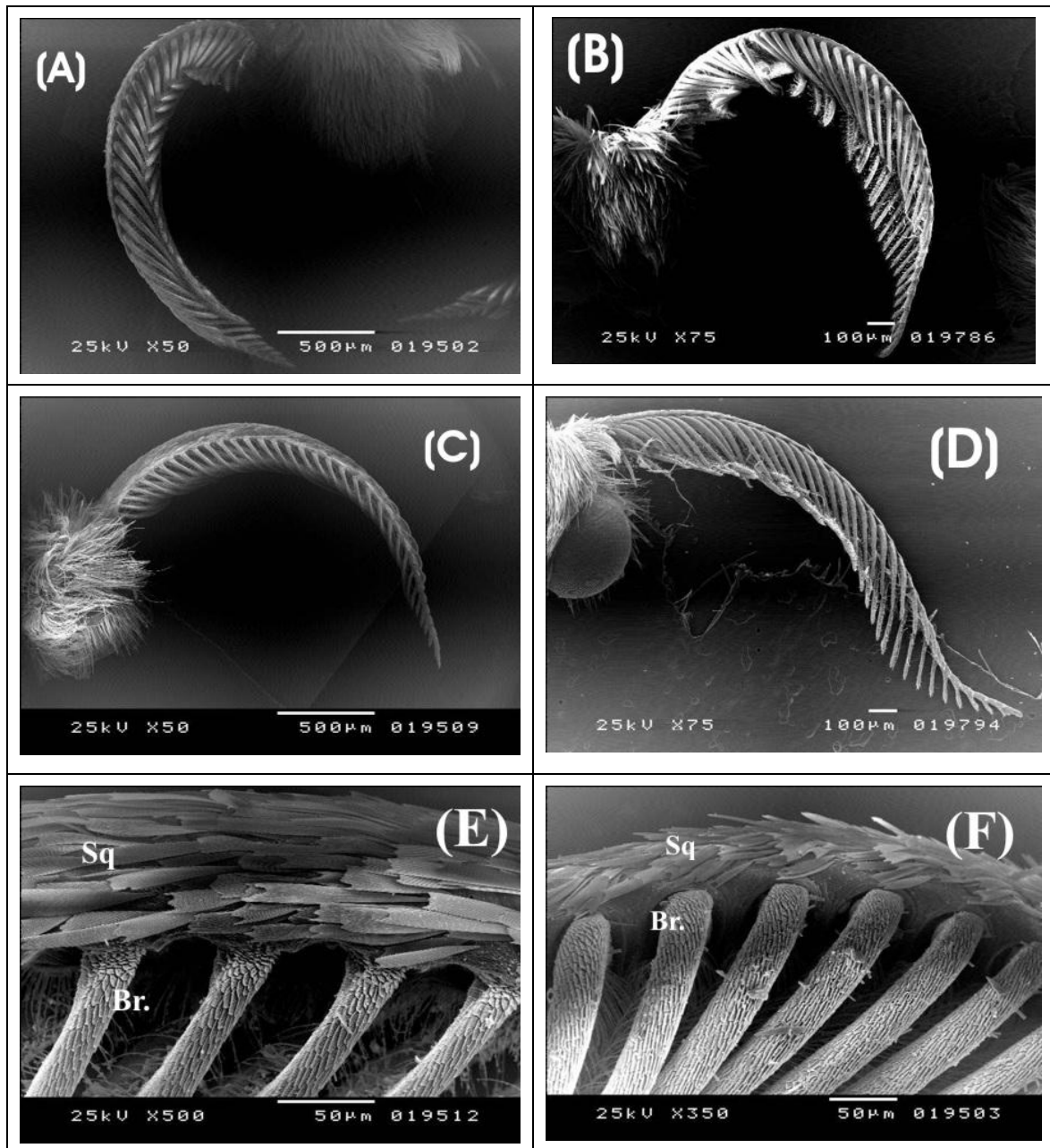
(Fig. 1): External morphology of silkmoth antennae by means of scanning electron microscopy (SEM). (A) External aspect of the head male and female capsule and the antennae (A&C as control) (B&D) feeding on fetilized mulberry by chemical fertilizer 400 gN +300 gP + 250 gK, respectively. Structure of a branch of female and male the flagellum (E&F) bearing numerous sensillar hairs, respectively.
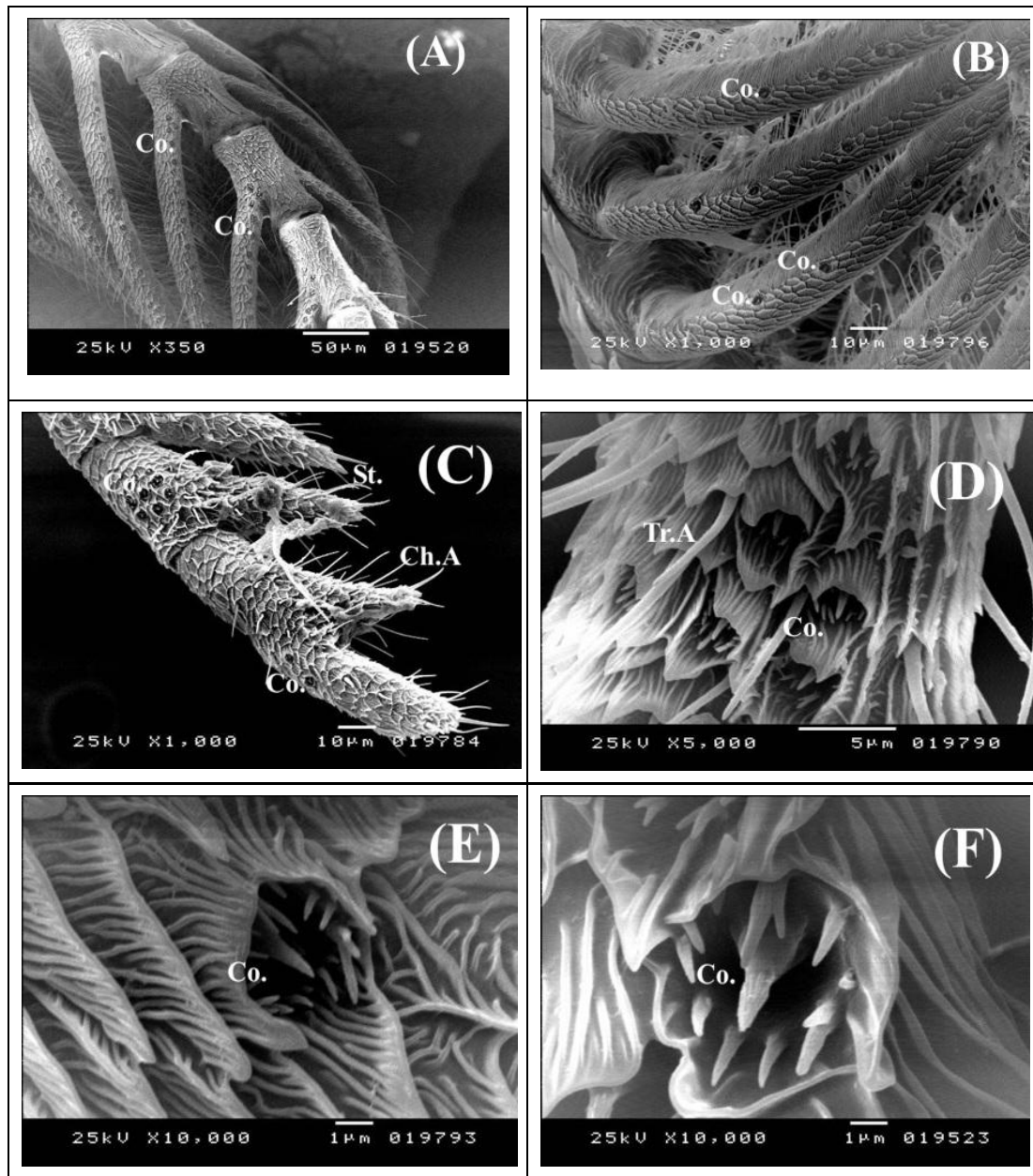
Fig. 2): *B. mori*, scanning electron micrograph showing tip of antennal branch and branches with sensilla coleonica (Co.) in the grooves, sensilla basiconic (Ba.) and sensilla tricodium (Tr.) this samples treated with mixtures fertilizers (260gN+195gP +250gK+500gNitroben+ 150g Phosphorin) female (B&C) and male fertilized by chemical fertilizers 400gN+300gP+250gK (A,D,E&F).

Their number increases reaching to the maximum in the distal third of the antenna. The total number of pit pegs per antenna is between 580 and 790 (mean of 5 male and 5 female antennae: 694 $\pm$48).The numbers of sensilla on the right and left antenna of the same individual differed by 3-5 % of the total. The sensilla coeloconica in the silk moth, *B. mori,* show no sexual dimorphism (Hunger and Steinbrecht, 1998). Bio-fertilizers increased the size of sensilla coeloconica (Tables 2&3).

-Sensilla styloconica

The sensilla styloconica of *B. mori* are slender located in narrow grooves at the very tip of the antennal branches (Fig. 3 E&F). Only the most proximal branches of the antenna bear no styloconic sensilla. There may be one, two or three on a single branch tip. A few of them are located on a prominent stylus amidst the sensillum field of the sensilla trichodea and sensilla basiconica (Steinbrecht 1989). Sensilla styloconica per antenna are about 86 sensilla. Different fertilizers and their mixtures affected the size of sensilla styloconica of both sexes of mulberry silkworm (Tables 2&3). The size of sensilla showed significant difference between control, chemical, bio-fertilizers and their mixture. Bio-fertilizers increased the size of sensilla styloconica compared with the control (Tables 2&3).

-Sensilla basiconica

Sensilla basiconica are peglike or conelike process characterized by: short, have a thinner cuticular wall pitted by a higher density of pores, have a larger number of pore tubules and have many distal dendritic branches. They are arranged in oblique linear rows along the base of deep furrows. The antennal flagellum of female and male *B. mori* bears two types of basiconic sensilla. The first type averaged 22.1& 23.3 μm in length, while the second type averaged 15.1&16.11μm in length, respectively.There are two types of basiconic sensilla, type-A and B. The fine structure of the basiconic sensilla of female *M. sexta* most closely resembles that of the large basiconic sensilla of female and male *B. mori* (Steinbrecht, 1973,1980; Steinbrecht and Gnatzy, 1984), males of *M. sexta* (Keil, 1989; Lee and Strausfeld, 1990), *Antheraea polyphemus* and *A. pernyi* (Keil, 1984a), and *Y. vigintipunctatus* and *A. orana* (Cuperus, 1985a &b). Bio-fertilizers increased the size of sensilla basiconic type A&B compared with the control (Fig. 3B&D. Tables 2&3).

Comparisons between type-A and type-B basiconic sensilla reveal a sexual dimorphism. Type-A basiconic sensilla of males are longer, with wider base diameter and typically are innervated by either two or three sensory cells with branched dendrites. Type-B basiconic sensilla of males are shorter and typically innervated by one, two, or three branched sensory cells. In contrast, both types of basiconic sensilla of females are innervated by three sensory cells with branched dendrites. The basiconic sensilla of female *A. orana* (Den Otter *et al*., 1978) and *Yponomeuta* species (Van der Pers and Den Otter, 1978) are reported to be sensitive to plant odors, as are similar sensilla of both sexes of *B. mori* and male *A. pernyi* and *Sphinx pinastri* (Schneider and Steinbrecht, 1968). The two types of basiconic sensilla of female *M. sexta* were not physiologically characterized. Based on their similarity to morphologically or physiologically characterized female olfactory basiconic sensilla of other insects, these sensilla of female *B. mori* may also be olfactory and possess sensory cells tuned to plant odors.

-Sensilla trichodeum

The trichoid sensilla are hair like with single multiporous walled (Fig 3 A-E). The antennal flagellum of female and male *B. mori* bears two typs trichoid sensilla. The first type averages 34.1&35.04 μm in length and is innervated by two sensory cells. The second type averages 26.1&27.06 μm in length, respectively. The sensilla have a diameter of 4 μm near the base of the sensillum, and tapers to 1 μm at the tip (Fig. 3 B&D). The type-B trichoid sensillum is slightly shorter than the type-A trichoid sensillum and averages 26.1 μm in length. This sensillum has a diameter of 2 μm near its base, and tapers to 0.4 μm at the tip. The fine structure of type-A trichoid sensilla in females is very similar to the long, pheromone sensitive type A trichoid sensilla of males. In that both are taller than type-B trichoid sensilla of females or type B trichoid sensilla of males, have cuticular shafts with a similar base diameter, have cuticular walls that are thinner than those of type-B trichoid sensilla, have similar transverse plates on the cuticular shaft that are pitted by pores, each pore associated with a similar number of pore tubules that extend into the shaft lumen and are innervated by two sensory cells with unbranched distal dendrites (Sanes and Hildebrand, 1976; Keil, 1989; Lee and Strausfeld, 1990).

The fine structure of the trichoid sensilla of female *M. sexta* very closely resembles that of the trichoid sensilla of female and male *B. mori* (Steinbrecht, 1973, 1980; Steinbrecht and Gnatzy, 1984), males of *M. sexta* (Keil, 1989; Lee and Strausfeld, 1990), *Antheraea polyphemus* and *A. pernyi* (Keil, 1984a), *Yponomeuta vigintipunctatus, Adoxophyes orana*, and female *Yponomeuta cagnagellus* (Cuperus, 1985a).

The fine structure of type-B trichoid sensilla of females is comparable to that of type-B trichoid sensilla of males. Both are shorter than the type-A trichoid, they are similar in length, have a similar base diameter, have a wall thickness similar to that of type-A trichoid and have pores that are associated with a similar number of pore tubules that do not extend to the shaft lumen (Sanes and Hildebrand, 1976; Keil, 1989; Lee and Strausfeld, 1990). Studies by Cuperus (1983) and Cuperus *et al*. (1983) revealed a sexual dimorphism with respect to the number of trichoid and basiconic sensilla in eleven species of small ermine moths (*Yponomeuta* spp). Bio-fertilizers increased the size of trichoid sensilla A&B (Tables 2&3).

- Sensilla chaetica

Sensilla chaetica are long, stiff, ridged hair-like structures without wall pores that are set into a socket at the base and radially distributed in whorls at the flagellar margins. They are scattered at the proximal (long) and distal (short) branches of each flagellar segment (Fig. 3 F, G&H. Table 2&3 indicated that the Bio-fertilizers increased the size of sensilla chatica. Sensilla chaetica (both long and short) are
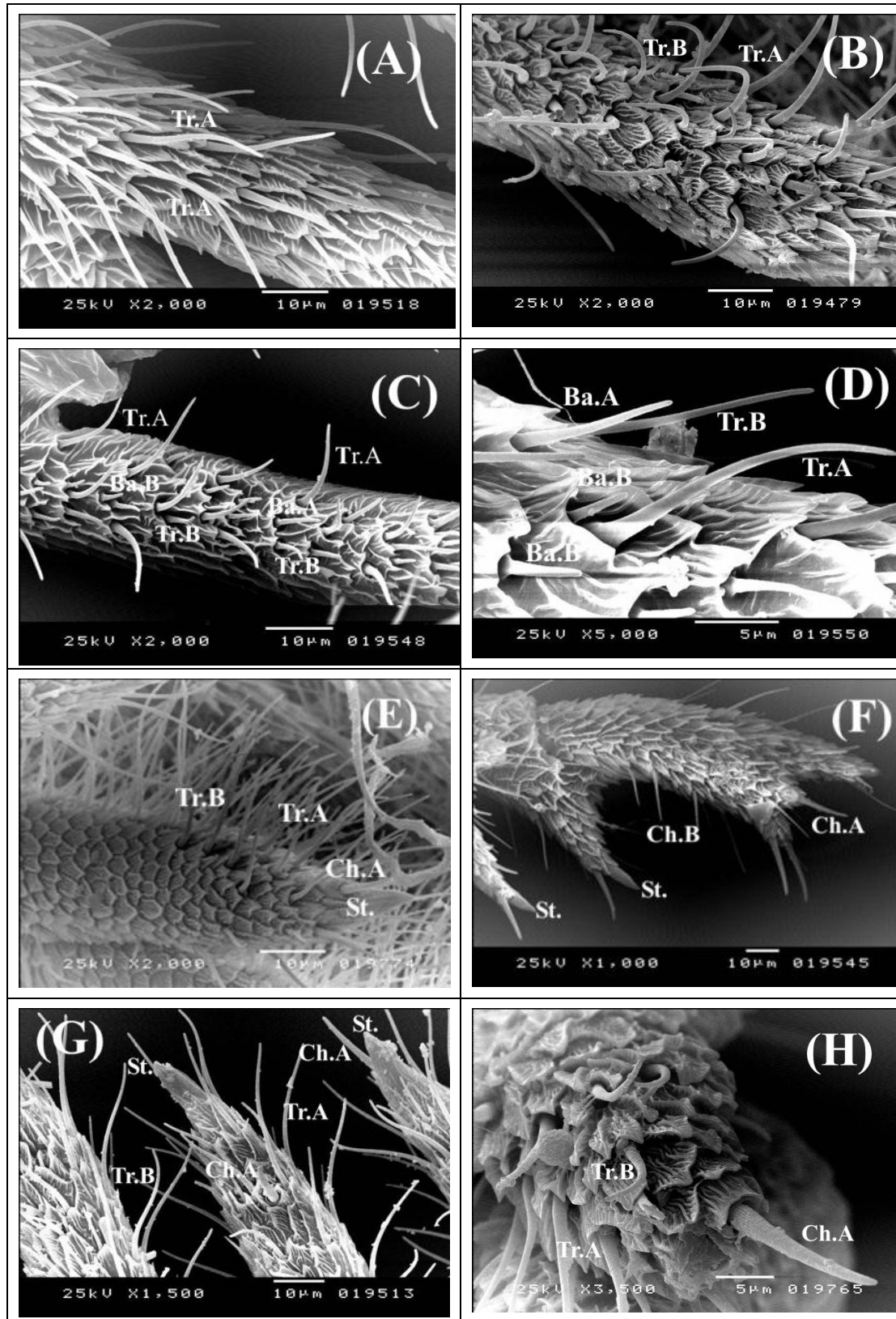
Fig. (3): *B. mori*, scanning electron micrograph showing tip of female antennal branch and branches with sensilla trichodium (Tr.) and basiconic (Ba). as male control (A) and the treated by biofertilizer 500g Nitrogen + 150g Phosphorin (B). Female sensilla trichodium and basiconic A&B (C&D) treated by chemical fertilizer 400gN+300gP+250gK. Male sensilla styloconica (St.) which are protected by s. chaeticum (ch), trichodea (Tr.) (E, F, G & H).

Table (1): Ingredients of different fertilizers on Mulberry (*Morus alba* L.) used for feeding larval silkworm (*B. mori* L).

			Type of Fertilizer					Average Fresh cocoon weight (g)		Average Shell cocoon weight (g)		Pupal weight PW (g)	
			Urea (46 %N) (g)	super phosphate (16 % P_2O_5) (g)	potassium sulphate (48 % K_2O) (g)	(Bio-N) (g)	(Bio- P) (g)	2014	2105	2014	2105	2014	2105
bio-fertilizers (1)	T1		0	0	0	700	300	1.88 ±0.13	1.51 ±0.11	0.45 ±0.09	0.34 ±0.08	1.43 ±0.12	1.17 ±0.1
	T2	(Recommended dose)	0	0	0	500	150	1.77 ±0.14	1.45 ± 0.12	0.44 ±0.09	0.32 ±0.068	1.33 ±0.13	1.13 ±0.08
	T3		0	0	0	250	75	1.64 ±0.48	1.54 ±0.61	0.40 ±0.09	0.34 ±0.078	1.24 ±0.1	1.2 ±0.07
chemical fertilizer (2)	T4		800	600	500	0	0	1.89 ±0.13	1.39 ±0.11	0.45 ±0.08	0.34 ±0.09	1.44 ±0.12	1.05 ±0.09
	T5	(Recommended dose)	400	300	250	0	0	1.95 ±0.17	1.59 ±0.11	0.46 ±0.09	0.34 ±0.084	1.49 ±0.11	1.25 ±0.12
	T6		200	150	125			1.84 ±0.14	1.44 ±0.12	0.44 ±0.09	0.33 ±0.073	1.4 ±0.18	1.11 ±0.08
Mixed fertilizer (1+2)	T7		520	390	500	700	300	1.66 ±0.13	1.48 ±0.11	0.39 ±0.076	0.31 ±0.081	1.27 ±0.12	1.17 ±0.09
	T8	(Recommended dose)	260	195	250	500	150	1.70 ±0.15	1.39 ±0.12	0.41 ±0.08	0.29 ±0.07	1.29 ±0.09	1.1 ±0.09
	T9		130	97.5	125	250	75	1.63 ±0.12	1.29 ±0.14	0.39 ±0.07	0.27 ±0.081	1.24 ±0.1	1.02 ±0.08
Control			0	0	0	0	0	1.71 ±0.14	1.29 ±0.11	0.40 ±0.08	0.26 ±0.08	1.31 ±0.1	1.03 ±0.1
F								1.041	1.034	1.121	1.114	1.442	1.424
LSD								0.18	0.17	0.1	0.12	0.1	0.1

Table (2): Effect of different fertilizers on length (µm) of different types of sensilla female *B. mori* L. during 2014 & 2015 seasons

Type fertilizer		Sensilla trichodea				S. basiconica				S. Coeloconica		S. chaetica				S. styloconica	
		Type A		Type B		Type A		Type B				Type A		Type B			
		2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Bio fertilizer (1)	T1	34.2	34.4	26.1	26.4	22.3	22.3	15.06	15.4	10.09	10.2	202	204	168.6	168.5	10.23	10.5
	T2	34.1	34.3	26.1	26.4	22.1	22.4	15.2	15.4	10.1	10.2	203	204	168	168.3	10.3	10.54
	T3	34.1	34.5	26.2	26.2	22.2	22.3	15.07	15.43	10.2	10.3	204	205	168	168.1	10.2	10.42
	Mean	34.13	34.4	26.13	26.33	22.2	22.33	15.11	15.41	10.13	10.23	203	204.33	167.2	168.3	10.24	10.48
Chemical fertilizer (2)	T4	34.06	34.3	26.1	26.3	22.09	22.1	15	15.4	10.2	10.2	201.3	205	167.3	167.2	10.2	10.4
	T5	34.1	34.2	26.2	26.34	22.2	22.3	15.1	15.2	10.1	10.3	203	204	167.2	167.1	10.1	10.5
	T6	34.1	34.1	26.09	26.2	22.1	22.4	15.2	15.4	10	10.1	202	203	167.1	167	10.3	10.4
	Mean	34.086	34.2	26.13	26.28	22.13	22.14	15.1	15.33	10.1	10.2	202.1	204	167.2	167.1	10.2	10.43
Mixture (1+2)	T7	34.08	34.2	26.1	26.3	22.3	22.2	15	15.3	10.1	10.2	202	203	167.4	167.2	10.16	10.3
	T8	34.1	34.24	26.08	26.2	22.05	22.2	15.1	15.32	10.1	10.1	202	204	167.2	167.1	10.2	10.41
	T9	34.14	34.13	26.2	26.1	22.06	22.1	15.07	15.1	10.2	10.3	203	204	167	167	10.07	10.32
	Mean	34.106	34.19	26.12	26.2	22.13	22.16	15.056	15.24	10.13	10.2	202.33	203.66	167.2	166.8	10.143	10.343
Control		34	34.1	26	26.1	22	22.1	15	15.1	10	10.1	201	201.1	166.2	166.4	10.1	10

Table (3): Effect of different fertilizers on length (µm) of different types of sensilla male *B. mori* L. during 2014 & 2015 seasons

Type fertilizer		Sensilla trichodea				S. basiconica				S. Coeloconica		S. chaetica				S. styloconica	
		Type A		Type B		Type A		Type B				Type A		Type B			
		2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Bio fertilizer (1)	T1	35.4	35.6	27.3	27.5	24.4	24.4	16.46	16.5	11.19	11.3	205	206	169.6	170.6	11.85	11.94
	T2	34.84	35.14	26.71	27.4	23.3	24.4	16.4	16.42	11.14	11.25	204	205	169	170.2	11.7	11.5
	T3	34.1	34.4	26.2	26.3	22.2	23.3	15.27	15.41	10.61	10.51	204	204	168	169	11.52	11.2
	Mean	34.78	35.04	26.73	27.06	22.3	23.3	16.04	16.11	11.096	11.02	204.33	205	168.86	169.93	11.69	11.54
Chemical fertilizer (2)	T4	34.86	35.5	27.3	27.3	23.19	23.3	16	16.4	11.2	11.4	202.3	206	168.3	170.2	11.6	11.4
	T5	34.51	34.8	26.9	27.14	23.1	23.3	16.1	16.2	11.1	11.3	203.8	205	168.2	169.1	11.1	11.5
	T6	34.31	34.8	25. 9	26.82	22.91	23.23	15.92	16.2	11	11.1	203.5	203	168.1	168	11.3	11.4
	Mean	34.56	35.03	26.7	27.08	23.07	22.14	16.01	16.26	11.1	11.2	203.2	204.66	168.2	169.1	11.33	11.43
Mixture (1+2)	T7	34.88	34.52	27.1	27.3	22.93	22.2	16	16.3	11.1	11.2	203	205	168. 4	168.6	11. 6	11.83
	T8	34.43	34.24	26.98	26.76	22.85	22.2	16.1	16.32	11.1	11.1	202.8	204	168.2	169.1	11.2	11.21
	T9	34.34	34.43	26.2	26.31	22.66	22.1	15.87	16.11	10.92	10.39	203	204	168	168	10.97	10.92
	Mean	34.55	34.39	26.76	26.79	22.81	22.16	15.99	16.24	11.04	10.89	202.93	204.33	168.2	166.56	11.25	11.31
Control		34.3	34.4	26.4	26.5	22.5	22.3	15.4	15.5	10.4	10.8	201.5	202	167	166.5	11.1	11.5

classified as nonolfactory sensilla in *B. mori* (McIver 1973, 1974; Boo and McIver 1975, 1976; Davis and Sokolove 1975; Steinbrecht, 1988).

Sensilla squamiformia

The dorsal side of *B. morei* flagellum bears squamiformia sensilla (scale like procces) which average 65 μm in length (Fig 1 E&F).

Most sensilla on silkmoth antennae are olfactory, in the male; about 70 % of these are sensitive to sex pheromone. The s. trichodea sensitive to pheromone contain two sensory neurons of which one is sensitive to bombykol while the other is sensitive to bombykal (Kaissling *et al.,* 1978). In females, the most abundant type of olfactory s. trichodeum contains two receptor cells highly responsive to two plant odours, linalool and benzoic acid (Heinbockel & Kaissling, 1996 and de Brito Sanchez and Kaissling, 2005). Other olfactory sensilla also respond to plant odours. The male sensilla trichodea has a receptor cell responding to the bombykol pheromone component. The sensilla trichodea of the females contained specialized receptor cells responding to certain terpenoids, the most frequent being the nerolidol-sensitive cell. Cells of the sensilla coeloconica responded to aliphatic aldehydes andacids de Brito Sanchez, 1996 and Ziesmann *et al.*, 2000.

Effect of different fertilizers and their mixtures on the fresh cocoon weight, shell cocoon weight and size of sensilla of mulberry silkworm is presented in Tables 1, 2&3. No significant differences were found between control, chemical, bio-fertilizers and their mixture for weights of fresh cocoon, cocoon shell, while the size of sensilla showed significant difference. Bio-fertilizers increased fresh cocoon weight; cocoon shell weight compared with the control and was reflected in the size of sensilla styloconica (Tables 2&3). The application Bio-fertilizer resulted in better values for silk gland length, pupation ratio, cocoon percentage and larval weight (ElKahyat 2013). These results are in agreement with those obtained by Rao *et al.,* (2008), Mary and Saravanan (2010) and Waktole and Bhaskar (2012) who reported that nutrition plays a pivotal role in sericulture. It improves the growth, development, health, feed consumption and conversion of silkworm thereby improving the commercial traits.

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