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Protein profiling of *Apis* species (Hymenoptera: Apidae) adult worker honey bees from North-western region of India

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ABSTRACT: In *Apis*, biodiversity studies at genetic and molecular level were done to identify the proteins responsible for polymorphism in different species of *Apis viz*. *Apis florea* F., *Apis dorsata* F., *Apis cerana* F. and *Apis mellifera* L. from north-west region of India using SDS-Polyacrylamide gel electrophoresis (PAGE). Some species specific protein bands were obtained by SDS-PAGE technique followed by Commassie BB staining and silver staining. The present results revealed that the total content of proteins was highest in *A. dorsata* with highest number of protein bands unique to species followed by *A. mellifera*, *A. florea* and *A. cerana*. Most prominent were at 38.7 kDa and 35.3 kDa in *A. dorsata*; 46.4 kDa and 29.0 kDa in *A. florea*; 83.9 kDa and 40.4 kDa in *A. mellifera* and 61.0 kDa and 27.9 kDa in *A. cerana* respectively. Some species specific protein bands with silver staining were as 33.6 and 59.2 kDa in *Apis florea*; 57.2 kDa in *A. dorsata;* 49.6 and 63.6 kDa in *A. mellifera* and 36.1 and 38.8 kDa in *A. cerana*. Based on identification of these unique bands, it is concluded that the species with wide geographic distribution have large number of protein bands as compared to species with least distribution area.

KEY WORDS: *Apis*, polymorphism in honey bees, gel electrophoresis, species specific bands

INTRODUCTION

Honey bees are efficient pollinators and honey producers and also as wax, propolis and venom providers. The performance of these services varies from species to species due to their differences at the molecular level. Changes in the environment and exposure to different pollutants like poisonous gases, radiations *etc.* cause mutations at the genetic level and thus get reflected at the protein levels. Biodiversity can be studied at molecular level by analyzing the variations in number and type of proteins in one organism (Kumar and Kumar, 2013; Mestriner, 1969; Nunamaker *et al*., 1984; Sheppard, 1988; Sheppard and Berlocher, 1984; Sylvester, 1982). SDS PAGE protein profiling has an advantage that the degree of genetic correspondence between populations or taxa of a biological species can be quantified. Iftikhar *et al*. (2011) established biodiversity of honey bee species on the basis of physiochemical analysis of honey and suggested that in addition to the morphometric methods, molecular techniques should be used for phylogenetic studies. El-Bermawy *et al.* (2012) characterized the Egyptian, the Italian and the Carnolian subspecies of *A. mellifera* workers using protein, isozymes and RAPD-PCR. They have reported low level of polymorphism and Egyptian, Italian and Carniolan subspecies were distinguished on the basis of 18, 5 and 4 unique bands respectively. Surendra *et al*. (2011) studied the toxic potential of venom from three honey bee species

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viz. *Apis cerana*, *Apis dorsata* and *Apis florea* on bacteria and fungi by using SDS-PAGE and reported highest susceptibility of the pathogens to the venom of *A. cerana followed by A. dorsata* and *A. florea*. Priya *et al*. (2013) have reported odorant-binding proteins and pheromone-carrying proteins from sting and mandibular glands of *A. cerana indica* using GC-MS and SDS-PAGE profile. Sixteen volatile compounds in the sting and 19 in mandibular glands of the worker honey bee were reported. But SDS-PAGE protein profiling has not been used to characterize or establish biodiversity in the four species of honey bees found in north-west India. There is little published data from north western region of India. Some literature is available on venom glands, hypopharyngeal glands or larval stages of workers, drones or queen of any single species of honey bee. Similarities and differences in protein and enzyme patterning of the whole body homogenate of *Apis mellifera* have been studied in different populations in other countries (Ivanova *et al*., 2011, 2012; Kandemir and Kence, 1995; Krieg and Marek, 1983; Lee, 1993; Li *et al*., 1986; Markert and Moller, 1959; Moradi and Kandemir, 2004; Sylvester, 1976). There are very few reports of biodiversity studies on honey bees using molecular techniques from India, therefore the present studies were done to find polymorphism in the honey bee species, using certain proteins for their applicability as biochemical markers for inter specific differentiation of four species of honey bees *viz*. *Apis florea*, *A. dorsata*, *A. cerana* and *A. mellifera* found in north western regions of India.

MATERIAL AND METHODS

Study area and Sample Collection

Honey bees, were collected from north western region of India. Collection of samples was made by insect trapping net and forager bees were collected. From the region, 25-50 bees of each species were collected and were frozen at -20ºC for biochemical analysis. The average individual body weight of honey bee was 27 mg of *A. florea,* 57 mg of *A. cerana,* 69 mg of *A. mellifera* and 117 mg of *A. dorsata* respectively.

Preparation of homogenates

Honey bees of each species were weighed and homogenized with homogenizer, POLYTRON PT 2100 in Phosphate buffer saline (PBS)-pH 7 for 5 minutes to prepare 10% homogenate. It was centrifuged at 5000 g in a refrigerated centrifuge (REMI) for 10 min at 4°C. Supernatant obtained was used for further analysis.

SDS PAGE profiling of Proteins

Protein content of the above samples was estimated by employing the method of Lowry *et al.* (1951). BSA (20mg/100 ml) was used as a standard. In order to calculate the protein content in the samples, a standard graph was plotted.

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on homogenates of four honey species, by the method of Laemmli (1970). Equal amount of protein (15 µg) from the homogenate of the four species was loaded in the wells. 10% resolving gel gave the best resolution of proteins after staining with Commassie Brilliant Blue R-250 (CBB) and 12% resolving gel was used for staining with Silver stain as the latter stain is highly sensitive and visualize the proteins present in extremely low quantities which cannot be seen by the former stain. Molecular weight markers (MWM) were also run on the same gel and were labelled as protein ladder. Bromophenol blue was used as a tracking dye.

Molecular weight calculation

To determine the molecular weight of each band obtained on gel, first a standard graph was plotted for the markers by taking R_f values of the markers on X-axis *vs* log molecular weight of respective standards on Y-axis. Best fit straight line graph was obtained as shown in Fig. 1. Then molecular weight of each band on the gel was calculated using the graph.

RESULTS AND DISCUSSION

In order to ascertain variations at inter species level sample proteins were resolved on gel as shown in

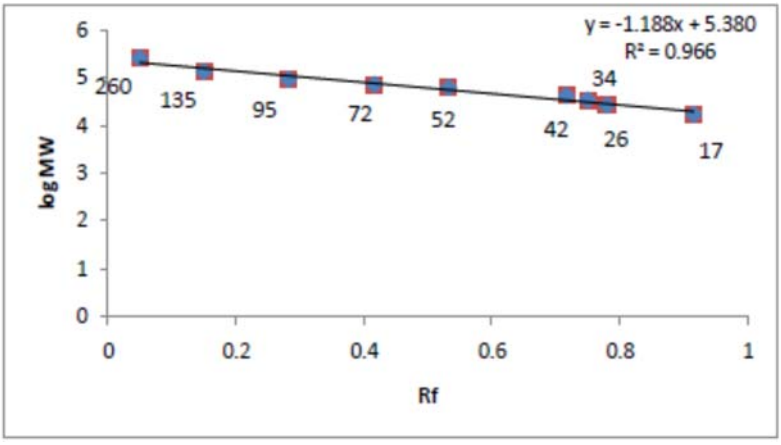

Figure 1. R_f vs log molecular weight (log MW) to determine the molecular weight of each band on the gel

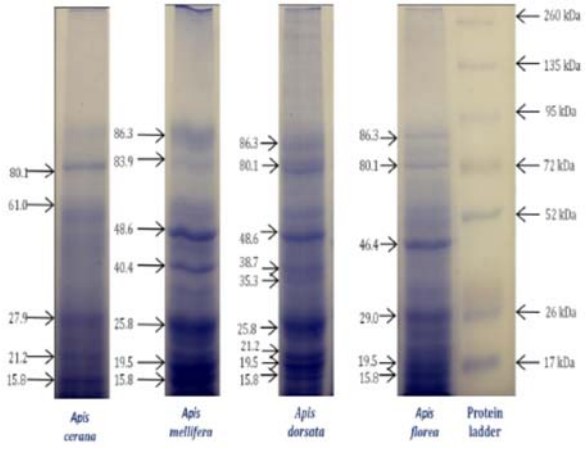

Figure 2. Protein profile of honey bee species on 10% SDS-PAGE [from left *Apis cerana*, *Apis mellifera*, *Apis dorsata*, *Apis florea* and marker]

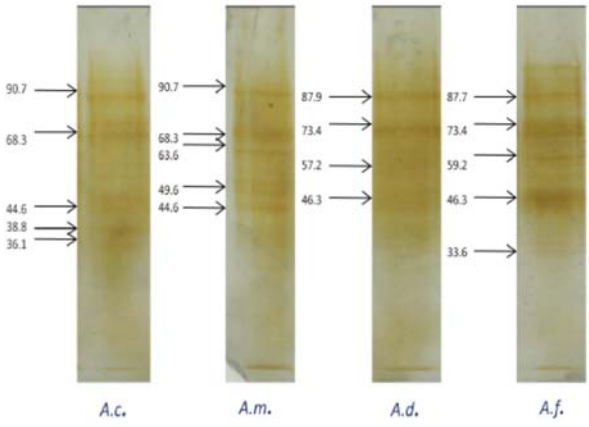

Figure 3. Protein profile of honey bee species on 12% SDS-PAGE [from left *Apis cerana*, *Apis mellifera*, *Apis dorsata*, *Apis florea* and marker]

Fig. 2 and 3 respectively. Protein profile of three native Indian species of honey bees *A. florea*, *A. dorsata* and *A. cerana* showed a common band of 80.1 kDa which was missing in the exotic species, *A. mellifera*. Protein bands of 19.5 and 86.3 kDa were observed in *A. florea* as well as in two other species of honey bees *viz*. *A. dorsata* and *A. mellifera*. *Apis mellifera* and *A. dorsata* exhibited similarity in banding pattern of proteins with molecular weights of 25.8 and 48.6 kDa. Rest of the Commassie stained protein bands were unique to different honey bee species. Protein bands common to all species studied (*A. florea*, *A. dorsata*, *A. mellifera* and *A. cerana*) were of molecular weights 15.8, 21.2, 25.8, 27.9, 29, 83.9 and 86.3 kDa respectively (Fig. 2). No such data on proteome analysis is available from India, however in studies reported by Surendra *et al*. (2011), common protein bands with molecular weight of 4, 5, 6, 7, 8, 9, 11, 15, 16, 31, 27, 30, 34, 35, 66 and 97 kDa were found in venom constituents of *A. cerana indica*, *A. dorsata* and *A. florea* using SDS-PAGE. During the present studies protein bands of 15, 21, 27, 61 and 80 kDa were observed in *A. cerana*. Priya *et al.,* (2013) also reported the presence of protein bands of 21 and 61 kDa in the sting and mandibular glands of *A. cerana*.

In the present studies protein bands with 15.8, 19.5, 40.4, 48.6, 83.9 and 86. 3 kDa molecular weight

were observed in *A. mellifera* from Chandigarh. In previous studies, protein bands with MW 14, 21, 31, 45, 66, 97, 116 and 200 kDa have been reported in labial glands of *A. mellifera* (Sutherland *et al.*, 2006).

Using silver staining, molecular weights of proteins on SDS PAGE of the four honey bee species were calculated (Fig. 3). Three bands were observed to be common in *A. mellifera* and *A. cerana.* These had molecular weights of 44.6, 68.3 and 90.7 kDa. While *A. florea* and *A. dorsata* shared three protein bands of 46.3, 73.4 and 87.9 kDa, these were not found in *A. mellifera* and *A. cerana*. The silver stained protein bands unique to each species of honey bee were 33.6 and 59.2 kDa (*A. florea*), 57.2 kDa (*A. dorsata*), 49.6 and 63.6 kDa (*A. mellifera*) and 36.1 and 38.8 kDa (*A. cerana*). Sibele *et al.* (2007) through silver staining of SDS-PAGE reported protein bands with molecular weight less than 25 kDa and more than 205 kDa in testes, seminal vesicles and accessory glands during different pupal stages of *A. mellifera* drones. In our results the protein bands differed in number, width and intensity among the different species studied thus exhibiting variation in number and quantity of proteins and enzymes, there by confirming polymorphism of proteins at interspecific level.

This paper can be considered as a study on protein profile of *Apis* species and polymorphism. The future implication include that the species specific protein bands can be isolated, sequenced, and searching similarities will give a clear picture of phylogeny which leads to biodiversity studies.Species specific protein bands of four honey bee species obtained from North-west region of India confirmed biodiversity at interspecific level and can be used as molecular markers for identification of species.

ACKNOWLEDGEMENTS

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First record of the genus *Caviceps* Malloch (Diptera: Chloropidae: Oscinellinae) from India with description of a new species

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ABSTRACT: Genus *Caviceps* Malloch is recorded for the first time from India and a new species, *C. aristalis* is described. *Dasyopa orientalis* Cherian from India is transferred to genus *Caviceps* with replacement name *Caviceps nartshukae* Cherian as *Caviceps orientalis* is a preoccupied name.

KEYWORDS: Chloropidae, Tricimbini, *Caviceps*

INTRODUCTION

Caviceps Malloch (1924) is a small genus known by 4 species from the world. Nartshuk (1987) placed this genus under the tribe *Tricimbini*, along with 9 other genera. Ismay (1993) synonymised some of these genera and recognized only 4 of these genera namely, *Aphanotrigonella* Nartshuk, *Aprometopis* Becker, *Siphunculina* Rondani and *Tricimba* Lioy under *Tricimbini* to which he added *Pseudotricimba* erected by him in 1993 and *Tricimbomyia* Cherian (1989). Ismay (1998) synonymised *Aprometopis* with *Strobliola* Czerny and there by recognized 6 genera under *Tricimbini.* Later Ambily and Cherian (2013) added *Indometopis* Cherian to the tribe. However, Ismay without explanation and Cherian inadvertently had left out *Caviceps* Malloch from the list of genera placed earlier by Nartshuk (1987) under the tribe.

The distinct identity of this genus had long been recognised by earlier workers including Sabrosky (1980), Kanmyia (1983) and others, though because of its rareity it hardly found mention in recent literature. Thus 8 genera are recognized under tribe Tricimbini.

While revising species of *Dasyopa* Malloch from the Oriental Region it was realised that *Dasyopa orientalis* Cherian (1990) with its indistinct frontal triangle, broad frons, long arista and other deviating characters, though originally placed with stated reservations under *Dasyopa, shows* closer affinities to the genus *Caviceps* to which it belongs. Hence it is transferred to that genus. Besides, a new species, *Caviceps aristalis* from India is also described. This is the first record of this genus from India.

MATERIAL EXAMINED

The type specimens are retained at present in the collections of the Department of Zoology, University of Kerala, Trivandrum and shall later be transferred to the National Zoological Collections, Western Ghats Regional Centre, Zoological Survey of India, Kozhikode (Calicut).

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The morphology nomenclature is followed after Mc Alpine (1981).

RESULTS AND DISCUSSIONS

Genus *Caviceps* Malloch

1924. *Caviceps* Malloch, *Proc. Linn. Soc*. N.S.W., **49**: 355.

Type species: *Caviceps flavipes* Malloch. By original designation

Diagnostic characters:

Head much wider than long with wide frons moderately compressed from front to rear; frons hardly produced beyond anterior margin of eye; frontal triangle not developed, only restricted to the small ocellar tubercle; face as high as wide; facial carina reaching lower facial margin as a distinct ridge, forming two deeply concave antennal foveae; basal antennal segments short; *ant* 3 nearly rounded, wider than long; arista slender, as long as head or a little shorter, with short hairs; gena narrower anteriorly, width in the middle less than half that of *ant* 3; vibrissal corner not projecting beyond anterior margin of eye; parafacialia not developed; labella slender, shorter than proboscis; eye fairly large, densely pubescent with nearly oblique long axis; *oc* and *pvt* upright and cruciate; orb 8-10 short, reclinate, anterior ones hair-like; *ovt* longer than *ivt*; scutum convex, often with dusty black longitudinal stripes, densely tomentose with short fine hairs; scutellum of *Conioscinella*-type with convex disc and rounded margin; pleura with maculae; *npl* 1+1,1+2 or1+3; *h* 1, *pa* 1, *pa* 2 and 1 *dc* well developed; *as* widely separated at base, longer than scutellum; *ss* 1-3; wing hyaline with dark maculae in some species; R_{2+3} long; anal area developed; haltere yellow, abdomen often as wide as thorax; female cerci very long; epandrium small with posteriodorsal notch often not well demarcated; stylus attached to posterioventral aspect of epandrium, pregonite invisible; postgonite well developed; hypandrium open distally.

Distribution: Australian and Oriental Regions.

Key to species of *Caviceps* Malloch from India.

Arista as long as head; hairs on scutum and scutellum yellow; *npl* 1+2; second sector of costa 2x as long as third sector; coxae black to brownish black..... *nartshukae* Cherian

Arista shorter than head; hairs on scutum and scutellum brownish black; *npl* 1+1; second sector of costa less than 2x the third sector; coxae entirely yellow……. *aristalis* sp.n

Caviceps nartshukae Cherian **N. name** (Pl. 1)

urn:lsid:zoobank.org:act:36D1B8D7-32C6-4CA9-8069-E34DF562B221

Caviceps nartshukae Cherian (for *Dasyopa orientalis* Cherian). Type locality: India: Tripura: Paratia. **N. name.** *Dasyopa orientalis* Cherian, 1990, *Oriental insects***, 24**: 356-358. (*Caviceps*; preocc. *Caviceps orientalis* (Becker) (=*Aprometopis orientalis* Becker 1924). Type locality: Taiwan: Maruyama.

Diagnostic characters:

Female (Pl. 1): Head nearly as long as high; frons slightly narrowing anteriorly, width half that of head and 0.9x its own length, with dense, short, black *fr*; frontal triangle extremely short, hardly extending beyond black ocellar triangle; facial carina reaching epistomal margin as a low ridge; antennae yellow with dark tinge on upper margin of *ant* 3; arista as long as head, slender with short brownish black hairs; gena yellow, less than half as wide as ant 3 with a row of whitish yellow hairs on lower margin; vibrissal corner not projecting beyond eye; eye large, pubescent, with nearly oblique long axis; head bristles black; *orb* about 10, anterior 5 shorter; rest of head bristles as described under the genus; scutum black with short, punctate yellow hairs; three longitudinal lines of punctae, one each along *dc* lines and third in mid longitudinal line, faint; pleura mostly dark brown with yellow tinge in certain areas; scutellum of *Conioscinella*-type, tomentose, pubescent and punctate like scutum; thoracic bristles mostly black; *npl* 1+2; *as* widely separated at base; *as* as long as scutellum; a pair of *prsc* distinct; legs almost whitish yellow but for brownish

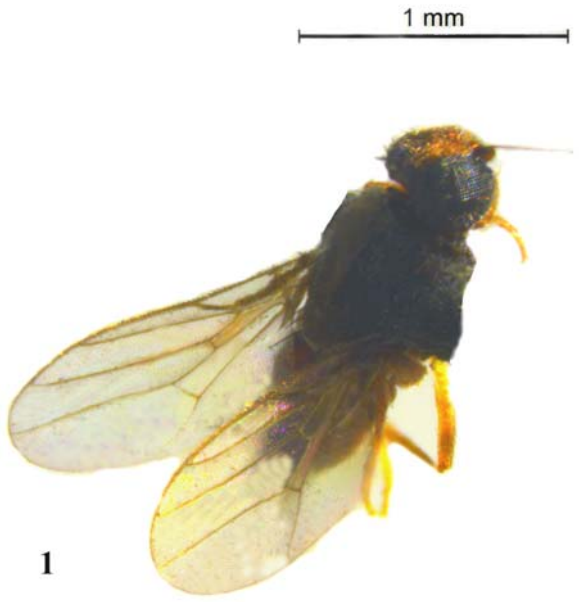
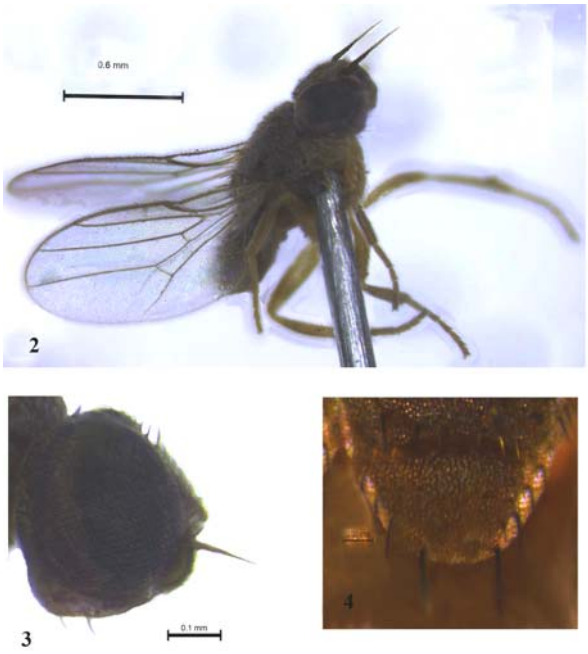


PLATE 1. *Caviceps nartshukae* Cherian: 1- Male fly



PLATES 2-4. *Caviceps aristalis* sp. n. 2- Female fly; 3- head, in profile; 4- wing

tinge on fore tibia and tarsi; tibial organ narrow; wing hyaline with brownish yellow veins; r-m cross vein joining discal cell opposite 0.62 of its length; terminal sector of M_{1+2} slightly convex above in the middle; haltere yellow; abdomen nearly as wide as thorax, basally brownish yellow with dark tinge at sides of second segment and on dorsam of distal segments.

Specimen studied: *Holotype*: Female, India: Tripura: Paratia, 10.x.1977. Coll. N. Muraleedharan (ZSI).

Length: Female 1.38 mm; wing 1.38 mm

Remarks*: C. nartshukae* shows affinities t*o C. orientalis (Becker)* but in the former parafacialia is not developed, gena is less than half as wide as *ant* 3, scutum is black, pleura is with black macula only on part of anepst, r-m cross-vein joins discal cell far distad of its middle and penultimate section of R_{4+5} is normally developed. But in *orientalis* (Becker) parafacialia is developed, gena is more than half as wide as *ant* 3, scutum is yellow with four black longitudinal stripes, pleura is with brown maculae on *anepst, kepst* and meron, r-m cross-vein joins discal cell far basad of its middle and penultimate section of R_{4+5} is extremely short. This species has not been collected since it was originally described.

***Caviceps aristalis* Cherian sp. n. (Plates. 2-4)**

urn:lsid:zoobank.org:act:FFDC470C-7950-4ADA-B79A-E196106EECF9

Female (Pl. 2): *Head* (Pl. 3): Length, height and width ratio 10:11:17. Frons a little widened at vertex and slightly narrowing anteriorly, width at point of widening about half that of head and 0.8x its own length, some what rugulose, finely tomentose, dull brownish yellow with fine, dense, punctate short brown *fr*; two lateral corners of frons at vertex dull black and densely tomentose as in *nartshukae* and this colouration extends narrowly along vertex margin; frontal triangle extremely short, almost indistinct as in *nartshukae*, its maximum width less than half that of frons, subshiny black, fairly densely tomentose especially around area of ocellar tubercle, hardly projects beyond anterior ocellus

and ends with obtuse apex; ocellar tubercle black, densely silvery grey tomentose, projecting a little above frontal triangle. Face concave, dull dark brown medially in the area of antennal fovea and yellowish brown along epistomal margin; facial carina running as a triangular ridge between bases of antennae whence continues as a low linear ridge which widens and joins epistomal margin. Basal antennal segments brownish yellow; *ant* 2 with a row of hairs on anterior margin; ant 3, 1.4x as wide as long, yellowish brown along lower half of base and part of lower margin and blackish brown in upper half and distal margin; arista a little shorter than head and in *nartshukae*, brownish black with conspicuous concolourous pubescence. Gena brownish yellow, becoming a little infuscated around postgena, gradually becoming narrower anteriorly, width in the middle about one-fourth that of *ant* 3, finely tomentose with a few short hairs; vibrissal corner receding, does not reach anterior margin of eye; postgena brownish around posterior part of gena and infuscated above. Parafacialia not developed. Eye large, densely and conspicuously pubescent with nearly oblique long axis. Palpi slender, yellow with pale hairs; proboscis brownish yellow, labella very short. Head bristles brownish black; *orb* about 10, hair-like, rest of cephalic bristles as in *nartshukae*.

Thorax: Scutum nearly as wide as long, fairly densely tomentose with gently convex blackish brown dorsum bearing fairly dense black to brownish black punctate hairs. Humeral callus yellowish brown. Scutellum (Pl. 4) of *nartshukae* type, width at base 1.5x the length, with gently convex disc which is less convex than in *nartshukae* but is concolourous with and tomentose, punctate and pubescent like scutum. Pleura concolourous with and tomentose like scutum but for diffused yellow tinge covering especially part of *ppl* and *anepst*. Thoracic bristles brownish black; *npl* 1+1; *prsc* short, hardly distinguishable from scutal hairs; other thoracic bristles as in *nartshukae* with basally widely separated *as*, two pairs of *ss* bristles but *as* is 1.3x as long as scutellum and relatively a little longer than in *nartshukae.*

Wing (Pl. 4): 2.15x as long as wide, hyaline with dark brown costa and brown veins; proportions of costal sectors 2- 4 in the ratio 13:7:6; *r-m* cross-vein distad of middle of discal cell, opposite 0.6 of its length; terminal sectors of R4+5 and M1+2 nearly subparellel except that the latter is convex above especially medially and the two diverge a little before joining costa, the latter at apex of wing; anal area moderately developed. Haltere yellow.

Legs: Slender, almost whitish yellow except for dorsum of distal part of midfemur and with diffused brown tinge on tibiae and some tarsi in certain angles of illumination. Tibial organ distinct.

Abdomen: A little longer than wide, subshiny dark brown except for light yellow tinge on dorsum of some basal segments, partly finely tomentose. Ovipositor short, slender dull black, with a few slender hairs.

Length: Femae 1.6 mm; wing 1.4 mm

Holotype: Female: India: Kerala: Trivandrum: Kariavattom, 25 m, 20. xi. 2006, Coll. Jyothi Tilak.

Etymology: The species derives its name from its long arista.

Remarks: *C. aristalis* shows affinities to *nartshukae* Cherian. However in *aristalis* arista though relatively long is a little shorter than head, there are only 1+1 *npl,* hairs on scutum and scutellum are entirely black to brownish black and coxae are yellow. But in *nartshukae* arista is as long as head, there are 1+2 *npl*, hairs on scutum and scutellum are yellow and coxae are entirely black.

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Abbreviations

anepm - anepimeron; *anepst* - anepisternum; *ant* 2 - second antennal segment; *ant* 3 - third antennal segment; *as* - apical scutellar bristle; 1 *dc* - first dorsocentral bristle; *fr* - frontal hair; *h* - humeral bristle; *if* - interfrontal bristle; *ivt* - inner vertical bristle; *kepst* - katepisternum; *npl* - notopleural bristle; *oc* - ocellar bristle; *orb* – fronto-orbital bristle; *ovt* - outer vertical bristle; *pa* - postalar bristle; *ppl* - propluron; *prsc* - prescutellar bristle; *pvt* - postvertical bristle; *ss* - subapical scutellar bristle.

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Repellent activity of plant essential oil extracts against malaria vector *Anopheles arabiensis* Patton (Diptera: Culicidae).

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ABSTRACT: Repellent activity of essential oils extracted from the leaves of *Otostegia integrifolia* and *Stephania abyssinica*, roots of *Echinops kebericho* and seeds of *Millettia ferruginea* and *Datura stramonium* were tested against malaria vector, *Anopheles arabienses*. The repellent activity was determined at 125, 250, 500 and 1000 ppm concentration by human volunteer. The dorsal side of each human arm 153.86 cm^2 of the skin was exposed for twenty minutes by covering the remaining area with rubber glove. The control and treated arm were introduced simultaneously into mosquito cage under laboratory condition maintained at 27 ± 1°C, 65–70% RH. Among the four different concentrations tested, maximum repellent activity was observed at 1000 ppm of *O. integrifolia*, *S. Abyssinica and M. ferruginea* and also *E. kebericho* has strong repellent properties in all concentrations.*O. integrifolia, S. abyssinica, M. ferruginea* and *E. kebericho* may contain repellent chemicals which can be used for the development of safer mosquito repellent product.

KEY WORDS: Repellent activity, volatiles, malaria vector, *Anopheles*

INTRODUCTION

Mosquitoes are responsible for transmission of malaria which is one of the important and fatal diseases worldwide (Yohannes and Boele, 2011). In sub-Saharan Africa, children under the age of five years and pregnant women are highly affected by malaria (Morlais *et al.,* 2005). In Ethiopia, 68 per cent of the populations live in malaria prone areas covering almost 75 per cent of the land (FDROEMOH, 2006; PMI, 2010).

The diverse eco-climatic condition in Ethiopia is much favourable for malaria transmission pattern seasonal and unstable. The widely distributed malaria vector in Ethiopia includes *Anopheles arabiensis, Anopheles pharoensis, Anopheles funestus* and *Anopheles nili.* These vector breeds in small, temporary, sunlight pools and in low land as well as highland areas up to 2000 m. a. s. l. (Nyanjom *et al.,* 2003; Ashenafi Woime, 2008).

According to the World Health Organization, mosquito control using insecticides is the most efficient means for short term being widely exploited in the treatment of bed nets and indoor residual spraying (Yakob *et al.,* 2011; Bigoga *et al.,* 2012). Chemical control of mosquitoes is highly complicated because of persistent chemical insecticides lead to environmental pollution, killing non-target organism and insecticides resistance development among the vector populations, especially in the *Anopheles gambiae* complex (UNICEF, 2000). Despite, considerable effort is

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made through control programs to curb the disease burden; it is still remains a major public health problem in many countries including Ethiopia. These problems have warranted the need for search and development of alternative eco-friendly strategies.

Bioactive compounds from plants are eco-friendly, environmentally safe, biodegradable and cost effective without altering natural habitat (Redwane *et al.,* 2002; Mittal and Subbarao, 2003). The larvicidal and repellent effects of essential oils extract from various plants are reported to be effective against different mosquito species (Duangkamon *et al.,* 2011; Raghavendra *et al.,* 2011). Secondary metabolites of bioactive plants reported to inhibit insects' development and production behaviour repellence (Viglianco *et al.,* 2006), anti-feeding effect (Eriksson *et al.,* 2008), growth regulation (Wheeler and Isman, 2001), feeding deterrence (Koul, 2004), and oviposition deterrence (Banchio *et al.,* 2003). The plant secondary metabolites are evaluated against insects as volatile chemicals (Bobadilla *et al.,* 2005), essential oils (Pérez-Pacheco *et al.,* 2004) and powders (Silva *et al.,* 2003). Mudalungu *et al.* (2013) reported potential larvicidal activity of both essential oil and non-volatile compounds from *Fagaropsis angolensis* leaves against *A. gambiae* larvae. The larvicidal and repellent effects of the essential oils from the seeds and leaves of *Chenopodium ambrosoides* was reported against larvae and adults of *A. gambiaes* mosquitoes (Bigoga *et al.,* 2013). There is no report on repellent properties *O. integrifolia, S. abyssinica, M. ferruginea, D. stramonium* and *E. kebericho* against *A. arabiensis* mosquitoes. These valuable medicinal plants are widely distributed and abundance in Ethiopia. Therefore, present study was initiated to evaluate repellent properties of selected plant essential oil extracts against malaria vector, *A. arabiensis.*

MATERIALS AND METHODS

The laboratory study was conducted from February 2015 to June 2015 at General Entomology laboratory, Department of Biology, College of Natural and Computational Sciences, University of Gondar. The study area is located in the North West of Ethiopia with 12° 36" N latitude and 37° 28" E longitude with an elevation of 2133 meter above sea-level.

a) Anopheles arabiensis culture establishment

Eggs of *Anopheles arabiensis* were collected from pastor campus, Addis Ababa University and reared in the laboratory at 27 ± 1° C, 65–70% RH and 12:12 h light: dark cycle. Once the larvae reached the pupal stage they were transferrd to adult emergence cage. The adult mosquitoes emerged from the pupa were provided *ad libitum* access of 10 percent sugar solution (w/v) and kept in Bugdorm cages (30 cm x 30 cm x 30 cm). The starved female adults were allowed to feed blood meal from the arms of human volunteers. Blood fed female mosquitoes were allowed to oviposit in 30 ml cups filled with 15 ml of distilled water. The cup edges were covered with a filter paper for egg deposition. Filter papers that contain eggs were transferred to plastic trays (25 cm x 25 cm x 7 cm) filled with distilled water. After egg hatching larvae were provided with finely powdered Tetramin® fish food. The mosquitoes cultures were maintained continuously to get adequate adult female mosquitoes to conduct repellent bioassay.

b) Extraction of essential oils

Otostegia intergrifolia and *S. abyssinica* leaves, *E. kebericho* root and *D. stramonium* and *Millettia ferruginea* seeds were collected during autumn season from Kola Deba region, Ethiopia. The plant parts were thoroughly cleaned with water and shade dried by spreading on a clean and well-ventilated surface. After drying, plant parts were grounded by using electric blender in order to get fine powder for essential oil extraction. Two hundred grams of powder from each plant sample was mixed with 1000 ml of distilled water in a conical flask and subjected to essential oil extraction by hydro-distillation method at 100° C using Clevenger apparatus for 3 h. The essential oils were separated from water, dried over anhydrous sodium sulphate and stored at 4° C for further experimentation (Mudalungu *et al.,* 2013).

Figure 1. Medicinal plants and parts used for essential oil extraction

c) Preparation oil concentration

Stock solution of 10,000 ppm concentration was prepared by adding 1 ml of pure essential oil mixed with 1 ml of acetone and make up to 100 ml in 250 ml conical flask by adding distilled water. Four concentrations viz., 1000, 500, 250 and 125 ppm were prepared through dilution of the stock solution with distilled water. Three replicate for each concentration were made for repellent response of mosquitoes against non-blood fed of female *A. arabienses*. In addition, control contains 1 ml of 100 percent acetone and the amount of distilled water varied according to parallel concentration prepared (Xue *et al*., 2001).

d) Experimental design

Repellent properties of essential oils against mosquitoes were conducted in dark room by maintaining at 27 ± 1° C and relative humidity of 60-70 percent. The whole arm of volunteer was covered with glove except specific area with the diameter of 7 cm removed at the back of palm. The exposed part of arm was applied four drops of essential oils in one hand as a treatment and four drops diluted acetone on the other hand as a control. Both arms simultaneously inserted in to mosquito cage for 20 minute and monitored for number of mosquitoes landed on treatment and control arms.

e) Data analysis

The percentage repellence index (R) was estimated by using the formula $R = (C-T)/C \times 100\%$, where C and T are the data of mosquitoes landed on the control and treated arms, respectively (Chio and Yang, 2008). The SPSS Version 20 software was used to calculate LC_{50} values and LC_{90} values and 95 per cent of upper and lower confidence limit (UCL). The results were subjected to Chi-square analysis for statistical significant at 5 percent level ($p<0.05$).

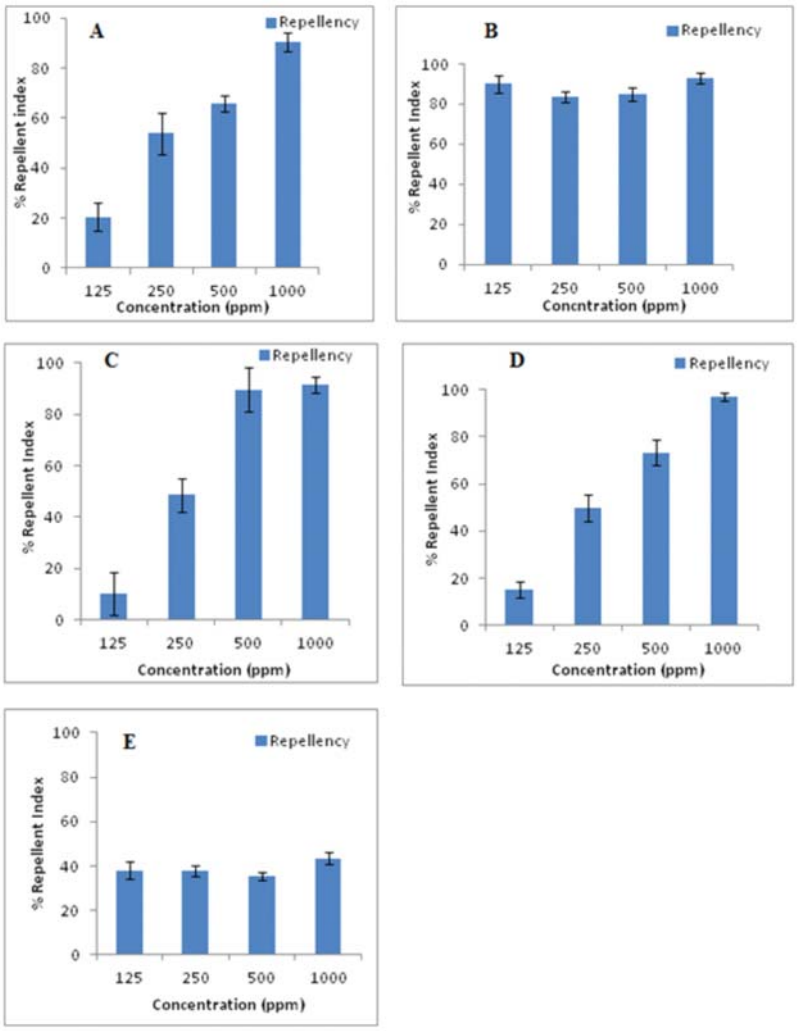

Figure 2. Mean percentage repellent activity of plant essential oils against *Anopheles arabiensis*, A. *Otostegia integrifolia,* B. *Echinops kebericho,* C. *Stephania abyssinica,* D. *Millettia ferruginea,* E. *Datura stramonium*.

RESULTS

a) Repellent activity of *Otostegia integrifolia* essential oil against *A. arabienses*

Mean percentage repellent response of non-blood fed female *A. arabiensis* exposed to essential oil of *O. integrifolia* treated arms of human volunteers after 20 minutes was presented in Fig 2A. Results revealed that the mean percentage repellent activity ranged from 20.54 ± 5.82 to 90.47 ± 3.63 at 125 and 1000 ppm respectively. The calculated LC_{50} and LC_{90} value was 1.886 ppm and 4.865 ppm respectively. The chi-square analysis results showed statistical difference at 5% level (χ^2 = 74.180; $p< 0.05$). The calculated range of 95 percent lower and upper confidence limit of LC_{50} and LC_{90} value was 1.630 - 2.139 ppm and 3.986 - 6.644 ppm respectively.

b) Repellent activity of *Echinops kebericho* essential oil against *A. arabienses*

Mean percentage repellent response of non-blood fed female *A. arabiensis* landed on essential oil of *E. kebericho* treated human volunteer after 20 minutes was presented in Fig 2B. Results revealed that the mean percentage of repellent activities ranged from 90.31 ± 4.34 - 93.16 ± 2.62 was observed at 125 and 1000 ppm respectively. The

calculated LC_{50} and LC_{90} value was 0.28 and 0.71 ppm respectively. The chi-square analysis results showed statistical significant difference at 5% level ($\chi^2 = 71.58$; $P< 0.05$).

c) Repellent activity of *Stephania abyssinica* essential oil against *A. arabienses*

Mean percentage repellent response of non-blood fed female *A. arabiensis* landed on essential oil of *S. abyssinica* treated human volunteer arms after 20 minutes was presented in Fig 2C. Results revealed that the mean percentage repellent activity ranged from10.27 ± 4.09 – 87.73 ± 4.46 at 125 and 1000 ppm respectively. The calculated LC_{50} and LC_{90} value was 1.89 and 3.52 ppm respectively. The chi-square analysis results showed statistical difference at 5% level ($\chi^2 = 263.80$; $p< 0.05$). The calculated range of 95% lower and upper confidence limit of LC_{50} and LC_{90} value was 1.52 – 2.26 and 2.87 – 5.09 ppm respectively.

d) Repellent activity of *Millettia ferruginea* essential oil against *A. arabienses*

Mean percentage repellent response of non-blood fed female *A. arabiensis* landed on essential oil of *M. ferruginea* treated human volunteer arms after 20 minutes was presented in Fig. 2D. Results revealed that the mean percentage of repellent activities ranged from 15.33 ± 3.40 – 97.12 ± 1.67 at 125 and 1000 ppm respectively. The calculated LC_{50} and LC_{90} value was 1.89 and 3.85 ppm respectively. The chi-square analysis results showed statistical significant difference at 5% level ($\chi^2 = 82.05$; $p< 0.05$). The calculated range of 95 percent lower and upper confidence limit of LC_{50} and LC_{90} value was 1.67 – 2.11 and 3.31 – 4.78 ppm respectively.

e) Repellent activity of *Datura stramonium* essential oil against *A. arabienses*

Mean percentage repellent response of non-blood fed female *A. arabiensis* landed on essential oil of *D. stramonium* treated human volunteer arms after 20 minutes was presented in Fig. 2E. Results revealed that the mean percentage repellent activity ranged from 38.10 ± 3.68 – 43.47 ± 2.64 at 125 and 1000 ppm respectively. The calculated LC_{50} and LC_{90} value was 13.07 and 26.42 ppm respectively. The chi-square analysis results did not show statistical difference at 5 percent level ($\chi^2 = 19.16$; $P>0.05$).

DISCUSSION

Bioactive products of plant have been used to control mosquitoes for a very long time. Natural plant products reported to be effective against the mosquito vector species and considered as plausible alternatives to synthetic chemical insecticides. Secondary metabolite produced by diverse plant species contains unique biological principles, such as toxin for physiological activities and attractant or deterrents for behavioural response of insect (Arivoli *et al.*, 2011; Muthu *et al.,* 2012). Essential oil extract from leaves, flowers seeds and roots of various plants not only exhibit inhibitory activity against bacteria, fungi and termites but also showed strong mosquito repellent and larvicidal activities (Sosan *et al.,* 2001; Cheng *et al.,* 2004). Larvicidal and repellence effects of essential oils from various botanicals against different mosquito species were reported (Bigoga *et al*., 2013).

The local communities in Ethiopia traditionally adapt various methods to repel the insects/ mosquitoes. Application of smoke by burning the plant parts is one of the most common practices. In addition, spraying extracts of *O. integrifolia* and *E. kebericho* after crushing and grinding; hanging and sprinkling on the floor as a protestant against mosquito bites by believing repellent properties (Kidane *et al*., 2013). Previous studies, confirmed that extracts of smoke from burning leaves were repellent to host-seeking non-blood-fed female *A. arabiensis* (Due *et al*., 2011). Moreover, Karunamoorthi *et al*. (2008) observed that *O. integrifolia* were burnt to repel mosquitoes, have also demonstrated a large reduction in the number of mosquitoes landing. There are also many other examples of burning leaves to decrease the number of mosquitoes in the house, some of which have also resulted in the reduction of other arthropod vector densities indoors, such as the sand fly and

black fly (Moore and Debboun, 2006 and Biran *et al*., 2007).

The present results confirmed repellent activities of *O. integrifolia, E. kebericho, S. abyssinica* and *M. ferruginea* essential oils against *A. arabiensis*. Mean percentage repellence of essential oil was 90.47, 93.16, 87.75 and 97.12 percent at 1000 ppm after 20 minutes exposure period in *O. integrifolia, E. kebericho, S. abyssinica* and *M. ferruginea* respectively. The smoke from *Otostegia integrifolia* leaves was previously suggested as a strong mosquito repellent in controlled semi-field studies using volatiles expelled through heating the leaves on metal plate (Seyoum *et al*., 2003 ; Due *et al.,* 2011). The leaves of many plant species reported to contain repellent compounds (Carroll and Loye, 2006). The smoke produced by burning dry roots of *E. kebericho* act directly as a natural insect repellent to provide protection against mosquitoes and other harmful arthropods (Fokialakis *et al*., 2006; Tariku *et al.*, 2011). These essential oils are used currently in many commercially available products like; perfume, soap and deodorant, appear to be within the repellent activity against mosquito species tested in the laboratory (Carroll and Loye, 2006). The result of this study is an indication that the essential oil of tested five species of plants has potential repellent action against *A. arabiensis*. These essential oils can be utilized for the development of mosquito repellent products.

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Population dynamics of mango leaf gall midge, *Protocontarinia matteiana* and its correlation with weather parameters

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ABSTRACT: Highest leaf gall midge damage (60.49 %) was observed on 7th standard week (SW) coinciding with pea cum marble sized fruit stages of the crop. It was positively influenced by sunshine and negatively by temperature (minimum and average), relative humidity, rainfall and wind velocity. The lowest leaf damage (31.69 %) was observed during 36 SW coinciding with emergence of new flush stage.

KEY WORDS: Population dynamics, weather parameters, mango, leaf gall midge, *Protocontarinia matteiana*

INTRODUCTION

Mango (*Mangifera indica* L. : Anacardiaceae), occupies a pride place amongst the fruits grown in the country. It has been found attacked by as many as 492 pest species in India (Butani, 1974). Of these, leaf gall midge, *Protocontarinia matteiana* Kieffer & Cecconi (Cecidomyiidae: Diptera) earlier considered a minor pest, has recently assumed a major pest status in the mango growing tracts of south Gujarat. It is commonly called mango midge fly due to its close association with leaves as well as fruits.

It is observed throughout the year causing galls in new flush leading to defoliation and reduction of the photosynthetic activity. So the present investigation on the population dynamics of mango leaf gall midge and its correlation with weather parameters was carried out at the Regional Horticultural Research Station (RHRS), Navsari Agricultural University (NAU), Navsari.

MATERIALS AND METHODS

A field experiment with 12 randomly selected 15 year old mango (cv. Kesar) trees in an insecticide free 1 ha. plot was conducted at the RHRS, NAU, Navsari, Gujarat during 2009-11. To study the population dynamics, the number of healthy as well as damaged leaves was counted on each of the ten terminal twigs from the lower canopy of each experimental tree at weekly interval throughout the year and was calibrated as per cent leaf damage. Important meteorological data *viz*., temperature (maximum and minimum), relative humidity (morning and evening), rainfall, rainfall days, sun shine and wind velocity were recorded at weekly interval during October 2009-June 2011.

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RESULTS AND DISCUSSION

The damage was assessed in terms of percentage of leaves damaged. Highest leaf damage (60.64, 62.64 and 60.49 % in 2009-10, 2010-11 and in pooled results, respectively) was observed during 6 (5-11 Feb.), 7 (12-18 Feb.) and 7 SW which coincided with peak flowering and pea cum marble sized fruit stages of the crop (Table 1). High to very high (> 50 %) leaf damage was also observed during the periods of 52-9 SW (24 Dec.- 4 March). Comparatively lower leaf damage of < 30 % (32.56, 30.18 and 31.69 per cent during 36 (3-9 Sep.), 35 (27 Aug. - and 2 Sep.) and 36 SW, respectively) observed coincided with emergence of new flush.

Peak leaf damage was observed during 7th SW (12-18 February) (60.49 % in pooled results) which happened to be the pea cum marble sized fruit stage of the crop. However, the damage (31.69 % in pooled results) started at the emergence of new flush (36 SW) (3-9 Sep.). This may be attributed to the thin leaf epidermis of the new flush which facilitated easy oviposition leading to puncturing of leaf epidermis and hyperplasia (initiation of swelling symptoms). As the leaves matured, the swellings became warts and ultimately the galls. The increase in swelling size may be due to the metamorphic changes in the maggots, requiring more inner epidermal space leading to the formation of galls in thick mature leaves at pea/marble sized fruit stage (7 SW) (12-18 Feb.).

As per the population dynamics of leaf gall midge, Patel (2011) reported highest leaf damage (62.51 %) and gall intensity (116.56 galls/sq.cm. on infested leaves) caused by *P. matteiana* in Alphonso during 5-6 (29 January-11 February) and 1-2 (1-14 January) standard weeks and 56.80 per cent with 105.69 galls during 7-8 (12-25 February) and 1-2 (1-14 January) standard weeks in Kesar. In the present findings, highest leaf damage (60.49 % in pooled result) was observed during 7th SW (12-18 Feb.).

Table- 1. Population dynamics of mango leaf gall midge, *Procontarina matteiana*

Std. week	Std. Period	Crop Stage	Leaf gall midge (Leaf Damage %)		
			2009-10	2010-11	Pooled
48	26 Nov- 2 Dec 2009	Bud/bud burst	44.12	42.24	43.18
49	3-9 Dec	Bud/bud burst	46.26	44.27	45.27
50	10-16 Dec	Bud/bud burst	47.35	48.35	47.85
51	17-23 Dec	Bud/bud burst	49.12	48.54	48.83
52	24-31 Dec	Bud/bud burst	50.72	51.38	51.05
1	1-7 Jan 2010	In Flowering	52.12	53.58	52.85
2	8-14 Jan	In Flowering	52.94	52.42	52.68
3	15-21 Jan	In Flowering	54.62	56.12	55.37
4	22-28 Jan	In Flowering	56.72	56.88	56.80
5	29 Jan- 4 Feb	Peak Flowering	58.18	58.64	58.41
6	5-11 Feb	Peak Flowering	60.64	58.98	59.81
7	12-18 Feb	Pea/Marble	58.34	62.64	60.49
8	19-25 Feb	Pea/Marble	52.24	56.68	54.46
9	26 Feb-4 March	Pea/Marble	50.36	51.24	50.80
10	5-11 March	Pea/Marble	45.46	48.68	47.07
11	12-18 March	Stone Size	42.18	46.82	44.50
12	19-25 March	Stone Size	44.26	44.00	44.13
13	26 March-1 Apr	Stone Size	42.22	40.26	41.24

Std. week	Std. Period	Crop Stage	Leaf gall midge (Leaf Damage %)		
			2009-10	2010-11	Pooled
14	2-8 Apr	Stone Size	40.28	38.36	39.32
15	9-15 Apr	Stone Size	38.22	36.74	37.48
16	16-22 Apr	Stone Size	37.48	34.28	35.88
17	23-29 Apr	Stone Size	39.63	36.83	38.23
18	30 Apr-6 May	Stone Size	41.18	42.68	41.93
19	7-13 May	Fruiting	43.12	46.73	44.93
20	14-20 May	Fruiting	45.27	48.36	46.82
21	21-27 May	In Ripening	46.36	48.88	47.62
22	28 May-3 June	Rip/Harvest	48.52	50.26	49.39
23	4-10 June	Harvest	50.58	50.72	50.65
24	11-17 June	Harvest	52.42	54.58	53.50
25	18-24 June	Vegetative	46.22	48.22	47.22
26	25 June-1 July	Vegetative	44.28	46.00	45.14
27	2-8 July	Vegetative	42.72	44.56	43.64
28	9-15 July	Vegetative	42.12	40.83	41.48
29	16-22 July	Vegetative	40.88	38.42	39.65
30	23-29 July	Vegetative	40.24	38.08	39.16
31	30 July-5 Aug	Vegetative	38.46	36.28	37.37
32	6-12 Aug	Vegetative	37.24	35.46	36.35
33	13-19 Aug	Vegetative	36.92	33.82	35.37
34	20-26 Aug	Vegetative	36.18	32.34	34.26
35	27 Aug-2 Sep	Emerge New Flush	34.28	30.18	32.23
36	3-9 Sep	Emerge New Flush	32.56	30.82	31.69
37	10-16 Sep	Emerge New Flush	35.38	32.35	33.87
38	17-23 Sep	Emerge New Flush	36.52	34.82	35.67
39	24-30 Sep	Emerge New Flush	38.48	36.18	37.33
40	1-7 Oct	Emerge New Flush	38.92	36.72	37.82
41	8-14 Oct	Emerge New Flush	40.52	38.18	39.35
41	15-21 Oct	Emerge New Flush	41.36	38.8	40.08
43	22-28 Oct	Emerge New Flush	41.82	40.22	41.02
44	29 Oct- 4 Nov	New twigs	42.72	40.72	41.72
45	5-11 Nov	New twigs	42.88	41.47	42.18
46	12-18 Nov	New twigs	43.92	41.83	42.88
47	19-25 Nov	New twigs	44.08	42.58	43.33

Effect of abiotic factors on population build-up of leaf gall midge

The results based on correlation studies of leaf gall midge oriented leaf damage (Y) with major weather factors (X_1 to X_{10}) indicated significant positive correlation with sunshine (X_8)('r' = 0.3200, 0.4991 and 0.4214 during 2009-10, 2010-11 and in pooled results, respectively), however it was significant but negative with minimum temperature (X_2)('r'= -0.6396, -0.5771 and -0.5934), average temperature (X_3)('r'= -0.5290, -0.4847 and -0.4976), morning relative humidity (X_4) ('r' = -0.7611, -0.4736 and -0.5719), evening relative humidity (X_5) ('r' = -0.6232, -0.5488 and -0.5747), average relative humidity (X_6) ('r' = -0.6852, -0.5497 and -0.5874) and rainfall (X_9) ('r' = -0.4455, -0.5119 and -0.2985) in 2009-10, 2010-11 and pooled results, respectively (Table- 2).

Table 2. Correlation and regression coefficients of mango leaf gall midge damage with weather parameters

Weather parameters		Correlation coefficient ('r')			Regression coefficient		
		2009-10	2010-11	Pooled	2009-10	2010-11	Pooled
Maximum temp.	(X_1)	-0.0244	-0.0050	-0.0140	—	—	—
Minimum temp.	(X_2)	-0.6396**	-0.5771**	-0.5934**	-0.0663	-0.0103	1.0459
Average temp.	(X_3)	-0.5290**	-0.4847**	-0.4976**	0.0249	-1.0981	-2.8339
Morning Relative humidity	(X_4)	-0.7611**	-0.4736**	-0.5719**	-87.8139	-194.9628	-52.6196
Evening Relative humidity	(X_5)	-0.6232**	-0.5488**	-0.5747**	-87.2292	-194.8343	-52.6335
Average Relative humidity	(X_6)	-0.6852**	-0.5497**	-0.5874**	174.4309	389.7815	104.9645
Wind Velocity	(X_7)	-0.1073	0.0799	0.0012	—	—	—
Sunshine	(X_8)	0.3200*	0.4991*	0.4214**	-0.7345	0.3714	-0.5420
Rainfall	(X_9)	-0.4455**	-0.5119**	-0.2985*	-0.0207	-0.3714	0.0019
Evaporation	(X_{10})	0.1562	0.2700	0.2095*	—	—	2.8601
R^2		—	—	—	0.5670	0.3969	0.5007
Variation explained (%)		—	—	—	56.70	39.69	50.07
R		—	—	—	0.7915	0.6926	0.7345
Constant (A value)		—	—	—	103.7050	75.2048	110.0238

* Significant at 5 % level

* * Significant at 1 % level

The multiple correlation coefficients (R) were significant (R = 0.7915, 0.6926 and 0.7345) in respective years and in pooled observations. The regression equations developed are:

2009-10: $\hat{Y} = 103.7050 - 0.0663 (X_2) + 0.0249 (X_3) - 87.8139 (X_4) - 87.2292 (X_5) + 174.4309 (X_6) - 0.7345(X_8) - 0.0207 (X_9)$

2010-11: $\hat{Y} = 75.2048 - 0.0103 (X_2) - 1.0981 (X_3) - 194.9628 (X_4) - 194.8343 (X_5) + 389.7815 (X_6) + 0.3714 (X_8) - 0.3714 (X_9)$

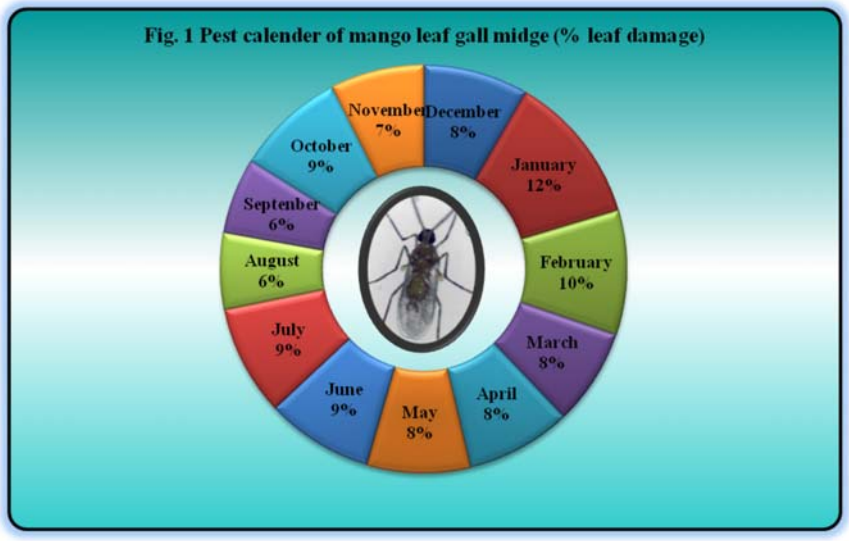
Pooled : $\hat{Y} = 110.0238 + 1.0459 (X_2) - 2.8339 (X_3) - 52.6196 (X_4) - 52.6335 (X_5) + 104.9645 (X_6) - 0.5420 (X_8) + 0.0019 (X_9) + 2.8601 (X_{10})$

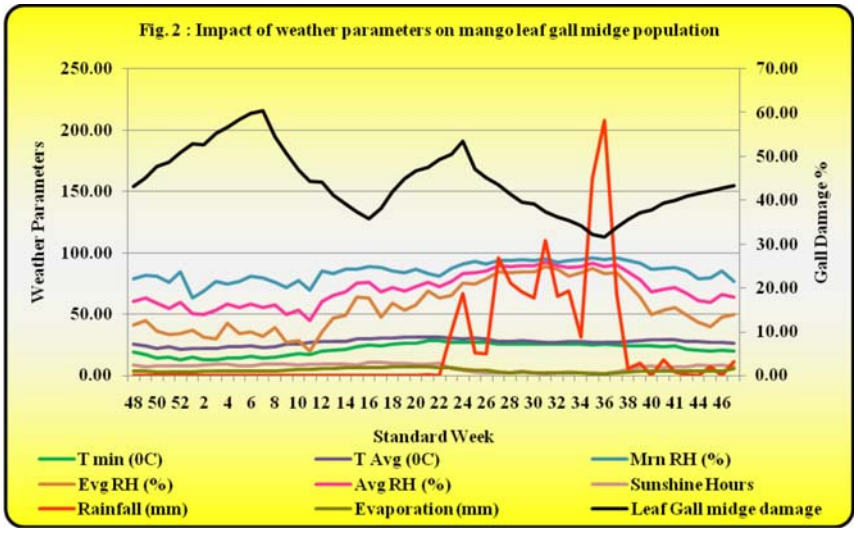
Where,

$\hat{Y}$ = Leaf damage (%)
X_2 = Minimum temperature
X_3 = Average temperature
X_4 = Morning relative humidity
X_5 = Evening relative humidity
X_6 = Average relative humidity
X_8 = Sunshine
X_9 = Rainfall
X_{10} = Evaporation

The regression analysis also explained total contribution of all the weather factors on seasonal cyclicity of leaf damage to the tune of 56.70, 39.69 and 50.07 per cent in 2009-10, 2010-11 and pooled observations, respectively.

So, looking to the impact of weather factors on overall abundance of mango leaf damage, it may be concluded that leaf midge damage was





directly influenced by sunshine implying higher the sun shine or longer the day length, higher was the leaf damage. Whereas, temperature (minimum and average), relative humidity (morning, evening and average), and rainfall had a negative impact indicating higher leaf damage when these factors had a minimum range and vice-versa. Patel *et al.,* (2011) reported leaf damage was directly influenced by sunshine, whereas relative humidity, temperature, rainfall and rainy days had a negative impact on their abundance. Kumar and Patel (2012) recorded highly significant positive correlation of sunshine hours with infestation of mango gall fly. Infestation was negatively correlated with temperature (minimum and average), relative humidity (Max., Min. and Av.), wind velocity, rainfall and rainy days. Jadhav *et al.* (2014) recorded infestation of mango leaf gall midge had significant positive correlation with sunshine hours. While, temperature, relative humidity, wind velocity and rainfall had significant negative correlation with the infestation of mango gall fly showed that when temperature, relative humidity, wind velocity and rainfall increased infestation of gall fly was decreased and vice-a-versa while, sunshine hours increased infestation of mango gall fly also increased and vice-a-versa. In the

present investigation, the damage increased during 11-29 (12 March – 22 July), 18-25 (30 April – 24 June) and 18-26 SW (30 April-1 July).

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In vitro rearing of brinjal shoot and fruit borer, *Leucinodes orbonalis* (Guenée) (Lepidoptera: Crambidae) on artificial diet

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ABSTRACT: The brinjal shoot and fruit borer (BSFB), *Leucinodes orbonalis* (Guenée) (Lepidoptera: Crambidae) is a key pest of brinjal. Studies were carried out on the development of its *in vitro* rearing on artificial diet on a large scale. An artificial diet without natural ingredients of brinjal is found to support the growth and development as good as or better than natural brinjal fruits. The BSFB takes 26.25 days to complete life cycle on artificial diet as compared to 25.42 days on natural brinjal fruits (var. Pusa Kranti). On the contrary, other traits viz., 13 day old larval weight, 2 day old pupal weight, % pupation, % adult emergence, adult longevity and fecundity of the BSFB on artificial diet are either better or is at par with those on the natural brinjal fruits. The artificial diet has a shelf life of 75 days at 4 ^{0}C. Thus, the artificial diet is useful for quality and economic production of insects under aseptic conditions on the basis of rearing at 27 ^{0}C, 60-75% rh and 13 hr photophase for more than 56 generations without fortification with field populations. The prospects of this method being useful for studies on various aspects of BSFB management including insect resistance management in insect protective transgenic brinjal are discussed.

KEYWORDS: Brinjal, *Leucinodes orbonalis*, artificial diet, rearing.

INTRODUCTION

Brinjal (*Solanum melongena* L.), also called aubergine or egg plant, is an important vegetable crop grown in India and many other parts of the world. It is tasty and nutritious vegetable rich in minerals (Choudhary and Gaur, 2009). India is the second leading producer of brinjal crop in the world after China with an annual production of 12.2 million tons from about 7,00,000 ha (FAO, 2012, 2013). Brinjal has a wide spectrum of insect pests (Srinivasan, 2009). The brinjal shoot and fruit borer (BSFB), *Leucinodes orbonalis* (Guenée) (Lepidoptera: Crambidae) is the key pest that causes heavy damage throughout the crop life (Atwal, 1976). The pest is primarily monophagous in nature, but sometimes has been reported on some other crops (Dhankar, 1988; Srinivasan, 2009; Onekutu *et al*., 2013). The infestation starts from the seedling stage till the final harvest. Within few hour of the egg hatching, BSFB larvae bore into and feed on the tender shoots and the fruits of the brinjal plant. It damages the young shoots, reducing plant growth thereby adversely affecting productivity. The boring nature of BSFB, with a barely visible sign during early infestation is the

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major reason that impairs the effective control of the pest (Butani and Verma, 1976). Despite a large scale use of insecticides (15-40 applications in a crop season), an annual loss of 60-70% in production was reported (MoEF, 2009). The pest is perennial in nature and its incidence varies with temperature, relative humidity (rh) and other environmental factors (Katiyar and Mukharji, 1974; Singh, 1977; Mahyco, 2006; Shukla and Khatri, 2010). Hence, the knowledge of its biology, ecology and susceptibility to insecticides is an essential requirement for integrated pest management including deployment of insect protective transgenic crops. The basic need for these studies is the availability of adequate number of high quality insects throughout the year. Rearing of BSFB is carried out on the natural fruits to study its biology and also elucidate the insect plant relationship including crop resistance (Wankhede *et al*., 2009; Maravi *et al*., 2013; Onekutu *et al*., 2013). However, non-availability of fruits of suitable varieties could be constraint in its rearing, as brinjal varieties are also known to be resistant to some extent (Dhankar, 1988; Ranjithkumar *et al*., 2013; unpublished). Besides continuity of regular supply of brinjal fruits; storage and associated contamination will adversely affect its rearing. Hence, Talekar *et al*. (1999) developed an artificial diet for its rearing using 1part of dried brinjal fruits and 9 parts of cotton bollworm artificial diet. Similar artificial diets with brinjal constituents with varying degree of success were also developed for BSFB rearing (Mahyco, 2006; Rahman *et al*., 2011; Ghante, 2012; Ranjithkumar *et al*., 2013). However, the use of natural fruits or its ingredients will limit the efforts to identify the suitable allelochemics responsible for resistance to BSFB, as the pest will adapt to such chemicals on their rearing over generations. Hence, the development of artificial diet with all nutrients in right proportion without natural ingredients of brinjal is essential (Patil, 1990; Cohen, 2004).

The present study reports the *in-vitro* rearing of BSFB *en masse* based on the development of artificial diet devoid of any nutrients of brinjal fruits for mass rearing over generations along with biological traits.

MATERIALS AND METHODS

(i) Collection and maintenance of test insects

Infested brinjals were collected from the wholesale vegetable market from Delhi (India) in 2010 and sliced to collect different stages of BSFB larvae. The collected larvae (69 larvae) were kept singly in sterile plastic containers (2 cm h × 5 cm dia) with brinjal slice at 27±2 °C, 60-75% rh and 13L: 11D photoperiod. The newly emerged adults were transferred to the mating jars (20 cm h × 15 cm dia), having a fresh brinjal twig dipped in water for egg laying and 10% honey solution fortified with multivitamins for feeding. Twigs containing the eggs were collected and kept in plastic jar (11 cm h × 7.5 cm dia) for hatching. Twig was replaced every 2nd day for more egg laying. The insect culture was maintained on the brinjal (var. Pusa Kranti) for first generation at 27±2 °C and 60-75% rh and 13 hr photophase for multiplication. These environmental conditions were maintained throughout studies.

(ii) Preparation of artificial diet

The key ingredients of the test diet were divided into three fractions (Table 1). Fraction A (chickpea, dried yeast powder, casein, L-ascorbic acid, methyl parahydroxybenzoate, sorbic acid, streptomycin sulphate, cholesterol, Wesson salt mixture), Fraction B (multivitamins, tocopherol, formaldehyde), Fraction C (Agar) and double distilled water. All the dietary ingredients used were of high quality and weighed accurately.

Diet was prepared by weighing the different fractions separately. The fraction A of the diet was mixed in one part of double distilled water (300 ml) and blended for 2 min in a one liter blending jar. Meanwhile, Fraction C was mixed with other part of double distilled water (150 ml) and boiled for 1-2 min in a microwave. The boiled fraction was allowed to cool down to 50±5 °C in order to reduce degradation of heat sensitive ingredients of artificial diet. Fraction B (except formaldehyde) was added to the finely mixed Fraction A and blended for 2-3 min. The cooled Fraction C and formaldehyde was added to the mixture of fraction A and B in the

Table 1. Cost of production of 450 ml of artificial diet for brinjal shoot and fruit borer

Fractions	Diet ingredients	Quantity (g)	Price (INR) per Unit pack	Cost (INR) as per quantity required
	Distilled water	450 ml	70 (5000 ml)	6.80
A	Bengal gram	84	23.25 (500 g)	4.20
	Dried Yeast granules	11	110 (500 g)	2.24
	Casein	5	781.5 (500 g)	7.82
	L-Ascorbic acid	3	2280 (500 g)	27.36
	Methyl parahydroxy benzoate	2	1015 (500 g)	6.10
	Sorbic acid	1	1008 (500 g)	2.02
	Streptomycin sulphate	0.2	4950 (500 g)	1.96
	Cholesterol	0.5	12190 (500 g)	12.19
	Wesson salt mixture	0.5	4035 (500 g)	8.07
B	Multivitamins[#]	1 ml	29.90 (15 ml)	1.90
	Vitamin E[##]	1 capsule	17 (10 capsule)	1.70
	Formaldehyde (10%)	1 ml	194 (500 ml)	0.39
C	Agar	11	4080 (500 ml)	89.76
				172.51 (Total)

[#]Each ml contains approximately: Energy (0.66 Kcal), Carbohydrate (0.15 g), Protein (0 g), Fat (0.007 g), Vitamin C (40 mg), Zinc sulphate (13.3 mg),Vitamin B_3 (10 mg), Lysine Hydrochloride (10 mg), Vitamin B_5 (1 mg), Vitamin B_2 (1 mg), Vitamin B_1 (1 mg),Vitamin B_6 (0.5 mg), Pine bark extract (500 mg), Vitamin A (2500 I.U.), Vitamin D_3 (200 I.U.) and Vitamin E (2.5 I.U.). ## Vitamin E capsule USP: Tocopheryl acetate IP (400 mg each).

blender, and blended again for 2-3 min. The diet was poured in the sterile Petri plates (17 cm dia × 3 cm h) and allowed to solidify at the room temperature. After solidification, the diet was stored in the refrigerator (at 4 °C) until use.

(iii) Evaluation of the artificial diet vis-à-vis brinjal fruits

The artificial diet was evaluated in comparison with natural brinjal oblong fruits and fruit slices (var. Pusa Kranti) at 27±2 °C, 60-75% rh and 13L: 11D photoperiod. The neonates of BSFB, five in number, were transferred on the artificial diet in cuboid form (3.4 cm length × 1 cm width× 1.5 cm h) weighing about 7 ± 1 gm in a sterile plastic container (2 cm h × 5 cm dia) or on to the brinjal fruit slice of ca. 7 gm or a fruit with a soft bristle brush. At least 30-40 replicates were maintained for each treatment. The brinjal fruit slice or fruit, as above, was replaced every alternate day to avoid decay while the artificial diet was added for growing larvae every 7th day. The observations of larval and pupal periods, % pupation and adult emergence, 13 day old larval and 2 day old pupal weights were recorded. The per cent pupation or adult emergence was estimated on the basis of the number of respective pupae formed or adults emerged out of neonates. The sex ratios were noted. The newly emerged adults were kept in the mating jars (19 cm h × 14.5 cm dia) with fresh brinjal twigs dipped in water for egg laying and 10% honey solution fortified with multivitamins for feeding of moths. Ten pairs of adults were kept per mating jar and the mouth of the jar was covered with rough cotton cloth. The eggs laid on the twig and rough cotton cloth were collected and kept in plastic container (11 cm h × 7.5 cm dia) until they hatch. The observations on

various biological traits including adult longevity, oviposition and incubation periods, fecundity and total life cycle for each treatment were noted and subjected to analysis of variance (ANOVA) using software (SAS, 1999).

In another set of experiments related to the effect of neonate density on insect growth and development; 2, 5 and 10 neonates (belonging F_{18} generation) were carefully transferred separately to each artificial diet (of the size as above) treatment in the plastic container of uniform size (2 cm h × 5 cm dia); with 30 replicates per treatment. The observations on various biological traits were recorded after every 24 hr without disturbing or removing the diet. The larvae were allowed to grow and pupate in the same container. The pupae formed in each treatment of different neonatal density were counted and collected in plastic Petri plates (11 cm dia × 2.5 cm h), lined with blotting paper at the bottom, until adult emergence. Observations on larval and pupal periods, % pupation and % adult emergence were subjected to ANOVA.

(iv) Evaluation of shelf life of artificial diet

Shelf life of the test diet was evaluated with neonates of BSFB belonging to $F_{21\text{-}22}$ generations. The test diet was prepared, as mentioned above. The freshly prepared diet was poured in 4 aliquots (100 gm each) in small Petri plates (11 cm dia × 2 cm h). The aliquots were labeled and date of diet preparation was mentioned on the covering lid. Once properly solidified, the 4 plates were covered, sealed with cling film and stored at 4 °C. One aliquot, labeled as 0 day prepared, was used for evaluating the biological traits viz. larval period, pupal period, % pupation and % adult emergence. The stored diet aliquots were taken out at different intervals (days) of 8, 18, 32, 75 and fed to the neonates as mentioned. Ten to twelve replicates were made for each shelf life treatment, with 5 neonates per diet replicate in a each plastic container (2 cm h × 5 cm dia). The 0 day prepared diet was kept as control and first observations were recorded on the 7th day. The rest of the stored diet was brought to room temperature before use. The rearing method was same as described above, and each time, 50-60 neonates were used to evaluate the stored diet quality by rearing of BSFB and analyzing their biological traits.

(v) Evaluation of microbial contamination

Prior to the evaluation of shelf life of diet, the stored diet aliquots were tested for contamination. A small fraction of the diet was diluted in autoclaved double distilled water and streaked on the nutrient agar (NA) medium. The plates were kept at 27 °C and 37 °C. Observations were recorded at 24 hr, 48 hr and 72 hr. Every time, six plates were streaked.

(vi) Biology of BSFB during different generations on artificial diet

BSFB was reared on the artificial diet for successive generations and in conditions as mentioned, except, that the brinjal twig was no longer used for egg laying as these were laid on the rough cotton cloth. The artificial diet was often not more than a week old, stored at 4 ^{0}C. The rough cotton cloth containing eggs was moistened with distilled water using spray bottle and stored in the plastic container. The biological traits were estimated the same way as described above. The larval density was 5 neonates on each diet cuboid in a plastic container (11 cm h × 7.5 cm dia) until they pupated at 27±2 °C, 60-75% rh and 13 h photophase.

(vii) Cost of artificial diet

Cost of artificial diet in Indian Rupees (INR) was calculated at the rates at which the ingredients (from the reputed supplier of chemicals) were purchased during the month of May 2013 and for the F_{40} generation. The rate of brinjal fruits were ascertained from the market.

(viii) Statistical analysis

The significance of difference in various traits of BSFB growth and development was determined by one way ANOVA at 95% degree of confidence using SAS software Enterprise guide 4.2 and Tukey's HSD ($p < 0.05$) test. The per cent data on pupation and adult emergence were subjected to

angular transformation prior to ANOVA. The cost of the diet was calculated in INR.

RESULTS

(i) Evaluation of artificial diet

The biological traits of BSFB on artificial and brinjal fruit diets showed differences (Table 2). The larval durations of BSFB fed on the artificial diet and the control diet (brinjal) differed ($F_{1,171}$ = 34.25, p < 0.0001). The pupal period was more on artificial diet than on control ($F_{1,142}$ = 4.91, p < 0.05). The per cent pupation was higher on artificial diet ($F_{1,59}$ = 46.65, p < 0.0001) and adult emergence was at par on either foods ($F_{1,59}$ = 23.38, p < 0.0001). There was no significant difference in the 13th day old larval weight on either diets ($F_{1,27}$ = 0, ns). The pupal weight of BSFB reared on brinjal fruit was significantly higher than that on artificial diet ($F_{2,27}$ = 53.13, p < 0.0001). Male and female longevities were at par on either diet, so were oviposition and incubation periods (Table 2). Female moths were more in number than male moths on either food source. The total life cycle of BSFB was shorter on brinjal than on artificial diet ($F_{1,40}$ = 5.51, p < 0.05). However, there were no differences in the fecundity of insects reared on either diets (Table 2).

(ii) Effect of Larval rearing density

The larval density affected all biological traits significantly (larval period, $F_{2,156}$ = 51.12 p < 0.0001; pupal period, $F_{2,131}$ = 5.89, p < 0.005; % pupation: $F_{2,89}$ = 10.14, p = 0.0001; % adult emergence, $F_{2,89}$ = 13.23, p < 0.05) (Table 3). The neonates grew and pupated in the same plastic container. The treatment of larval density of 5 neonates per diet cube (ca. 7±1 gm) in the plastic container (2 cm h × 5 cm dia) was the best in terms of % pupation and adult formation.

(iii) Effect of Shelf life of the diet

The F_{20} and F_{21} generation neonates acclimatized and adapted to the test artificial diet at 27±2 °C, 60-75% rh and 13L: 11D were used for evaluation of shelf life of the artificial diet (Table 4). The larval and pupal periods were significantly affected due

Table 2. Biological traits of brinjal shoot and fruit borer reared on artificial diet and natural brinjal fruits

Development parameters		Artificial diet	Brinjal*	F	P
Development duration (days)	Larval	15.89±0.08[a]	15.08±0.12[b]	34.23	<0.0001
	Pupal	7.21±0.09[a]	6.88±0.11[b]	4.91	0.03
% Survival	%Pupation	54.99±1.11[a]	43.81±1.21[b]	46.65	0.000
	%Adult emergence	46.61±1.45[a]	38.58±1.48[b]	23.38	0.000
Body weight (mg/insect)	Larva[#]	53.29±3.07[a]	53.07±1.52[a]	0	0.95
	Pupa[##]	37.50±1.02[b]	53.80±1.99[a]	53.13	<0.0001
Adult Longevity (days)	Female	3.95±0.15[a]	4.00±0.16[a]	0.05	0.824
	Male	3.35±0.15[a]	3.45±0.14[a]	0.25	0.62
Oviposition period (days)		2.6 ± 0.16[a]	2.5 ± 0.17[a]	0.18	0.67
Incubation period (days)		3.86 ± 0.18[a]	3.75 ± 0.16[a]	0.19	0.67
Fecundity (eggs/♀)		152.25±2.65[a]	147.95±1.62[a]	1.91	0.17
Total life cycle (days)**		26.25±0.25[a]	25.42±0.25[b]	5.51	0.024
Sex ratio (♂:♀)		1:1.5	1:1.5		

*Values (mean±s.e.) within the row, followed by same letter are not significantly different; [#] 13 day old larva and [##]2 day old pupa; *Brinjal was offered in form of slices of ca. 7 gm each; **Egg to adult stage*

Table 3. Effect of larval density on the biological traits of brinjal shoot and fruit borer

Development Parameters	No. of neonate/container			F	P
	2	5	10		
Total neonates	60	150	300		
Larval period (days)	14.96±0.15[b]	15.90±0.08[a]	14.33±0.12[c]	51.12	<0.0001
Pupal period (days)	6.71±0.14[b]	7.21±0.09[a]	7.5±0.10[a]	5.89	0.003
% pupation	42.27±4.78[b]	54.99±1.11[a]	36.85±1.25[b]	10.14	0.001
% adult emergence	35.45±4.68[b]	48.61±1.45[a]	26.19±2.19[c]	13.23	0.000

Values (mean±S.E.) within the row, followed by same letter are not significantly different.
n=number of replicates for each treatment

Table 4. Biological traits of brinjal shoot and fruit borer on artificial diet stored for different periods at 4 ^{0}C

Biological traits	Diet shelf life (days)					F	P
	0	8	18	32	75		
Larval period (days)	15.62±0.08[b]	14.22±2.22[d]	14.64±0.13[c]	15.33±0.18[b]	17.74±0.15[a]	81.45	<0.0001
Pupal period (days)	6.67±0.09[c]	6.58±0.09[c]	6.44±0.10[c]	7.52±0.09[b]	8.39±0.10[a]	65.77	<0.0001
% pupation	57.80±4.69[b]	58.09±3.63[b]	58.09±3.63[b]	64.14±4.07[a]	52.26±2.84[c]	1.21	0.32
% adult emergence	57.08±0.30[b]	57.86±1.07[b]	57.49±0.71[b]	62.91±0.52[a]	59.58±1.99[ab]	4.79	0.02

Values (mean±S.E.) within the row, followed by same letter are not significantly different.

to use of the artificial diets stored for different durations at 4 ^{0}C (larval period: $F_{4,175} = 81.45$, $p < 0.0001$; pupal period: $F_{4,126} = 65.77$, $p < 0.0001$). The % pupation and adult emergence were differently affected with dietary treatment (pupation: $F_{4,49} = 1.21$, $p > 0.05$; adult emergence : $F_{2,14} = 4.79$, $p < 0.05$). The BSFB rearing is unaffected on the diet stored until 32 days and possibly even more at 4 °C.

(iv) Biological fitness of BSFB of different generations on artificial diet

The biological traits of the BSFB in 2nd, 10th, 37th and 56th generation on the artificial diet at 27 ^{0}C, 60-75% rh and 13 h photophase differed in terms of larval and pupal periods and %pupation and adult emergence (Table 5). However, the larval and pupal weights were the highest in the 37th generation (larval weight: $F_{3,40} = 5.7$, $p = 0.003$; pupal weight: $F_{3,42} = 7.84$, $p = 0.0003$), suggesting that inbreeding did not affect adversely these traits.

(v) Cost of the artificial diet

The artificial diet produced significantly more adults per 100 gm diet than sliced or oblong brinjal fruits ($F_{2,16} = 436.89$, $p < 0.0001$)). Although sliced brinjal per INR cost produced more insects; nevertheless, artificial diet was much better in terms of higher number of adults, less drudgery and better insect quality (Table 6).

Table 5. Biological parameters of brinjal shoot and fruit borer reared on the artificial diet over 56 generations

Biological traits	Generations				F	P
	2	10	37	56		
Larval period	15.25±0.08^{b}	15.36±0.09^{b}	15.31±0.08^{b}	15.88±0.11^{a}	10.22	<0.0001
Pupal period	6.21±0.08^{b}	6.32±0.09^{b}	6.52±0.09^{a}	6.97±0.13^{a}	10.41	<0.0001
% pupation	48.57±2.38^{b}	54.57±1.94^{a}	53.30±1.69^{ab}	55.84±2.07^{a}	2.43	0.00001
% adult emergence	43.85±1.88^{b}	47.42±2.54^{ab}	49.73±2.15^{ab}	50.99±2.55^{a}	1.88	0.00001
Larval weight (mg)	48.5 ± 1.51^{b}	53.1 ± 2.21^{b}	59.3 ± 0.75^{a}	56.63±2.53^{ab}	5.70	0.003
Pupal weight (mg)	37.5 ± 1.02^{b}	43.5 ± 0.95^{a}	45.9 ± 0.79^{a}	42.92±1.59^{a}	7.84	0.0003

Values (mean±S.E.) within the row, followed by same letter are not significantly different.
[#] 13-day old larva and [##] 2 day old pupa

Table 6. Cost of production for each 100 gm diet and its equivalent natural food

Food Source (100 g)	Cost of diet/ 100 g (INR)	Adult production /100 g	Insect/ per INR	F value	Pr > F
Artificial diet	38.33	50.2 ± 1.5^{a}	1.3	436.89	<0.0001
Brinjal (oblong)*	3.00	4.2 ± 0.4^{b}	1.4		
Brinjal (sliced)*	3.00	6.8 ± 0.8^{b}	2.3		

Values (mean±S.E.) within the column, followed by same letter are not significantly different.
** Pusa Kranti*

(vi) Contamination test

The NA medium plates streaked with diluted stored diet aliquots did not show any microbial growth even at 37 °C.

DISCUSSION

The development of insect protective Bt brinjal and its consequent failure to get approval for commercialization in India and the successful commercialization in Bangladesh during 2013-14 has revived the need for studies on its biology, ecology and integrated pest management (http://www.barc.gov.bd/bt_brinjal.php). The rearing of BSFB has become a necessity to develop baseline susceptibility data for xenobiotics and also toxins present in insect protective transgenic brinjal, besides other studies (Rao *et al*., 1999; Wankhede *et al*., 2009; Ghante, 2012; Kalia *et al*., 2013; Ranjithkumar *et al*., 2013). The European corn borer, *Ostrinia nubilalis* was the first lepidopteran reared on artificial diet under controlled environment (Bottger, 1942), followed by many economical important insects (Singh, 1977; Cohen, 2004). The development of artificial diet for insect rearing is better than the use of natural diet in view of large scale availability round the year, economy of cost, less labour need and production of insects of uniform quality. The rearing of BSFB has been reported for limited generations on the natural fruits for studies on development of artificial diet as well as varietal screening for resistance in the laboratory (Kumar, 2004; Wankhede *et al*., 2009; Rahman *et al*., 2011; Maravi *et al*., 2013; Onekutu *et al*., 2013). The limitation in rearing on natural host or its parts is of a short shelf life and consequent decay (Maravi *et al*., 2013). Patil (1990) reported only 20-25% pupation of BSFB when reared on brinjal fruit slices (variety, not mentioned) which were

changed every alternate day. We in the present studies observed 43% pupation on brinjal fruit of var. Pusa Kranti. Further, the artificial diet was proved to be the best alternative to brinjal fruits or its slices for mass rearing of BSFB under controlled conditions (27±2 °C, 60-75% rh, 13L; 11D). Patil (1990) developed the mass rearing technique for BSFB and observed larval period of 18.50 days, 53.3% normal adult emergence and 66.7% pupation on artificial diet. Talekar *et al*. (1999) has reported the artificial diet comprising of one part of dried eggplant powder and 9 parts of the *Spodoptera exigua/Helicoverpa armigera* diet (commercially prepared, sold by BioServe Inc., USA) for rearing of BSFB. Our artificial diet appeared to be more successful in terms of % pupation and % adult emergence on the basis of neonates.

The larval density is also important constraint for their normal growth and development and consequently on mass rearing. The crowding of larvae leads to shortage of food, cannibalism and poor biological traits of the insects (Morton, 1979; Bhavanam *et al*., 2012). In the present studies, the cannibalism was observed in the treatment wherein 10 neonates/container were kept resulting in drastic reduction in % adult emergence. The cannibalism has been reported in other lepidopterans like cotton bollworms (Morton, 1979; unpublished). Hence, 5 neonates per plastic container was optimum density for efficient rearing of BSFB under controlled conditions. The artificial diet showed a good shelf life at 4 ^{0}C and without microbial contamination. Thus, we developed artificial diet and associated rearing techniques for successful rearing of BSFB for more than 56 generations without any infusion of wild population and without use of natural brinjal constituents based up the basic principles of nutrition and environment (Katiyar and Mukharji, 1974; Morton, 1979; Cohen, 2004). Interestingly, we did not observe any inbreeding depression, unlike in cotton bollworms (unpublished). At present, the rearing is in its F_{56} generation for more than 4 years. Although a100 gm artificial diet costs INR 34.70/-, it successfully supported 50 neonates to reach adult stage while the same amount of natural of brinjal fruit pieces, although cheaper, produced only 4-7 adults. This *in vitro* rearing technology may provide further impetus to the perspective studies on various aspects of biology, ecology, physiology and toxicology of xenobiotics.

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Population characteristics of phthirapteran ectoparasites infesting cattle in Rampur district

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ABSTRACT: Three hundred cattle were sampled for the presence of phthirapteran ectoparasites in Rampur district. Three phthirapteran species (*Damalinia bovis, Linognathus vituli, Haematopinus eurysternus*) were recovered from the cattle. *Solenopotes capillatus* and *Haematopinus quadripertusus* were not noticed. As many as 16.3%, (n = 300) cattle were found infested with one or other species of Phthiraptera, during 2007, in Rampur district. Single species infestation was more common than double (16.3%) and triple species (14.2%) infestation. Prevalence of Phthiraptera on two sexes remained similar ($\chi^2 = 0.48$, P = 0.05). It was higher in younger cattle than adults and aged ones ($\chi^2 = 10.48$, P = 0.05). Correlation between mean monthly intensity and mean monthly relative humidity was significant. Out of the three species recovered, the biting louse, *D.bovis* remained the most prevalent louse (11.3%), followed by anopluran, *L.vituli* (11%). The other anopluran louse, *H.eurysternus* was least prevalent (5.07%). Intensity of infestation (recorded by counting total number of lice noted on 20 anatomical sites measuring per square inch) remained 4.6 for *D.bovis*, 3.7 for *L.vituli* and 2.8 for *H.eurysternus*. In case of all the three cattle lice, sex ratios were female biased (1:1.4 to 1:1.5). Nymphal population dominated over adults (A: N = 1:1.7 to1:1.9).

KEY WORDS: Phthiraptera, Lice, *Damalinia bovis, Haematopinus eurysternus, Linognathus vituli*

INTRODUCTION

Survey of literature shows that basic information regarding different bio-ecological parameters of different phthirapteran species parasitizing cattle, has been provided by Craufurd-Benson (1941), Matthysse (1946), Ourmazdi and Baker (1974), Chalmers and Charleston (1980) and Milness *et al.* (2003). As far as population ecology of cattle lice is concerned, selected workers have indicated the population levels of different species of cattle lice in different parts of the world, from time to time (Chalmers and Charleston 1980; Titchener 1983; Kennedy and Karlka, 1986; El-Metenawy *et al.,* 1997; Milnes and Green, 1999; Colwell *et al.,* 2001; Kakar and Kakarsulemankhel, 2009). Geden *et al.* (1990) noted the effect of housing types on the population of cattle lice. Gibney *et al.* (1985) noted the effect of various infestation levels of lice on cattle feeding efficiency. Lewis and Christenson (1962) recorded the indices of the population of *D.bovis*. Watson *et al.* (1997) gave an indication of distribution patterns of cattle lice on their natural host. Nafstad (1998) also recorded the effect of various factors on the population of lice while talking of about their eradication measures. In India, Rawat *et al.* (1992) have noted the prevalence of lice on cattle in Dehradun. Seasonal variation in the population of cattle lice has been noted by Cumming and Graham (1982), De Vaney *et al*. (1992) and Geden *et al.* (1990).

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MATERIALS AND METHODS

The hosts were examined during January to December 2007 by the hair parting method (Lewis *et al.,*1967), at 20 anatomical sites (measuring 1 square inch by placing the thick wire molded in square shape) normally inhabited by the lice. Each site was thoroughly checked for the presence of lice. Lice from infested hosts were placed in glass tubes containing 70% ethyl alcohol, using a separate vial for each host. Each tube contained information regarding host, sex age or stage, condition and locality. Later, identification and sexing provided the data for recording the lice population. Sampled specimens were treated with 10% KOH for 24 hrs, (to dissolve the visceral organs) washed in water and transferred to 10% acetic acid for 1 hours (for better differentiation). Specimens were then subjected to dehydration (ethanol series), clearing (clove oil) and mounting (Canada Balsam) for Microscopy.

RESULTS AND DISCUSSION

Out of the six phthirapteran species reported to occur on cattle (*Bos taurus* L.), only three species, *Damalinia bovis* (Linnaeus), *Linognathus vituli* (Linnaeus) and *Haematopinus eurysternus* (Nitzsch) have been found on the cattle in Rampur district, in 2007. *Solenopotes capillatus* (Enderlain) and *Haematopinus quadripertusus* could not be recorded.

Prevalence of phthirapterans on cattle was found to be 16.33%, as 49 animals (out of 300) carried three species of lice. Out of 300 cattle examined in different localities in Rampur district, forty were males and 260 females. Prevalence of Phthiraptera on males and females remained 12.5% and 16.9% respectively (χ^2 = 0.48, P< 0.05, nonsignificant). Hence, prevalence of three cattle lice on two sexes remained nearly similar. Furthermore, out of the 300 cattle examined, 70 were young (below 04 years), 160 adults (04 -10 years in age) and remaining 70 older ones (above 10 years age). The prevalence of cattle lice on three groups remained 28.5%, 13.8% and 10.0%, respectively. Statistical analysis showed that difference in prevalence rate in three groups was significant (χ^2 = 10.48, P < 0.05). The data shows that maximum percentage of infested cattle bore single species infestation (69.4%). Double species infestation was noticed on 16.3% infested cattle. Simultaneous infestation by all the three species was recorded on only 14.2% hosts.

Damalinia bovis

Prevalence of *D.bovis* on cattle was 11.3%, (n = 300). Intensity of infestation remained 4.63. The value of sample mean abundance was 0.52 per square inch. A total number of 3152 specimens of *D. bovis* were recovered from 34 infested cattle. The range of infestation remained 45-228 (mean numbers collected from all the 20 sites). For recording the population structure at different levels of infestation, entire data was divided into four categories. Six cattle carried 45-65 specimens of *D.bovis* (mean number, 55.5; 8.3M, 12.3F, 34.8N; M: F = 1:1.5; A: N = 1:1.6). Maximum number of cattle (11) carried 66-86 lice (mean number, 76.6; 10.6M, 15.9F, 50.0N; M: F = 1:1.5; A: N = 1:1.8) (Fig. 1). Eight cattle were infested with 87-107 lice (mean number, 96.5; 13.6M, 20.5F, 62.3N; M: F = 1:1.5; A: N = 1:1.8). Nine cattle carried more than 107 lice (mean number, 133.7; 17.3M, 24.5F, 91.8N; M: F = 1:1.41; A: N = 1:2.1). The overall mean number remained 92.7 (12.7M, 18.6F, 61.3N; M: F = 1:1.5; A: N = 1:1.9). The overall ratio of three nymphal instars was found to be 1 : 1.2 : 2.

Back region was found to be the most heavily infested site (harboured 28.2% of the population obtained), followed by nape (20.8%). These two areas carried nearly 50% of total *D.bovis* population. Neck, abdomen and head were the next preferred sites (15.1%, 9.8% and 9.2%, respectively). The forelegs were the minimally infested site (3.9%) and carried lesser number of lice than tail and hind legs (7.1% and 5.6%, respectively). Our fields observations reveal that in case of heavy infestations, *D.bovis* could occur on any of the body parts but the back (also neck and nape) remain most lousy areas, during most parts of year.

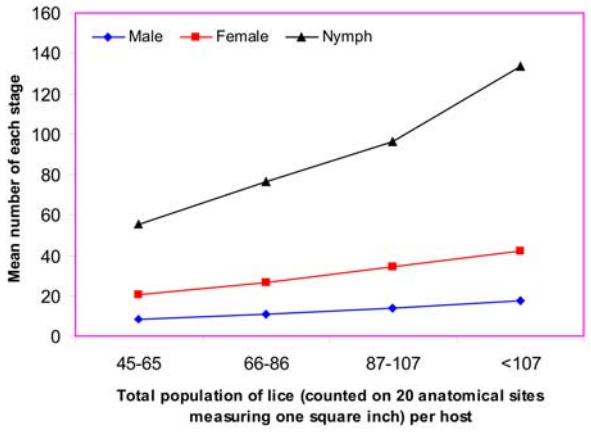

Fig. 1. Population composition of *Damalinia bovis* on cattle

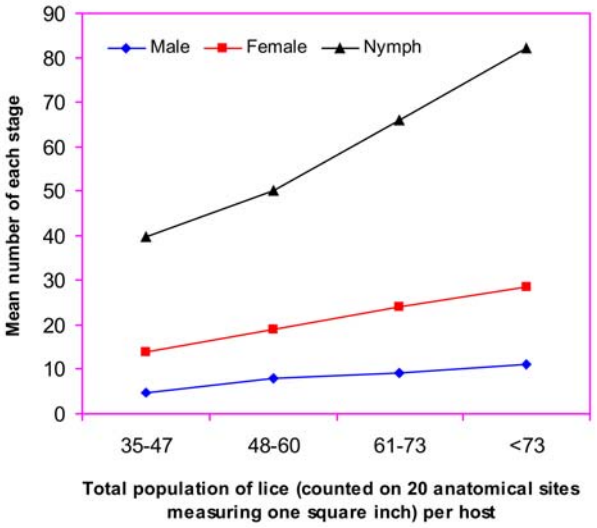

Fig.2. Population composition of *Haematopinus eurysternus* on cattle

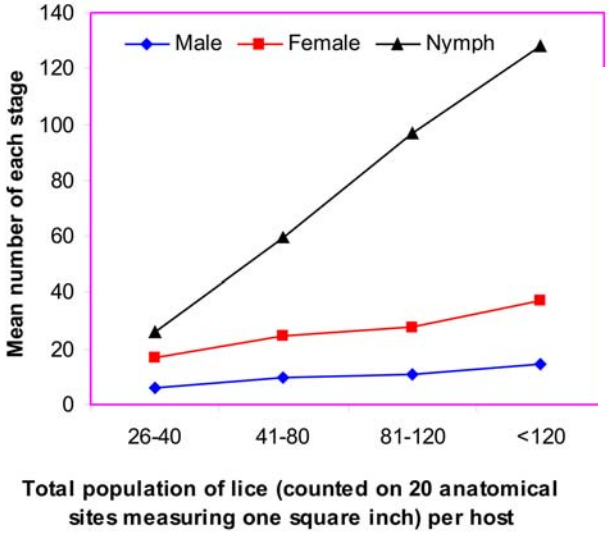

Fig. 3. Population composition of *Linognathus vituli* on cattle

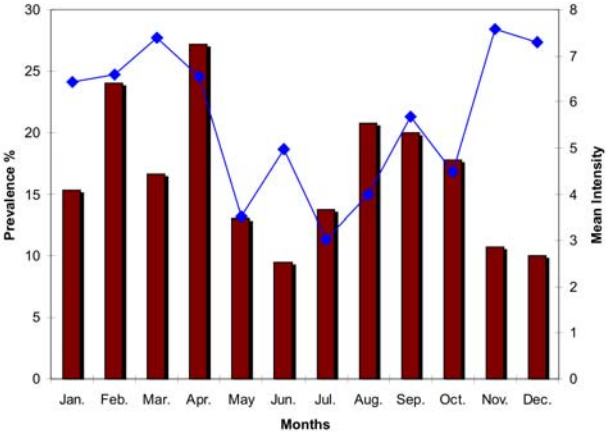

Fig. 4. Prevalence and intensity of infestation (based on mean of numbers counted on 20 sites per host) of phthirapterans on 300 cattle during different months of the year 2007, in the Rampur district (U.P.)

Haematopinus eurysternus

Prevalence of *H.eurysternus* on cattle was 5.3%, as 16 animals (out of 300) carried this louse. As many as, 892 lice were collected from the infested cattle (mean intensity, 2.8 per square inch) and value of sample mean abundance remained 0.14 per square inch.

For the sake of description, entire data was divided into four classes. Five cattle carried 35-47 (mean numbers collected from all the 20 sites) lice (mean number, 39.6; 4.8M, 9.0F, 25.8N; M: F = 1:1.8; A: N = 1:1.8). Four cattle carried 48-60 specimens of *H.eurysternus* (mean number, 50.2; 8.0M, 11.0F, 31.2N; M: F = 1:1.3; A:N = 1:1.6) (Fig. 2). Five cattle were infested with 61-73 lice (mean number, 65.8; 9.2M, 14.8F, 41.8N; M: F= 1:1.6; A: N= 1:1.7). Only two cattle carried more than 73 lice (mean intensity, 82.0; 11.0M, 17.5F, 53.5N; M: F = 1:1.5; A: N = 1:1.8). Thus, overall sex ratio of

H.eurysternus population remained 1:1.5. The adult nymph ratio was 1:2 and the ratio of the nymphal instars remained 1: 1.6: 1.7.

Maximum percentage of *H.eurysternus* (23.9%) occurred on the back region, narrowly followed by neck region (17.3%). Abdomen (13.0%), nape (11.7%) and head (9.6%) were the next preferred sites. The tail was minimally infested site (7.3%) as it harboured lesser number of lice than fore and hind legs (8.0% and 8.9%, respectively). In case of heavy infestation, *H.eurysternus* could occur on any of the body parts but the back region remains most lousy area.

Linognathus vituli

Twenty one (7.0%) of the cattle was found infested with *L.vituli.* A total number of 1545 lice (all stages) were recovered from the infested cattle (mean intensity, 3.6 per square inch). The sample mean abundance was found to be 0.25 per square inch (mean of total numbers collected from all the 20 sites).

Two cattle were infested with 26-40 lice (mean number, 26.0; 6.0M, 11.0F, 9.0 N; M: F = 1:1.8; A:N = 1:0.5) (Fig. 3). Maximum number of cattle (11) carried 41-80 specimens of *L.vituli* (mean number, 59.5; 9.6M, 15.23F, 34.6N; M: F = 1:1.5; A:N = 1:1.3). As many as, six cattle were found infested with 81-120 lice (mean number, 97.0; 10.6M, 17.0F, 69.3N; M: F = 1:1.5; A: N = 1:2.5). Only two cattle could be placed in more than 120 lice category (mean number, 128.0; 14.5M, 22.5F, 91.0N; M: F = 1:1.5; A:N = 1:2.4). In overall population composition, mean number (mean of lice collected from all the 20 sites) remained 73.6 (10.0M, 16.0F, 47.4N; M:F = 1:1.5; A:N = 1:1.8). The overall ratio of three nymphal instars was found to be 1: 1.4 : 1.8.

As far as the distribution of *L.vituli* on the body of host is concerned, the nape was found to be the most heavily infested site (harboured 25.6% of the population), followed by breast (22.8%) and neck (13.0%). Abdomen and head were the next preferred sites (11.6% and 10.6% respectively). The hind legs were minimally infested (4.2%) as it harboured lesser number of lice than tail and forelegs (5.9% and 5.6% respectively). In case of heavy infestation, *L.vituli* could occur on any of the body parts but the nape (also breast and neck) remain most lousy areas throughout the year.

The mean monthly prevalences and intensities of infestation of Phthiraptera on three hundred cattle in Rampur district during January to December 2007 have been depicted in Fig.4. The prevalence of lice on the cattle was maximum (27.2%) in April and minimum in June (9.52%). Likewise, the intensity of infestation was maximum in March (7.3 per square inch) and minimum in July (3.0 per square inch).

The correlation between prevalence and temperature (r = -0.034) and relative humidity were found nonsignificant (r = -0.31). The correlation between intensity and mean monthly temperature also remained nonsignificant at 5% level (r = 0.24).

Reports relating to prevalence of different phthirapteran species on the cattle in different parts of the world have appeared in literature from time to time. As far as, species wise prevalence is concerned, *D.bovis* reportedly occurred on 36.3% cattle in Alberta (USA) (Kennedy and Karlka, 1986), 52.8% (n= 1970) cattle in U.K (Milnes and Green, 1999), 23.6% cattle (n = 1752) in Turkey (El-Metenawy *et al.,* 1997), 36.9% cattle (n = 1230) in Canada (Colwell *et al.,* 2001) and 94% cattle (n = 33) in Norway (Nafstad and Gronstol, 2001). There is single report on the prevalence of Phthiraptera on Indian cattle (Rawat *et al.,* 1992). Latter, recorded the presence of *D.bovis* on 6.2% cattle (n = 1176) in Dehradun. Recently, Kakar and Kakarsulemankhel (2009) recorded the prevalence of *D.bovis* on cattle (38.3%, n = 990) in Pakistan. However, during present studies *D.bovis* was found to be more prevalent species on the cattle in Rampur district. This ischnoceran Phthiraptera is a minute non-haematophagous lice and feeds upon skin derivatives of its host. As far as, prevalence of *L.vituli* is concerned it is reported to occur on 37.2% cattle in Alberta, USA (Kennedy and Kralka, 1986), 1.6% cattle (n = 1752) in Turkey (El-Metenawy, 1997), 69% (n = 1230) in Canada

(Colwell *et al.,* 2001), and 42% cattle in Norway (n= 33) (Nafstad and Gronstol, 2001). Rawat *et al.,* (1992) found that 62.3% cattle (n = 1176) in Dehradun carried *L.vituli*. The haematophagous anopluran *L.vituli* is capable of causing considerable blood loss to host animals. However, its prevalence on the cattle in Rampur district appears to be quite low.

H.eurysternus is a robust haematophagous anopluran, capable of causing considerable annoyance to cattle. It is reported to occur upon 1.9% cattle (n = 1752) in Turkey (El-Metenawy 1997) and 4.4% cattle (n = 1230) in Canada (Colwell *et al.,* 2001). In India, as many as, 9.3% cattle (n = 1176) were found infested by this louse in Dehradun (Rawat *et al.,* 1992). During present studies *H.eurysternus* was found to be the least prevalent louse occurring on the cattle in Rampur district during the year 2007. However, another important louse *S. capillatus*, which is reported to occur upon cattle of most of the part, has yet not been recovered in India. Most of the workers agree that prevalence of lice increases on cattle during winter months (November to February) but during present studies correlation between mean monthly prevalences and mean monthly intensities with mean monthly temperature and R.H. was not found significant.

As far as, intensity of infestation is concerned different workers have adopted different coding system for describing the intensity. Hence, it is difficult to compare the results obtained during present studies. Data shows that mean intensity of *D.bovis* appeared to be higher (4.62 per square inch) in contrast that of *L.vituli* (3.7 per square inch) and *H. eurysternus* (2.8 per square inch). Present study further shows that in case of all the three species of cattle, females outnumbered the males in natural population, as the male, female ratio remained 1:1.4 to 1:1.5. The adult nymph ratio also appeared to be quite similar as it varied from 1:1.7 to 1:1.9. During present studies sex related differences in prevalence were not noted and the lice were more prevalent on younger cattle than older ones.

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New record of a genus and two species of whiteflies (Hemiptera: Aleyrodidae) from India

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ABSTRACT: The whitefly genus *Aleuroputeus* Corbett so far known from Malaysia is reported for the first time from India by reporting occurrence of *A. baccaureae* Corbett on *Tabebuia rosea* in Bengaluru, India. Further *Aleuroclava stereospermi* (Corbett) so far known from Malaysia has been reported for the first time from India on *Stereospermum colais.* The generic features of *Aleuroputeus* have been redefined. Both the species have been redescribed with illustrations.

KEYWORDS: Aleyrodidae, *Aleurputeus baccaueae, Aleuroclava sterospermi*

INTRODUCTION

Corbett (1935) erected the whitefly genus *Aleuroputeus* for *A. baccaureae* Corbett and *A. perseae* Corbett from Malaysia with the latter being the type species. This genus is so far known by these two species from Malaysia. In our survey on whitefly fauna of India *A. baccaureae* was found breeding on *Tabebuia rosea* in Bengaluru. Further *Aleuroclava stereospermi* (Corbett) so far reported from Malaysia was found breeding on *Stereospermum colais* in Bengaluru. Both the species have been redescribed with illustrations.

MATERIALS AND METHODS

The present study was based on the whitefly materials collected from various localities of south India during the period 2005-15 as well as the type specimens and other specimens of whiteflies available at the collections of Institute of Wood Science and Technology (IWST). The whitefly infested leaves were collected from the host plants and permanent mounts of the puparia were prepared by adopting the method suggested by David and Subramaniam (1976). The best mounts were obtained from puparia from which adults have emerged. Observations and illustrations were made using Nikon Optiphot T-2 EFD microscope and the identities of the whiteflies were confirmed.

RESULTS AND DISCUSSION

Aleuroclava stereospermi (Corbett) (Fig.1 – 3)

Aleurotuberculatus stereospermi Corbett1935**,** *J. fed. Malay. St. Mus.***17**: 832.

Aleuroclava stereospermi (Corbett) Jesudasan and David, 1990:4.

This species is reported for the first time from India.

Puparium: White, with no secretion of wax; elliptical, slightly constricted at thoracic tracheal pores and at the caudal end of abdomen; 0.64 -

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0.84 mm long, 0.46 - 0.64 mm wide; found singly on the under surface of leaves.

Margin: Finely crenulate, 23 crenulations in 0.1 mm; thoracic and caudal tracheal pores ending in a slight invagination. Anterior marginal setae 14 µm and posterior marginal setae 20 µm long.

Dorsum: Submargin with a row of papillae. Arrow-shaped microtubercles of varying sizes present on the subdorsum. Longitudinal moulting suture reaching margin and transverse moulting suture reaching submargin. Cephalothorax with four pairs of tubercles, on submedian area – one pair each on pro and mesothorax and two pairs on metathorax; base of cephalic setae granulated. Median tubercles on abdominal segments I - VI distinct, a submedian row of microtubercles extending from Vlll abdominal segment to I abdominal segment, ending with transverse moulting suture distinct. Base of I abdominal setae with distinct tubercle. Pores and porettes discernible. Thoracic tracheal furrow not indicated, caudal tracheal furrow funnel shaped with irregular markings, 50 - 76 µm long and 18 - 22 µm wide at the widest end.

Chaetotaxy: Four pairs of dorsal setae- cephalic setae 60 – 70 µm long, first abdominal setae 50 µm long, eighth abdominal setae 6 - 8 µm and caudal setae 60 - 80µm.

Vasiform orifice: Subcordate, wider than long, notched at caudal end, 38 - 44 µm long, 38 - 46 µm wide; operculum similarly shaped, 18 - 34 µm long, 18 - 36 µm wide, lingula not discernible.

Venter: A pair of ventral abdominal setae 10 µm long, 38 µm apart; thoracic and caudal tracheal folds not indicated. Antennae reaching base of prothoracic legs. Spiracles visible at the base of legs.

Material examined: India: Karnataka: Bengaluru, 5 puparia on *Stereospermum colais*,

24. xi. 2011, T.G. Revathi (IWST).

Host: *Stereospermum chelonoides* (Corbett, 1935); *S. colais* (new host record).

Distribution. Malaysia (Corbett, 1935); India: Bengaluru, Karnataka (new distribution record).

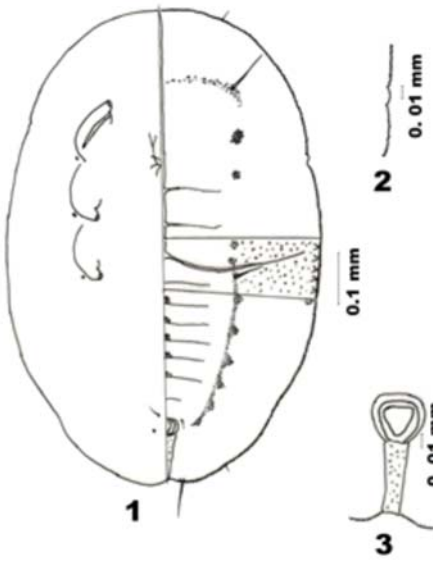
***Aleuroputeus* Corbett**

Aleuroputeus Corbett, 1935:846. Type species: *Aleuroputeus perseae* Corbett, 1935: 846-847, by original designation.

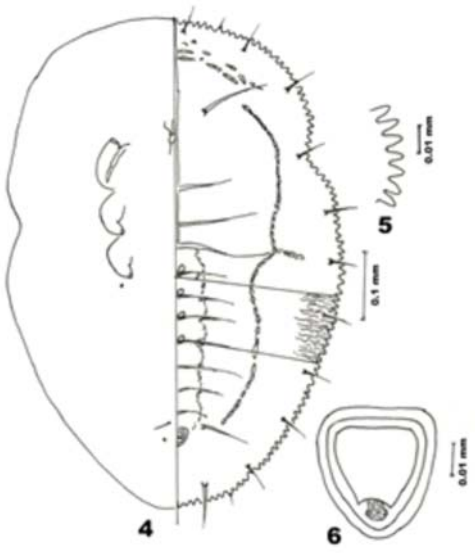
The genus is redefined here.

Diagnosis: Puparia white to yellowish, without any wax secretion; margin toothed without any indication of thoracic and caudal tracheal openings. A pair of conspicuous thin longitudinal interrupted lateral fold extending from anterior margin to laterad of vasiform orifice, demarcating submedian area from subdorsum; submargin with a row of ten pairs of long setae extending beyond margin. Median moulting suture reaching margin and transverse moulting suture reaching the lateral longitudinal fold. Abdomen with elevated submedian ridge and rachis, and with median tubercles. Cephalic, eighth abdominal and caudal setae present, first abdominal setae absent. Vasiform orifice cordate to subcordate, situated on elevated posterior end of submedian ridge, longer than wide; operculum recessed posteriorly, filling half to two-thirds of the vasiform orifice; lingula tip exposed but included.

Comments: Corbett (1935) differentiated *Aleuroputeus* from *Aleurocybotus* Quaintance & Baker in the possession of submarginal spines (setae) and in the presence of a chitinised fold (longitudinal lateral fold) differentiating the dorsal disc. It also essentially differs from *Aleurocybotus* by the absence of first abdominal setae and by the presence of elevated vasiform orifice. *Aleuroputeus* is also close to *Aleurotrachelus* Quaintance and Baker and *Cohicaleyrodes* Bink-Moenen in having pair of lateral longitudinal folds, elevated vasiform orifice and in the absence of first abdominal setae and not having any indication of thoracic tracheal openings but differs from them by the presence of ten pairs of long submarginal setae extending beyond margin and operculum filling only half to two-thirds of the vasiform orifice with posterior recession. The genus *Aleuroputeus* is so



Figs. 1-3. ***Aleuroclava stereospermi*** **(Corbett): 1. Puparium; 2. Margin at thoracic tracheal pore region; 3. Vasiform orifice**



Figs. 4-6. ***Aleuroputeus baccaureae*** **Corbett: 4. Puparium; 5. Margin; 6. Vasiform orifice**

Fig.7: **Mounted puparium of** ***Aleuroputeus baccaureae*** **Corbett**

far known by only two species both the species described by Corbett (1935) and in his description he indicated the presence of 11pairs of submarginal spines (setae), the posterior pair slightly longer. The posterior pair is caudal setae and the puparia are characterized by the presence of ten pairs of submarginal setae.

Key to puparia of the species of ***Aleuroputeus***

1. Puparium yellowish and subovate; abdominal segments I – II with median tubercles. . . . .*perseae* Corbett

-. Puparium white and elliptical; abdominal segments I – IV with median tubercles. . . . . *baccaureae* Corbett

Aleuroputeus baccaureae **Corbett** (Fig.4 – 7)

The genus *Aleuroputeus* and the species *baccaureae* Corbett are reported for the first time from India and the species is redescribed here.

Puparium: White, without wax secretion; elliptical, 0.65 - 0.76 mm long, 0.43 – 0.56 mm wide; found singly on the lower surface of leaves.

Margin: Smoothly crenulate, 13-15 crenulations in 0.1 mm; thoracic and caudal tracheal pores not indicated. Anterior marginal setae 14 - 20 µm long and posterior marginal setae 18 - 22 µm long.

Dorsum: Subdorsum with papillae-like irregular markings, submargin with faint striations. A pair of conspicuous thin interrupted longitudinal lateral folds

extending from anterior margin to laterad of vasiform orifice, demarcating submedian area from subdorsum. Median tubercles on abdominal segments I – IV distinct. Longitudinal moulting suture reaching margin and transverse moulting sutures reaching to the chitinised fold. Thoracic tracheal furrow and caudal tracheal furrow not indicated. Rows of pores and porettes distinct.

Chaetotaxy: Three pairs of dorsal setae- cephalic setae 84 µm long, eighth abdominal setae 62 – 74 µm long and caudal setae 40 µm long. First abdominal setae absent. Ten pairs of submarginal pointed setae, 20 µm long- 5 pairs on cephalothorax and 5 pairs on abdomen distinct.

Vasiform orifice: Subcordate situated on elevated posterior end of submedian ridge, 42 - 52 µm long, 40 - 48 µm wide, operculum similarly shaped, almost filling the vasiform orifice, recessed posteriorly, 32 - 40 µm long, 32 - 34 µm wide. Lingula tip knobbed, exposed and included.

Venter: A pair of ventral abdominal setae 18 - 20 µm long, 36 - 40 µm apart; thoracic and caudal tracheal folds not discernible. Antennae reaching the base of prothoracic legs. Spiracles visible at the base of legs.

Specimens examined: India: Karnataka: IWST campus (Bengaluru), 7 puparia on *Tabebuia rosea*, 7.ii.2011, T.G. Revathi (IWST).

Hosts: *Beccaurea motleyana* (Corbett, 1935); *Tabebuia rosea* (new host record).

Distribution. India: Karnataka, Bengaluru.

The family Aleyrodidae comprises of 438 species under 60 genera in India. Among the genera the genus *Aleuroclava* Singh is represented by 62 species (Sundararaj and Pushpa, 2010). Sundararaj and Pushpa (2011) described two new species of *Aleuroclava viz., A. sindhuiae* and *A. nigrus* and Chhakchhuak and Sundararaj described *A. mizoramensis*, *A. schimea* and *A. serchhipensis* as new species (Chhakchhuak and William, 2011) thus the number of species known under the genus *Aleuroclava from India is 67*. The new record of *A. sterospermi* brings the number of Indian species of *Aleuroclava* to 68. The genus *Aleuroputeus* Corbett is so far known only from Malaysia and its representation by the record of *A. baccaureae* brings the total number Indian whitefly genera to 61. Thus a total of 440 species of Aleyrodidae under 61 genera are now known from India.

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Determination of critical density in *Culex tritaeniorhynchus* Giles, 1901 (Diptera: Culicidae) as a deciding factor influencing the transmission of Japanese encephalitis virus in southern India

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ABSTRACT: Japanese encephalitis virus (JEV) causes a severe neurobiological health hazard, namely, Japanese encephalitis (JE) which has been rampant in Cuddalore district in the 1970s and 1980s, transmitted mainly by *Culex tritaeniorhynchus.* The authors have been working on various aspects of transmission dynamics of JE in Cuddalore district, south India, for past more than two decades and have analyzed varied data on ecology and biology of vector *Cx. tritaeniorhynchus,* to develop a model for depicting the critical density which is a primary requisite to develop control strategy against vector. Such a model of critical density for the vector will be helpful to forecast prospective outbreaks of the disease exacting heavy morbidity and mortality in many parts of India.

KEYWORDS: Cuddalore, JE virus, *Culex tritaeniorhynchus,* Critical density

INTRODUCTION

Japanese encephalitis (JE) is widespread over South East Asia and Pacific regions where 3 billion people are at risk of infection. It is a leading cause of viral encephalitis in Asia, caused by a virus from the family Flaviviridae and mainly occurs and prevailing in rural setting, especially in rural and suburban areas where rice growing and pig farming culture coexist (Campbell *et al.,*2011; Halstead and Jacobson, 2008). It is of greater public health importance since it produces mild infection to permanent brain damage with increasing case fatality rate through causing serious inflammation of the membranes around the brain (Bhowmik *et al.,* 2012). Japanese encephalitis virus (JEV) is transmitted to humans by infective bites of female mosquitoes mainly belonging to *Culex tritaeniorhynchus*, *Culex vishnui* and *Culex pseudovishnui*. In India, *Culex vishnui group (Culex tritaeniorhynchus, Culex vishnui and Culex pseudovishnui)* are the chief vectors of JE which breeds particularly in stagnant water in the flooded rice growing fields (WHO, 2001). JEV is maintained in an enzootic cycle between mosquitoes and amplifying vertebrate hosts, primarily pigs and wading ardeid birds. Humans are incidental or dead-end hosts, because they usually do not develop a level or duration of viremia necessary to infect mosquitoes (Susan *et al.,* 2012).

JE disease was first reported in Japan in 1924, was subsequently reported in other Asian countries whereas in India the first case was reported in the state of Tamil Nadu in 1955. Till 2012, about 17 states/UTs in India have reported incidence of JE.

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Since 2008, a maximum number of JE cases were reported in the years 2011 (1214) and 2012 (745) compared to previous years. It was estimated that an average incidence of JE cases and deaths were about 719 and 121 respectively can occur annually. Eastern and central regions of India are highly affected with maximum number of cases and deaths compared to other regions.

There are a number of studies related to serological and epidemiological aspects of JE in India. But there is lack of studies on entomological investigation, especially for the estimation of number of mosquito bites required (single host (pig)) to maintain virus transmission. CRME has field station at Cuddalore district in Tamil Nadu and has been working on vector borne diseases including JE during last two decades. In this paper, there was an attempt to develop a mathematical model on the basis of MacDonald's model on malaria and Gordon Smith's model on Western Equine Encephalomyelitis (WEE) with necessary inputs and refinements in order to determine the number of mosquito bites required (single host (pig)) to maintain JE virus transmission in Cuddalore district.

MATERIALS AND METHODS

Study area:

JE has been highly endemic in Cuddalore district in Tamil Nadu. The district is located in the South East of Tamil Nadu, Southern India. The district has an area of 3564 sq.km with a population of 2.6 million. Tamil Nadu is one of the leading rice growing states in India, in which Cuddalore district alone contributes 6.65% of production to the states. The major JE outbreak had occurred in Cuddalore district during 1981 (Kabilan *et al.,* 2004) and since then it has been reported to have high morbidity rate (Gajanana *et al.,* 1995; 1997). A prospective study conducted during 1989-91 in some villages of the study area was reported a high rate of seroconversion in sentinel pigs against flaviviruses and high vector densities with high infection frequency for JEV (MIR of JEV = 10.4).

Mosquito collection and estimation of infection rate:

Mosquitoes were collected during April 2011 to December 2012 by using aspirators and compared its results with the previous studies in order to understand the features and biological characteristics of the mosquitoes. The virus infection rate at mosquitoes was expressed as minimum infection rate (MIR) per 1000 female's tested (Chiang and Reeves, 1962).

$$\text{MIR/1000} = \frac{\text{Number of mosquito pools positive}}{\text{Total number of mosquitoes tested}} \times 1000$$

Daily survival rate (P = Proportion of mosquito survival one day) of *Cx. tritaeniorhynchus* was calculated by using a formula gc"p_i, where gc is the length of gonotrophic cycle and p_i is the parity rate (Davidson, 1954).

JE transmission modeling:

The life cycle of JEV is maintained between mosquitoes and hosts including pigs, birds and humans where humans are incidental or dead-end hosts since they do not develop required concentration for survival and replication of JE virus in their bloodstreams to infect feeding mosquitoes. In the mosquito borne diseases, the parasite transmission model was initially developed and estimated in 1957 by MacDonald for Malaria (Mac Donald, 1957). In 1970, Gordon Smith was estimated a transmission model for Western Equine Encephalomyelitis virus (WEE) (Smith, 1987) on the basis of Mac Donald's model with certain refinements since the virus transmission cycle for WEE was different from the cycle of Malaria parasite. Since the life cycle of Japanese encephalitis (JE) virus is closely related to WEE, West Nile and St. Louis encephalitis viruses, in this study, an attempt was made to estimate the model for JEV transmission on the basis of existing models (Mac Donald, 1957; Smith, 1987; Ebel and Kramer, 2009) of transmission with necessary changes. The various models estimated so far in mosquito borne disease are given in Table 1. There are factors like number of *Culex tritaeniorhynchus* bites/host,

Table 1. Comparison of inclusion/exclusion factors engendering in deriving various formulas for calibrating 'Critical Vector Density'

Sl. No	Author (Year)	Area (Species)	Models	Factors development of Model	Chronological
1	MacDonald G. (1957)	Malaria(*Anopheles)*	$R_0 = \frac{ma^2 b p^n}{-r(\log_e p)}$	**m** = the number of vectors per man **a** = the proportion of mosquito feeding on man /gonotrophic cycle **b** = probability that a human gets the infection after being bitten by an infective mosquito **p** = the proportion of vector surviving one day (Survival probability) **n** = the incubation period of parasite in the vector **r** = the daily rate each human recovers from infection	
2	Garrett-Jones (1964)	Malaria (*Anopheles gambiae)*	$C = \frac{ma^2 p^n}{-(\log_e p)}$	**m** = the number of vectors per man **a** = the proportion of mosquito feeding on man /gonotrophic cycle **p** = the proportion of vector surviving one day (Survival probability) **n** = the incubation period of parasite in the vector	Derived from Macdonald's Model but excluded certain parameters like 'b' and 'r'
3	Smith C.E.G. (1987)	Western Equine Encephalomyelitis (WEE) (*Culex tarsalis)*	$R_o = \frac{m b h S_m v S_v p^i}{-\ln p}$	**m** = the number of vectors per bird(critical vector density) **b** = no.of feeds by mosquito each day/ gonotrophic cycle **h** = proportion of blood meals taken from birds **Sm** = vector competence for WEE	Derived from Macdonald's Model with certain refinement on following parameters 1.Replaced denominator in variable 'm' (Replaced anthrpophilic into zoophilic)

Sl. No	Author (Year)	Area (Species)	Models	Factors development of Model	Chronological
			$m = \frac{-\ln p}{b\, h\, S_m\, V\, S_v\, p^i}$ (where $R_0=1$)	**V** = duration of infective viraemia in birds **Sv** = the proportion of birds susceptible to infection **p** = the proportion of vector surviving one day (Survival probability) **i** = the extrinsic incubation period of virus in the vector	2. **'a'** replaced with **(b). (h)** 3. '**b'** replaced as $\mathbf{S_m}$ (vector competence) 4. Added **V** = duration of infective viraemia in birds 5. Added **Sv** = birds susceptibility. 6. '**n'** replaced as '**i'**
4	Paulo *et al.* (2005)	Dengue (*Aedes albopictus*)	$C = \frac{ma^2 p^n}{(-\ln p)}$	**m** = the number of vectors per man **a** = the proportion of mosquito feeding on man /gonotrophic cycle **p** = the proportion of vector surviving one day (Survival probability) **n** = the incubation period of virus in the vector	Used similar model derived by Garrett-Jones (1964)
5	Ebel and Kramer (2009)	West Nile Virus (*Anopheles maculipennis)*	$V_C = \frac{ma^2 b\, p^n}{(-\ln p)}$	**m** = the number of vectors per host **a** = the proportion of mosquito feeding on host /gonotrophic cycle **b** = (Vector competence) **p** = the proportion of vector surviving one day (Survival probability) **n** = the incubation period of virus in the vector	Based on existing derivation except replacement of feature of parameter 'm' (Replaced denominator in variable 'm'. Replaced anthrpophilic into zoophilic)

Sl. No	Author (Year)	Area (Species)	Models	Factors development of Model	Chronological
6	Ciota *et al.* (2013)	West Nile Virus (*Culex pipiens)*	$V_C = \frac{mh^2 b p^n}{(-\ln p)}$	**m** = the number of vectors per host **h** = (blood feeding rate)the proportion of mosquito feeding on host / gonotrophic cycle **b** = (Vector competence) **p** = the proportion of vector surviving one day (Survival probability) **n** = the incubation period of virus in the vector	Based on existing derivation except replacement of feature of parameter '**m**' (Replaced denominator in variable 'm'. Replaced anthrpophilic into zoophilic) 1.The variable '$\mathbf{a^2}$' was replaced as '$\mathbf{h^2}$'
7	Garaza Hernandez *et al* (2013)	Dengue (*Aedes aegypti*)	$C = \frac{ma^3 b p^n}{(-\ln p)}$	**m** = the number of vectors per man **a** = the proportion of mosquito feeding on man /gonotrophic cycle **b** = (Vector competence) **p** = the proportion of vector surviving one day (Survival probability) **n** = the incubation period of virus in the vector	Derived on the basis of Garrett-Jones (1964)' s Model with following modifications 1.Included the variable '**b**' 2.Modified $\mathbf{a^2}$ as $\mathbf{a^3}$ based on the proportion of mosquito feeding on man/ gonotrophic cycle
8	Current Derivation	Japanese Encephalitis *(Culex tritaeniorhynchus)*	$m = \frac{-\ln(p)}{-a \cdot S_c \cdot V_c \cdot p^n}$	**a**= the proportion of mosquito feeding on pig /gonotrophic cycle $\mathbf{S_c}$=Proportion seroconversion rate of sentinel Pigs **Vc** = Vector competence **P** = Survival probability **n** = Extrinsic incubation period	Derived from Macdonald and Smith C.E.G's Model with certain refinements on following parameters 1. Replaced denominator in variable 'm' (birds replaced as pigs as source of blood feeding) 2.Replaced **'b. h'** as **'a'** 3.'**Sv'** replaced into '**Sc'** Seroconversion in pigs (Susceptibility) 4.'**Sm**' replaced into '**Vc'** (Vector competence) 5. Excluded the attribute '**V'** (duration of viraemia)6. Parameter '**i'** replaced as ' **n'**

mosquito survival rate, gonotrophic cycle, mosquito susceptibility, vector competence and incubation period for JEV in vector are influencing JEV transmission and required for its estimation. The factors responsible for influencing JEV transmission and required for estimation of transmission model was constructed by using two decadal research activities (published data) of CRME in Cuddalore district.

Critical vector (Culex tritaeniorhynchus) density:

The basic reproduction rate (R_0) is defined as "an average number of secondary infections produced when one infected individual is introduced into a host population where everyone is susceptible" and is commonly used term to predict epidemic dynamic of infectious diseases. If $R_0 > 1$, the disease is spread indefinitely; if $R_0 < 1$, the disease will die out where the value for $R_0=1$ the disease is likely to exist. The basic reproduction rate (R_0) for JEV transmission was expressed as

$$Ro = \frac{m.\ a\ .\ S_c\ .\ V_c\ .\ P^n}{-\ln(P)}$$

Where,

m: the number of JE mosquitoes bite/pig/night (Critical density).

P: the Proportion of mosquito surviving one day.

a: the proportion blood feeing on pig (h) / Gonotrophic cycle.

S_c: the seroconversion (JE) in sentinel pigs (proportion of pigs susceptible to infection).

V_c: the vector competence of *Cx. tritaeniorhynchus* (proportion of Vector susceptible to infection).

n: the incubation period of the JE virus in the vector.

The basic reproduction rate allows ($R_0 = 1$, endemic area) the calculation of the critical density (m) threshold of hosts necessary to maintain virus transmission. Thus the critical density (*Cx. tritaeniorhynchus*) for JEV transmission can be expressed as:

$$m = \frac{-\ln(P)}{a\ .\ S_c\ .\ V_c\ .\ P^n}$$

Data analysis:

Mathematical and statistical analysis of data for derivation of JE transmission model and its graphical presentation were done by using Microsoft Excel 2007 version and SPSS version 16.0. We have conducted online searches of published literatures on mathematical models related to vector density for JEV transmission through various database without restriction to languages or geography and finally ensures that the model for estimating critical density in *Culex tritaeniorhynchus* for transmission of JEV has yet to be derived. After initial search restricted to published data was done, a model was designed and derived on the basis of MacDonald, 1957 and Smith, 1987 models by including/excluding the parameters required for this estimation.

RESULTS

Mosquito abundance:

The mosquito vectors of JEV were longitudinally monitored for its abundance and virus infection in the villages of Cuddalore district. The *Culex vishnui* group, comprising *Cx. tritaeniorhynchus, Cx. vishnui* and *Cx. pseudovishnui* are proven vectors of JE in Southern India) (Reuben et *al.,* 1988). A three year longitudinal study was conducted during 1991-1994 with the objectives of monitoring vector abundance and JEV infection frequency in mosquitoes in the villages covered under the Nallur PHC, an area endemic for JE, in Cuddalore district. In which, a total of 422,621 female mosquitoes were collected and found that *Cx. tritaeniorhynchus* (62.6%) was the predominant species (Gajanana *et al.,* 1997). During April' 2011 to December' 2012, a total of 15,941 female mosquitoes representing 24 culicine species were collected. About 90.5% of total catch was contributed by the JE vectors, in which, *Cx.*

gelidus was 48.6%, *Cx. tritaeniorhynchus* was 40.7% and remaining 1.8% by *Cx. Vishnui*. A two decade research activities and the comparative analysis of the various studies related to vector abundance monitoring has revealed that *Cx. tritaeniorhynchus* was the principal vector. A maximum per man hour (PMH) density was observed (661.50) in October 2002 during the last ten years with reporting peak level during the months of October and November (Figure 1). A critical vector density was determined for *Cx. tritaeniorhynchus* since this particular species was found and reported as a predominant vector compared to other species in influencing the JEV transmission in Cuddalore district.

Blood feeding habit (h):

Assessment of blood feeding habits of *Cx. Vishnui* complex was done during the period of December 1988 and December 1990 and found out that a relatively high proportion of recognized vectors of JE virus were *Cx. tritaeniorhynchus* and *Cx. vishnui*, had fed mainly on cattle in addition to humans and pigs. Pig feedings (h) accounted for 4.4 – 5.4%, cattle feedings for 84.6 – 88% of the total feeding and human feedings for 2.4 – 6.2%, but reported that there were no ardeid - positive feeding (Reuben *et al.,* 1992). Though the ardeid was as one of host for JEV transmission, it was excluded while estimation of this model since there was no evidence of blood fed on ardeid in the study area.

Gonotrophic cycle (Gc):

The frequencies of blood meals taken and the survival rates of vector mosquitoes are important parameters influencing transmitting capacity of pathogens (Nat *et al.,* 1998). Mosquito gonotrophic cycle (Blood-feeding - egg maturation - oviposition) is repeated in several times throughout adult female mosquito's life cycle (Paaijmans and Mathew Thomas 2011). Mori (1983) and Somboon *et al. (*1989) estimated daily survival rate of JE vectors by using Davidson's method (Davidson, 1954) and found that the gonotrophic cycle duration was around 3-4 days. In Cuddalore district, JE cases mainly were occurred during the months of September – November. During these months, the gonotrophic cycle in females of the *Cx. vishnui* subgroup reached 3 days. Female mosquitoes infected after taking a viraemic blood meal and would become infective 9 day later after completing 3 gonotrophic cycles (Gajanana *et al.,* 1997).

Vector competence (Vc):

The virus transmitting capacity of a mosquito was influenced by various factors such as the ability of an ingested virus to survive and its development in the mosquito tissues and penetrates into the salivary glands in order to transmit into a new host. Vector competence is estimated as the proportion of mosquitoes with a disseminated infection to the total number of exposed mosquitoes, often expressed as dissemination rates within a vector population (Christofferson and Christopher, 2011). Philip Samuel *et al.,* 1998 have developed a system for assessing vector competence of mosquitoes and done experiments in three different areas (Cuddalore, Madurai and Alleppey) and reported that the estimated vector competence of *Cx. tritaeniorhynchus* (i.e. transmission rate) was ranged from 32 -74% ,in which, the range was about 32 % in Cuddalore.

Host (pig) susceptibility (Sc):

Gajanana *et al.,* 1995 have done a cohort prospective serological study (1989-1991) among primary school children and also monitored the prevailing status of JE virus infection among sentinel pigs in the study area. In this study, about 124 pigs were examined and 66 (53%) animals was found to be infected with JEV and seroconverted during the transmission season of JEV.

Survival probability (P):

The parous rate (Rate of parous mosquitoes) is one of the useful parameters to describe the age structure and net reproductive rate of the mosquito population. It is not only used to ascertain the daily survival rate of adult mosquitoes but also used to determine the recruitment rate of adults, the adult

longevity and the length of a gonotrophic cycle, therefore, any changes in the parous rate will reflect and bring changes in the population (Yoshio *et al.,* 1991). In Cuddalore district, human cases were mainly affected and reported during the months of September to November every year. It was estimated that the parity rate (PR= the proportion of parous from the total number of ovaries dissected Ndoen *et al.,* 2012) was 0.33 in September, 0.42 in October and 0.32 in the month of November and the probability of the vector surviving one day (survival probability (*P*) was 0.69, 0.75 and 0.69 respectively (Table 2) and an the average survival rate of *Cx. tritaeniorhynchus* (*P*) was 0.8 during this transmission season (Reuben, 1963). The survival probability of *Cx. tritaeniorhynchus* ranged between 0.64 – 0.76 during April 2011 to December 2012.

Extrinsic incubation period (EIP) in vector (n):

The prolonged development period of mosquito larval and the longer extrinsic incubation period of JE virus at cooler temperature will reduce the virus transmission rate (Solomon *et al.,* 2000). Due to prolonged viraemia, mosquitoes get the opportunity to pick up infection from pigs easily (NVBDCP). After an extrinsic incubation period of 9-12 days, infected female mosquito transmits the virus to other vertebrate hosts (NVBDCP, 2006). It was estimated that extrinsic incubation period of JEV in *Cx. tritaeniorhynchus* was 9 - 10 days (n = 9-10 days) in Cuddalore district. Female infective mosquitoes taking a viremic blood meal and would become infective 9d later after completing 3 gonotrophic cycles. The proportion of infective female mosquitoes among those infected would be about $P^n = 0.13$ ($P = 0.8$, n = 9, $P^n = 0.13$) (Gajanana *et al.,* 1997). The proportion of vector surviving in the incubation period (P^n, n = 9-12 days) was ascertained after 9 days during the years 2011 and 2012 and was given in Table 3.

Mosquito life expectancy: There are two factors such as gonotrophic cycle (gc) and parity rate (PR) were required and used here to estimate the life

Table 2. Parity Rate (PR) and survival probability (*P*) of *Cx. tritaeniorhynchus* in Cuddalore district (Transmission seasons)

Year	Month	Dissected	Parous	PR	*P*	Age (days)
2011	September	106	39	0.37	0.72	3.03
	October	102	40	0.39	0.73	3.24
	November	88	23	0.26	0.64	2.26
2012	September	27	9	0.33	0.69	2.73
	October	90	29	0.42	0.75	3.49
	November	50	21	0.32	0.69	2.66

Table 3. The proportion of *Cx. tritaeniorhynchus* surviving the virus - during in the transmission seasons (2011 and 2012)

Year	Month	*P*	P^9	P^{10}	P^{11}	P^{12}
2011	September	0.72	0.051	0.037	0.027	0.019
	October	0.73	0.062	0.046	0.033	0.025
	November	0.64	0.019	0.012	0.008	0.005
2012	September	0.69	0.037	0.026	0.018	0.012
	October	0.75	0.076	0.057	0.043	0.032
	November	0.69	0.034	0.023	0.016	0.011

expectancy and infective life of mosquito. The PR, proposed by Davidson, 1954 was used to arrive this derivation. The gonotrophic cycle (gc) value was found to be 3 days. The life expectancy (age) of *Cx. tritaeniorhynchus* was in the range value of 2.26 - 3.03 in 2011 and 2.66 - 3.49 in 2012 during the transmission season (Table 4) which likely to be change based on the survival probability (P = 0.60 - 0.80) of the mosquito. Therefore, based on the survival probability, the life expectancy (age) and the expected infective life (days) of *Cx. tritaeniorhynchus* was estimated and given in Table 5 (Figures 2 and 3).

Critical vector density (m):

Critical density is used to estimate the number of mosquitoes required to bite a single host (pig) in order to maintain virus transmission in an area .The values required for estimation of critical vector density (m) were collected from the results of various studies conducted on JE during the two decades in Cuddalore district (Tyagi *et al.,* 2011). The critical vector density for the study area was estimated to be around 595 which mean a minimum of 595 mosquito's bite/pig/night is necessary in order to maintain virus transmission in the particular area (Table 6).

DISCUSSION

Culex tritaeniorhynchus is the most abundant mosquito, accounting for 41 to 63 % of the total mosquitoes species in Cuddalore district since this district is a major rice growing area in Tamil Nadu where pig rearing culture also coexist. This species usually bite at night; preferably on cattle, pigs and humans in the study area. The blood feeding pattern of the mosquitoes were assessed and found that cattle feeding was predominant and accounted for around 88%, followed by human blood feeding (6%) and pig feeding (5%). Though 88% of blood feeding pattern of mosquito was depend on the cattle, it will not play an active role in influence the virus

Table 4. Life expectancy and expectation of infective life (days) of *Cx. tritaeniorhynchus* in Cuddalore district during the transmission seasons (2011 and 2012)

Year	Month	P	Expectation Life	Expectation of infective life (days)			
				n = 9	n = 10	n = 11	n = 12
2011	September	0.72	3.03	0.156	0.112	0.080	0.058
	October	0.73	3.24	0.201	0.147	0.108	0.079
	November	0.64	2.26	0.042	0.027	0.017	0.011
2012	September	0.69	2.73	0.102	0.070	0.049	0.034
	October	0.75	3.49	0.266	0.199	0.150	0.113
	November	0.69	2.66	0.090	0.062	0.043	0.029

P = probbility of *Cx. tritaeniorhynchus* survival through one day, n = Extrinsic Incubation period (JE virus in the vector). Life expectation is expressed as (1/-ln(p)) and expectation of infective life as (p^n/ -ln(p).

Table 5. Expectancy of life or age and infective life of *Cx. tritaeniorhynchus* in Cuddalore district

Survival probability (P)	Life Expectation (1/-ln(p))	Expectation of infective life (days)			
		n = 9	n = 10	n = 11	n = 12
0.60	1.96	0.020	0.012	0.007	0.004
0.65	2.32	0.048	0.031	0.020	0.013
0.70	2.80	0.113	0.079	0.055	0.039
0.75	3.48	0.261	0.196	0.147	0.110
0.80	4.48	0.601	0.481	0.385	0.308

Table 6. Computation of the critical density (m) of *Cx. tritaeniorhynchus* in Cuddalore district

Proportion of host (pig) blood feeding Index (h)	0.05
Gonotrophic cycle (gc) days	3
a = Proportion blood feeing on pig/ Gonotrophic cycle(h/gc)	0.017
Proportion of host (pig) Susceptibility (S_C)	0.53
Proportion of Vector Competence (V_C)	0.32
Survival probability (*P*)	0.8
Incubation Period (n) (days)	9
P^n = The proportion of *Cx. tritaeniorhynchus* surviving the virus	0.13
Critical Density (m)	595.3

transmission cycle (dead end host). Though the ardeid birds are one of the main hosts (reservoir) in influencing the JEV transmission cycle but in the study area, the assessment of blood feeding pattern of mosquitoes was revealed that the ardeid host was accounted for zero percentage (Reuben *et al.,* 1992). Therefore, ardeid was not considered as one of the parameter in influencing the virus transmission and excluded from the estimation of critical vector density (m). If ardeid would found be one of host, then this factor also need to be included and considered along with pigs for formulae derivation. Pigs are important amplifying host, in which naive pigs are highly susceptible to infection and reported with high mortality (CFSPH, 2007). The number of pigs in a region can affect and influence the incidence of JE cases. However, we could reduce the risk of infection through changing the type of husbandry practices and modern pig farming.

The two decadal studies of JE in Cuddalore district found that the *Cx. tritaeniorhynchus* was the predominant species during eighties and nineties, but during 2011 and 2012 *Cx. gelidus* was found to be the predominant species, perhaps shift in agricultural practices from paddy to sugarcane which has a direct correlation with this *Cx. vishnui* and *Cx. tritaeniorhynchus* in density of the two vector species. The assessment of biting behavior of *Cx. tritaeniorhynchus* was found that it bites throughout the year irrespective of dry and cool-wet (C-W) seasons and the mosquito abundance which influences the transmission of virus cycle and was found to be happening around the clock. The EIP in mosquito was found to be a minimum of 9 days. The duration of the gonotrophic cycle of *Cx. tritaeniorhynchus* was found as 3 days and completes its 3 gonotrophic cycles after 9 days (Gajanana *et al.,* 1997). The high parous rates imply that the probability of daily survival of the vectors for efficient transmission of infection is high (Ree and Hwang, 2000). High parity values would be evidence for high egg development and high blood intake. The interaction between the survival probability (*P*) and EIP decides and influences the infective life days. It was found that infective life was gradually increased when the survival probability (*P*) was increasing and decreasing in EIP and vice versa (Figure 3).

The Basic reproduction model was developed by MacDonald (1957) in 1957 for transmission of malaria by the *anopheline* mosquitoes (Smith, 1987) presented a modification of the Malaria reproduction rate formula of MacDonald, 1957 by replacing men by birds in order to estimate the reproductive rate of arboviruses (R) for WEE where birds are active host in disease transmission . However, this estimation was based on so many untested assumptions. In this study, we have tried and estimated the critical density for JEV transmission on the basis model of Smith *et al.,* 1987 by including and excluding the factors which required for estimation for JEV. This model was estimated that a minimum of 595 mosquito bites/

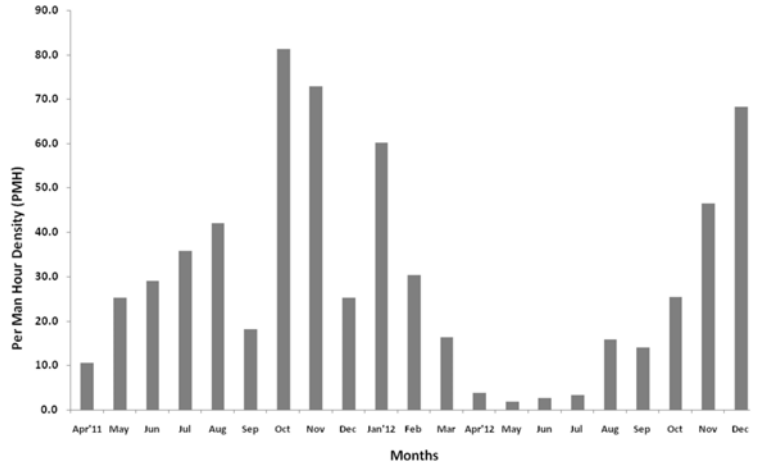

Figure 1. Cx. tritaeniorhynchus Per Man Hour density in Cuddalore district during

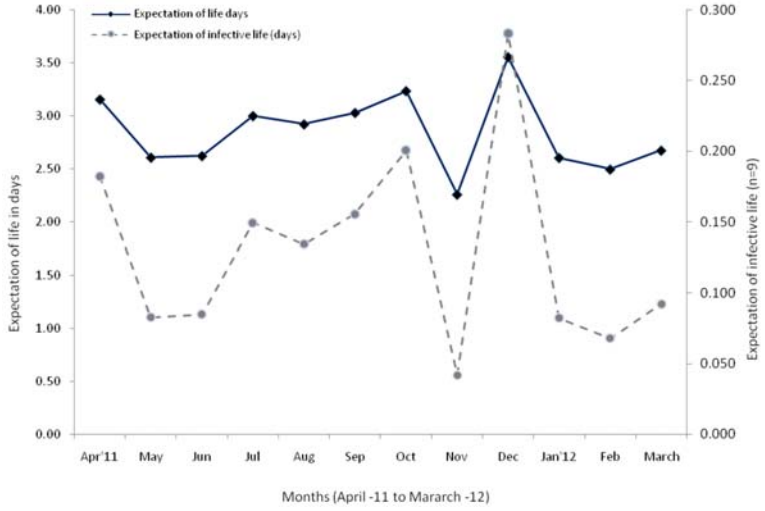

Figure 2. Expectancy of life days (age) and infective life (days) of *Cx. tritaeniorhynchus* in Cuddalore district during April 2011 to March 2012

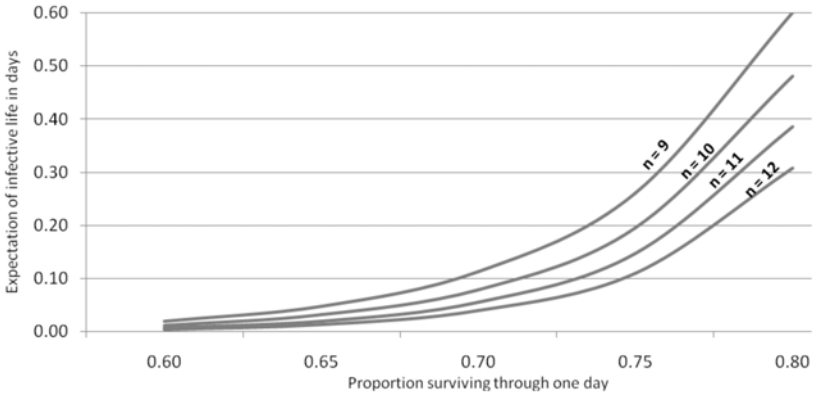

Figure 3. Survival probability and expectancy of infective life (days) of *Cx. traitaeniorhynchus* in Cuddalore district

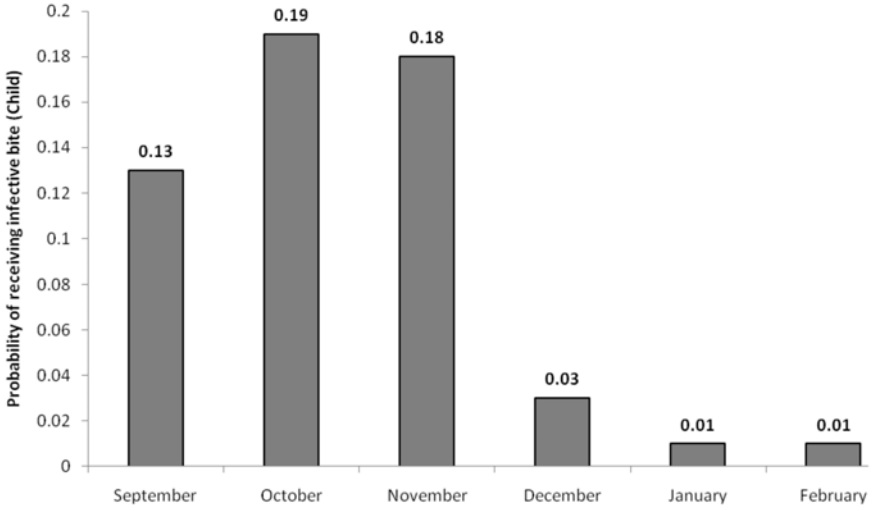

Figure 4. Monthly probability of receiving an infective bite in villages of Cuddalore district

pig/night are necessary to maintain JEV transmission.

Risk to human" Human is a dead end host in JE transmission cycle due to low and short-lived viraemia. Mosquitoes do not get infection from JE infected humans because humans usually do not develop a level or duration of viraemia sufficient to infect mosquitoes (Fischer *et al.,* 2010). Risk to humans depends on Infection rate in mosquito which relates to intensity of transmission between pigs and probability that a person is bitten by an infective mosquito. Majority of the cases were reported soon after monsoon, i.e. during August and September months. Upsurge of cases are reported during the rainy season (monsoon) (Gunasekaran *et al.,* 2012). JE cases started reporting during the months of April - May and reaching peak during late August to early September and subsequently decline from October (Ree and Hwang, 2000). The duration of the illness ranged from 1 to 14 days. The mean and median age were 5.7 +10.1 (S.D) and 7.5 years (range = 3 months – 15 years), respectively (Kabilan *et al.,* 2004). *The* distribution of cases was equal among both the sexes. In the study area, the probability of child receiving an infective bite during September and December was 0.53 which is reasonably close to the estimate of 0.50 – 0.75 was obtained from seroconversion rates in children in the same area (Figure 4) (Gajanana *et al.,* 1997). Minimum infection rate at mosquitoes (per 1000) in April 2011 to December 2012 was 0.24 (95 pools, 1 +ve pool) and average PMH was 26.8 during its peak season with 25.4 (October), 81.1(November) and 68.2(December) of the year (Figure 1). Susceptibility in humans may also depend on many other factors like age, immune status of the individual and other unknown factors. Hence, risk of the disease to humans will be evaluated with critical density of mosquitoes required to maintain the disease in pig population itself.

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Dissipation kinetics and effect of processing on clothianidin residues in cardamom (*Elettaria cardamomum* Maton)

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ABSTRACT: The dissipation behaviour of clothianidin (Dantop 50% WDG), a neonicotinoid insecticide, in fresh and cured cardamom capsules was studied following-application at 20 and 40 g a.i. ha^{-1}, respectively, in the plantation of Indian Cardamom Hills (ICH), Idukki, Kerala, India. A single laboratory UPLC-MS/MS method was developed and validated for the estimation of residues of clothianidin in fresh and cured cardamom. The recovery experiments were conducted at fortification levels of 0.01, 0.05 and 0.1µg g^{-1}.The average recoveries obtained were 91.04 to 94.44 and 89.22 to 91.41 % for fresh and cured cardamom, respectively. The LOD and LOQ in both fresh and cured cardamom were found to be 0.005 and 0.01µg g^{-1}, respectively. The initial deposits of clothianidin on fresh and cured cardamom were 1.96 and 5.24 µg g^{-1}, respectively, following application at the lower dose while the corresponding deposits were 4.13 and 10.61µg g^{-1} at the higher dose. For fresh and cured cardamom, the residues dissipated below the quantitation level of 0.01µg g^{-1} after 21 and 28 days at both the doses, respectively. The half-life of clothianidin in fresh and cured cardamom was 3.40 and 3.11 days at the lower dose and 3.42 and 3.45 days at the higher dose, respectively. The waiting periods of clothianidin on fresh and cured cardamom at the lower and higher doses were 18.41 and 22.09 days, and 21.16 and 27.54 days, respectively. The processing factor was evaluated at the lower dose and the mean processing factor was 2.90.

KEYWORDS: cardamom, clothianidin, dissipation, processing factor

INTRODUCTION

Cardamom (*Elettaria cardamomum* Maton), the queen of spices, is one of the most exotic and highly prized spices in the world. It thrives well in the tropical rain forests of Western Ghats of India. India accounts for the largest area under cardamom cultivation yet the productivity is low mainly due to the attack of diverse pests and diseases in all stages of the crop growth, necessitating frequent application of pesticides for their timely control (Kumaresan, 2008). It is reported that around 650 tons active ingredients of different pesticides were applied during 2009 season in Indian cardamom hills (Murugan *et al*., 2011). The residues of pesticides deposited during plant protection operations are a major concern and pesticide residue in spices have affected our exports (Bhardwaj *et al*., 2011). Management of pests in cardamom largely depend on the use of conventional, neurotoxic, broad-spectrum, synthetic chemical pesticides, such as organophosphates, carbamates, synthetic pyrethroids and a number of new chemical classes, such as neonicotinoids. Exposure to pesticides, both

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occupationally and environmentally, poses a range of adverse effects. The resistance build-up by pests to conventional pesticides demands the use of newer and safer products with low dose requirement and different modes of action (Sreelekshmi, 2014; Aravind *et al.,* 2015).

Neonicotinoids are relatively new class of insecticides with novel mode of action and chemistry. They act as agonists, which play an important role in synaptic transmission in the central nervous system of insects. Clothianidin ((E)-1- (2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine) is a second generation neonicotinoid insecticide with excellent plant systemicity, broad-spectrum activity and high crop safety, was discovered by the Agro Division of the Sumitomo Chemical Co. Ltd. (formerly Takeda Chemical Industries, Ltd.), and was co-developed with Bayer CropScience (Uneme, 2011). It acts selectively on insect nicotinic acetylcholine receptors (Thany, 2009). This pesticide is highly effective in controlling hemipterous insects coleopterous, thysanopterous, and certain lepidopterous pests (Chen *et al*., 2005). Being the member of biggest selling insecticide class worldwide, it is important to find out the residue dynamic characteristic of clothianidin in various agricultural and environmental commodities. However, studies on the residue and dissipation of clothianidin are limited. Uneme and co-workers (Uneme *et al.,* 2006) studied the efficacy of clothianidin against several pests including hemipterous, coleopterous, thysanopterous, dipterous and some lepidopterous in different crops including tea.

The monitoring studies of pesticide residues in market samples of cardamom in Kerala analyzed by Pesticide Residue Research and Analytical Laboratory of Kerala Agricultural University, Kerala, India indicated a high level of contamination with pesticides including neonicotinoid group insecticide; imidacloprid indicates the use of relatively new class of such insecticides. Since no information is available on the persistence of clothianidin in cardamom, the present study was undertaken to standardise the protocol for estimation of residues, assessment of dissipation kinetics and effect of curing on level of residues of clothianidin after application at the fruit maturing stage of cardamom.

MATERIALS AND METHODS

Analytical standard (≥ 95.0% purity) of clothianidin was procured from M/s. Sigma-Aldrich, USA. Dantop 50% WDG, formulation from Nagarjuna Agrichem Ltd, Hyderabad, acetonitrile of HPLC grade (LiChrosolv), magnesium sulfate, sodium chloride, sodium sulphate of GR grade from Merck (Mumbai) and methanol of HPLC grade (LiChrosolv) were procured and the solid reagents were activated before use. Primary secondary amine (PSA), endcapped C18 and graphitized carbon black (GCB) from Agilent Technologies, USA. The 0.22 µm pore sized polyvinylidene fluoride (PVDF membrane) syringe filters (Rankem, New Delhi) were used to filter the extracts. All the glasswares were thoroughly washed as per the standard operating procedure to avoid the interferences from any contaminant during analysis. The suitability of the solvents and other chemicals were ensured by running reagent blanks before actual analysis.

Preparation of standard solution:

A standard stock solution of clothianidin (1,000 mg L^{-1}) was prepared in methanol and stored at -20 °C. The working standard solutions were prepared by serially diluting the standard stock solution to obtain the suitable dilutions required for plotting a calibration curve (1.0, 0.50, 0.25, 0.10, 0.075, 0.05, 0.025, 0.0l and 0.005 µg mL^{-1}) and were used for studying the linear dynamic range of the UPLC–MS/MS analysis, preparing matrix-matched calibration standards and spiking samples. The working solutions were stored in refrigerator at 4 °C. The recovery study with the fresh and cured cardamom capsules were carried out at 0.01, 0.05, and 0.10 µg g^{-1} with five replications at each level. Matrix matched calibration standards, prepared by adding the extract of blank samples, were in the range of 0.01–1 µg mL^{-1} for the compounds under study.

Validation of analytical methods:

Single laboratory method validation was done to standardize the procedure for residue estimation of fresh and cured cardamom capsules. For method validation, the multiresidue estimation procedure recommended for fruits and vegetables using Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method as per AOAC Official Methods of Analysis (2007) was adopted for residue estimation in cardamom with slight modifications so as to suit the changed matrix. Control samples of fresh and cured cardamom with no history of pesticide application were collected and used for spiking insecticides at the three different levels. The samples were finely ground from which 12.5 g fresh and 5 g cured samples were taken and spiked with clothianidin at 0.01, 0.05 and 0.1µg g^{-1} levels in a 50-mL centrifuge tube. For cured cardamom, 4 g of sodium chloride and 5 mL water were added. The samples were mixed thoroughly in a vortex for 5 min. and 25 mL acetonitrile was added and kept in freezer at -20 °C for 20 minutes. The samples were homogenized (Heidolph Silent Crusher-M) at 14,000 rpm for 3 min. Sodium chloride (5 g) was added to the homogenized sample and thoroughly vortexed for 2 min. followed by centrifugation at 2500 rpm for 5 min. An aliquot of 12 mL of clear supernatant was transferred in to a 50-mL centrifuge tube prefilled with 6 g anhydrous sodium sulphate and mixed well for 2 min. for removing traces of moisture, if any. The extract was cleaned up by dispersive solid phase extraction (DSPE). For this, 8 mL of the supernatant was transferred to a 15-mL centrifuge tube containing 0.1 g primary secondary amine (PSA) sorbent and 0.6 g anhydrous magnesium sulfate, 0.05 g endcapped C18 and 0.025 g GCB. The mixture was again vortexed for 1 min. and centrifuged for 3 min. at 2,500 rpm. Five mL of the supernatant was transferred to a turbovap tube and evaporated to dryness under a gentle stream of nitrogen using the TurboVap set at 40 °C and 8 psi nitrogen flow. The residues were then reconstituted in 2 mL of methanol and filtered through 0.22µm polyvinylidene fluoride (PVDF) syringe filter for UPLC-MS/MS analysis.

Ultra Performance Liquid Chromatography (UPLC):

The residues of clothianidin were estimated using an Acquity UPLC (Waters, USA) - API 3200 MS/MS (AB Sciex) system by using a reversed phase 2.1 mm × 100 mm i.d. column (Atlantis d C18, Waters, USA) with 5 micron particle size in a column oven at 40 °C. The compound was separated in one single gradient run. The operation of the LC gradient involved the following two eluents: A: 10% methanol in water + 0.1% formic acid + 50 mM ammonium acetate; B: 10% water in methanol + 0.1 % formic acid + 50 mM ammonium acetate. The gradient elution followed was: 0 min. isocratic 5 % B, 0.0–3.0 min. linear from 5 % to 80 % B, 3.0 – 5.0 min. linear from 80 % to 100 % B, 5.0 - 7.0 min. linear from 100% to 50% B, 7.0 - 9.0 min. linear from 50% to 30 % B with 9.0 – 10.0 min. for initial conditions of 5 % B. The flow rate remained constant at 0.8 mL/min. and injection volume was 10 µL. The retention time of clothianidin was 1.65 min. (Fig. 1).

Mass spectrometry:

The effluent from the LC system was introduced into Triple quadrupole API 3200 MS/MS (AB Sciex) system. Analyte was detected by multiple reaction monitoring (MRM) using electrospray ionization mass spectrometry (ESI -MS) operating in the positive ion mode. The source temperature was 550 °C. Other important source parameters were; Ion source gas (GS1) 25 psi, ion source gas (GS2) 40 psi, ion spray voltage 2800 V and curtain gas 30 psi. For clothianidin, two MRM transitions were monitored and the quantitative and qualitative ion pairs selected were 250>169.1 and 250>132, respectively (Fig. 2). The parameters like declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) for the quantitative ion pair were 45, 5, 18, 20 and 1, respectively. The corresponding values in the case of qualitative ion pair were 45, 5, 18, 23 and 1, respectively.

Field Experiment and sample collection:

The field experiment was conducted in a full bearing stage cardamom plantation in a randomized block design (RBD) replicated thrice with a plot size of 3 x 3 m^2 at the plantation of Indian Cardamom Hills (ICH), Idukki, Kerala, India. The site is situated at an altitude of 1,068 m above mean sea level at 9° 472' 273" N latitude, and 77° 092' 283" E longitudes and enjoys humid tropical climate with an average rainfall of 92.9 mm and relative humidity of 88.6 %. A single spray of clothianidin (Dantop 50 % WDG) was applied at two doses of 20 and 40 g a.i. ha^{-1} using a Knapsack sprayer. Despite the lack of label claim of clothianidin in cardamom, the dosage has been fixed based on the recommendation of clothianidin 50 % WDG against jassids and whiteflies of cotton (www.cibrc.nic.in). The control plots were sprayed with water. Three replicates of 100 g each fresh cardamom capsules from the treated and control plots were taken at intervals of 0 (2 hours), 1, 3, 5, 7, 10, 14, 21, 28 and 35 days after application. The samples were divided into two equal portions of which one portion (50 g) was used for estimation of residues on fresh capsules and the other portion was set apart for curing. The curing process included drying of fresh cardamom capsules at 50 °C for 36 h and then keeping at room temperature for 6 h. The capsules were again dried at 70 °C for 3 h and then polished for removing the perianth parts. The samples were crushed well using a blender. In the case of fresh samples, 12.5 g each was used for the estimation of residues, while only 5 g was used in the case of cured samples. Residues were extracted, cleaned up, and analysed following the method described earlier.

The residue data obtained at different intervals were subjected to statistical analysis to determine the half-life ($t_{½}$) values as per the procedure outlined by Hoskin (1961). The waiting period of clothianidin in fresh and cured cardamom was calculated based on the EU MRL value of 0.05 µg g^{-1}. The processing / dehydration factor (PF) was calculated as

$$PF = \frac{\text{Residues in cured cardamom (µg g}^{-1}\text{)}}{\text{Residues in fresh cardamom (µg g}^{-1}\text{)}}$$

RESULTS AND DISCUSSION

Efficiency of the method:

The efficiency of the method was evaluated by carrying recovery experiment. The modified QuEChERS method was suitable for the residue estimation of clothianidin from fresh and cured cardamom which indicated good recovery and high sensitivity. To minimise the matrix effect, quantitation of residues was conducted using matrix matched calibration curves. The instrument response was linear in the range 0.01 to 1 µg g^{-1} levels with a correlation coefficient (r) of above 0.999. The limit of detection (LOD) and limit of quantification (LOQ) of the analyte in both fresh and cured cardamom were found to be 0.005 µg g^{-1} and 0.01 µg g^{-1}, respectively.

To evaluate the accuracy and precision, fortified recovery experiments at three levels (0.01, 0.05, 0.10 µg g^{-1}) were done for both fresh and cured cardamom. In fresh cardamom recovery of clothianidin ranged between 91.0 and 94.4 %, with relative standard deviation of repeatability (RSDr) between 3.3 and 6.2 (Table 1). The mean percentage recovery from cured cardamom ranged from 89.2 to 91.4 %, and RSDr from 5.1 to 8.1. All of these values of recovery indicated good method accuracy and repeatability, and are within the accepted range for residue estimation.

Persistence of clothianidin in fresh cardamom:

The data on the persistence of clothianidin residues in fresh cardamom capsules when applied at 20 and 40 g a. i. ha^{-1} revealed the persistence up to 21 days (Table 2). No residue of clothianidin was detected in control sample. When applied at the lower and the higher dose, clothianidin resulted in an initial deposit of 1.96 and 4.13 µg g^{-1}, respectively. The mean deposit at 1, 3, 5, 7, 10, 14 and 21 days after treatment were 1.57, 1.12, 0.93, 0.61, 0.26, 0.11 and 0.03 µg g^{-1}, respectively, when applied at the lower dose of 20 g a. i. ha^{-1}, while the corresponding levels were 3.26, 2.21, 1.92, 1.35, 0.61, 0.19 and 0.05 µg g^{-1} when applied at the higher dose. On the 5th day after application, clothianidin

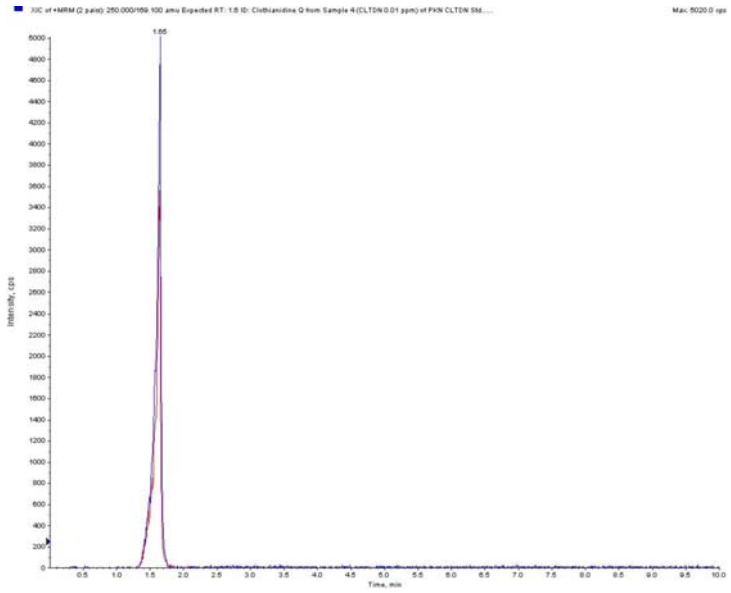

Fig. 1 LC-MS/MS MRM chromatogram of clothianidin in standard solution of 0.01 µgmL^{-1}

Fig. 2 MS/MS spectra of clothianidin

Table 1. Fortification and recovery in fresh and cured cardamom

Compound	Fortification level (µg g^{-1})	Fresh cardamom		Cured cardamom	
		Mean % recovery	RSDr	Mean % recovery	RSDr
Clothianidin	0.01	91.1	6.2	89.2	8.1
	0.05	94.4	5.8	90.1	6.9
	0.10	91.0	3.3	91.4	5.1

RSDr- Relative Standard Deviation of repeatability (n = 5)

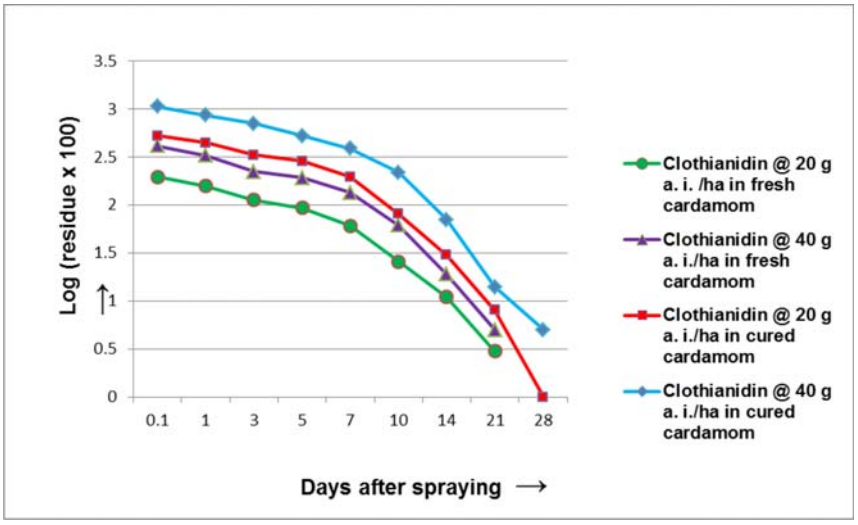

Fig. 3 Dissipation curve of clothianidin applied @ 20 and 40 g a.i. ha^{-1} in fresh and cured cardamom

showed more than 50 per cent dissipation at both the doses. Residues of clothianidin in fresh cardamom reached below the quantitation limit of 0.01 µg g^{-1} on the 28th day at both the lower and higher dosages (Fig. 3).

Persistence of clothianidin in cured cardamom

The data on the persistence of clothianidin residues in cured cardamom capsules when applied at 20 and 40 g a. i. ha^{-1} revealed the persistence up to 28 days which recorded 0.01 and 0.05 µg g^{-1}, respectively (Table 2). When applied at the lower and the higher doses, clothianidin resulted in an initial deposit of 5.24 and 10.61 µg g^{-1}, respectively. The mean deposits of 1, 3, 5, 7, 10, 14 and 21 days after treatment were 4.45, 3.30, 2.85, 1.95, 0.80, 0.30 and 0.08 µg g^{-1}, respectively, when applied at the lower dose of 20 g a. i. ha^{-1} while the corresponding levels were 8.56, 7.01, 5.23, 3.85, 2.17, 0.70 and 0.14 µg g^{-1} when applied at the double dose. In both the concentrations, the level of clothianidin residues dissipated on the fifth day was more than 45 per cent i.e., 45.61 and 50.71 %. Residues of clothianidin in cured cardamom reached below the quantitation limit of 0.01 µg g^{-1} on the 35th day at both the dosages (Fig. 3). A similar dissipation trend was reported by Pratheeshkumar and Chandran (2015) that the residues of acetamiprid, a neonicotinoid, reached below the quantitation limit of 0.01 µg g^{-1} within 28 days in both fresh and cured cardamom following the application of 10 and 20 g a. i. ha^{-1}.

Half-life and waiting period of clothianidin on fresh and cured cardamom:

The data on the level of residues of clothianidin in fresh and cured samples of cardamom is subjected to regression analysis for arriving half life ($t_{1/2}$) and pre harvest intervals. Accordingly, half life of clothianidin applied at 20 and 40 g a.i. ha^{-1} were 3.40 and 3.42 days, respectively, for fresh cardamom while the corresponding values were 3.11 and 3.45 days for cured cardamom (Table 2). Pesticide dissipation was reported to be dependent on physical and chemical factors, including environmental conditions, mode of application, plant species and growth rate, dosage, interval between applications and time of harvest (Khay *et al.,* 2008). The half-life values of clothianidin in tomato ranged between 7 and 11.9 days (Li *et al.,* 2012) whereas, Chen *et al.,* 2008 reported 1-2 days in cabbage. Thus, the half-life of clothianidn is influenced by the type of crop. The waiting period calculated for fresh and cured cardamom was 18.41 and 21.16 days and 22.09 and 27.54 days, respectively, for the lower and the higher doses. Pratheeshkumar and Chandran (2015) have reported waiting periods of 11.65 and 17.35 days and 14.86 and 20.40 days for acetamiprid in fresh and cured cardamom,

Table 2. Persistence of clothianidin residues in/on fresh and cured cardamom and processing factor

Days after spraying	Fresh cardamom				Cured cardamom				Processing Factor (Lower dose)
	Lower dose (20 g a.i. ha^{-1})		Higher dose (40 g a.i. ha^{-1})		Lower dose (20 g a.i. ha^{-1})		Higher dose (40 g a.i. ha^{-1})		
	Mean residue ± SD (µg g^{-1})	Dissipation %	Mean residue ± SD (µg g^{-1})	Dissipation %	Mean residue ± SD (µg g^{-1})	Dissipation %	Mean residue ± SD (µg g^{-1})	Dissipation %	
Before	BQL		BQL		BQL		BQL		
0 (2 hr after)	1.96 ± 0.132		4.13 ± 0.264		5.24 ±0.321		10.61± 0.624		2.67
1	1.57± 0.084	19.89	3.26 ± 0.202	21.06	4.45 ± 0.114	15.08	8.56 ± 0.551	19.32	2.83
3	1.12 ± 0.086	42.85	2.21 ± 0.133	46.49	3.30 ± 0.124	37.02	7.01 ± 0.451	33.93	2.95
5	0.93 ± 0.052	52.55	1.92 ± 0.101	53.51	2.85± 0.152	45.61	5.23 ± 0.217	50.71	3.06
7	0.61 ± 0.032	68.87	1.35 ± 0.092	67.31	1.95 ± 0.104	62.79	3.85 ± 0.103	63.71	3.20
10	0.26 ± 0.012	86.73	0.61 ± 0.044	85.23	0.80 ± 0.054	84.73	2.17 ± 0.106	79.55	3.08
14	0.11 ± 0.008	94.39	0.19 ± 0.008	95.40	0.30 ± 0.011	94.27	0.70 ± 0.031	93.04	2.73
21	0.03± 0.001	98.47	0.05 ± 0.003	98.79	0.08 ± 0.005	98.47	0.14 ± 0.007	98.68	2.67
28	BQL	-	BQL	-	0.01± 0.001	99.81	0.05 ± 0.002	99.53	-
35	BQL		BQL		BQL		BQL	BQL	-
Mean Processing Factor									2.90
Half life	3.40 days		3.42 days		3.11 days		3.45 days		
Waiting period	18.41 days		21.16 days		22.09 days		27.54 days		
Integrated rate equation	log ct = 2.3276 - 0.2037 t/2.303 R2= 0.99		log ct = 2.6666 - 0.2142t/2.303 R2= 0.98		log ct = 2.8378 - 0.2229 t/2.303 R2= 0.98		log ct = 3.1003 - 0.2007 t/2.303 R2= 0.99		

BQL – Below quantitation limit (0.01 µg g^{-1}), SD – Standard Deviation
log Ct = logarithm concentration of residue at 't' time; t - time in days

respectively, after application at 10 and 20 g a. i. ha^{-1}. Celik *et al*. (1995) concluded that under natural field conditions volatilization is the main process that affects pesticide dissipation. Huang *et al.,* 2010 reported that in agricultural fields, the growth dilution of treated plants may play a significant role in the diminution of pesticides in crop plants.

Effect of processing in dissipation of clothianidin in cardamom:

The initial deposit of clothianidin at the lower dose on fresh cardamom was 1.96 µg g^{-1} while it was 5.24 µg g^{-1} after curing registering a processing factor of 2.67. The processing factors arrived for 1, 3, 5, 7, 10, 14 and 21 days after treatment were 2.83, 2.95, 3.06, 3.20, 3.08, 2.73, and 2.67, respectively (Table 2). The residues were found to get magnified during drying presumably due to dehydration during curing/processing after accounting for the dissipation losses. During the curing process, 75–79 % of moisture got depleted which would have resulted in an accumulation of residues and a portion of residues also would have dissipated due to the effect of heat and rubbing. The mean processing factor 2.90 indicates that application of clothianidin in fresh cardamom followed by curing as per the farmers adopted practice followed by polishing resulted in 2.90 times more residues in the cured cardamom. A higher value of processing factor for clothianidin indicates its relatively high stability to dissipation during curing and that it cannot be dislodged easily by the action of curing. Pratheeshkumar and Chandran (2015) reported a mean processing factor of 2.77 for acetamiprid in cardamom after the application of 10 g a. i. ha^{-1}. George *et al.* (2013) reported that by the processing of cardamom, the residues of chlorpyrifos got magnified to the tune of 3.24-3.68 times and that of lambda-cyhalothrin to 2.98-3.46 times of initial residues, consequent to loss of weight due to dehydration during curing.

The information generated in the present study indicate moderate rate of dissipation of clothianidin on both fresh and cured cardamom capsules. As no Codex/FSSAI MRL is available for fresh or cured cardamom capsules, EU MRL of 0.05 mg kg^{-1} for dry cardamom is taken in to account for the calculation of pre harvest interval. Thus a pre harvest interval of 18.41 and 21.16 days are recommended for fresh cardamom for the lower and higher doses, respectively, while 22.09 and 27.54 days are recommended for the lower and higher doses, respectively, in cured cardamom. As the capsules are consumed after curing, waiting periods of 22.09 and 27.54 days may be followed to avoid the risk in terms of harvest time residues in the produce.

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Persistence and effect of processing on reduction of spiromesifen residues in chilli pepper (*Capsicum annum* L.) and soil

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ABSTRACT: Residue dynamics of spiromesifen (Oberon 240 SC) in chilli fruits was studied using Ultra high performance liquid chromatography coupled with tandem mass spectrometer (UPLC-MS/MS). The initial deposits of spiromesifen in chilli fruits after application at 96 and 192 g ai ha^{-1}, were found to be 0.62 and 1.20 ìg g^{-1}, which reached below quantitation level after 21 and 27 days, respectively. Half-life of spiromesifen at single and double recommended dose were 3.65 and 3.19 days, respectively and the corresponding waiting period calculated was 0.74 and 4.49 days. The removal of spiromesifen residues using different simple decontamination 2 h after spraying and 3 days of spraying were 77.74 -88.18 % and 55.46 -71.00 % respectively. Processing factor due to sun drying in dry chilli fruits ranged from 2.12 to 1.33 during 0 to 15th day after application.

KEY WORDS: Chilli, Spiromesifen, Dissipation,Decontamination

INTRODUCTION

Chilli is the most important condiment, which adds pungency, taste, flavor and color to various cuisines around the world. Currently, India is the world leader in chilli production followed by China and Pakistan. Among the different constraints contributing to low chilli productivity, the pest complex that attack chilli crop at different crop stages is most important. Chilli farmers often resort to the use of more frequent and higher doses of pesticides, to overcome the losses due to these pests in commercial cultivation, thereby leading to unusual levels of their residue in the final produce. Also, the sprays of the popular conventional insecticide chemistries like organochlorines, organophosphates, carbamates and synthetic pyrethroids become a threat to chilli ecosystem causing resurgence of pest and threat to beneficial fauna. Since, insects and other animals have similar reproductive, hormonal and nervous systems; these compounds have potential for non target effects, including humans. Spiromesifen is a non-systemic insecticide-cum accaricide belonging to the chemical class of cyclic ketoenoles effective against the sucking pest complex in chilli. Spiromesifen is an acetyl CoA carboxylase inhibitor and the biological activity of cyclic keto enoles correlates with inhibition of lipogenesis, resulting in decreased lipid contents, especially of triglycerides and free fatty acids, in treated insects (The United Kingdom, 2004). Owing to its unique chemical structure it is a useful tool in resistance management in many cropping systems including cotton, tea, vegetables, fruits and ornamentals (Nauen et al., 2002).

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Farmers often resort to the application of pesticides at recurrent intervals, resulting in substantial residues in the chilli fruits. Pesticide residue will be strictly monitored in future owing to the high concern about the toxic residues. There are reports of chilli consignment rejection from India in European countries due to the presence of pesticide residues of ethion, triazophos, chlorpyriphos, phosphamidon, cypermethrin, fenvalerate and dicofol (Rao, 2005). Chilli being consumed more as dried products rather than green chillies, the residue accumulation will be more on dry weight basis. Degradation studies in soil are required for evaluating the persistence of pesticides in the environment. Chillies can be subjected to some simple household practices e.g., washing, cooking, removal of non edible parts, etc. before actual consumption. The effects of these processing techniques on residue levels are extremely important in evaluating the risk associated with residues.

A literature survey reveals that data pertaining to the dissipation of spiromesifen in/on fresh, dry chilli fruit and soil in Indian condition and the effect of various household preparations in reduction of residues in chilli is scanty. Therefore, the present investigation was carried out with the objective to examine the persistence of spiromesifen in chilli fruits both fresh and dry and in the soil and the effect of culinary practices on the reduction of residues for ensuring consumer safety.

MATERIALS AND METHODS

Chemicals and reagents

Analytical standards of spiromesifen purity 98 per cent, sodium citrate tribasic dihydrate and sodium hydrogen citrate sesquihydrate were procured from M/S Sigma-Aldrich, India and the formulation Oberon 240 SC was from Bayer Crop Science India Ltd. Solvents like acetonitrile, water (HPLC grade), sodium chloride, anhydrous sodium sulfate, and anhydrous magnesium sulfate (ACS reagent grade) were obtained from Merck Germany. Primary secondary amine (PSA) was obtained from Agilent Technologies, USA. All the glass wares were thoroughly washed as per the standard operating procedure to avoid the interferences from any contaminants during analysis. The suitability of the solvents and other chemicals were ensured by running reagent blanks before actual analysis.

Preparation of standard solution

Single laboratory method validation was done initially to test the efficiency of extraction and clean up procedures and to standardize the procedure for residue estimation of fresh, dry chilli fruits and soil. Standard stock solution of spiromesifen (1000 µg ml^{-1}) was prepared in HPLC grade methanol and serially diluted with acetonitrile to obtain the solutions required for forming a calibration curve (1.0, 0.50, 0.25, 0.10, 0.075, 0.05, 0.025, and 0.0l µg ml^{-1}. The standard solutions were stored at 4°C before use. The fortification study of the untreated fresh, dry chilli fruits and soil was carried out by spiking at 0.01, 0.05, 0.1, and 0.5 µg g^{-1} levels.

Instrument Parameters

The chromatographic separation was achieved using Waters Acquity UPLC system equipped with a reversed phase Atlantis C-18 (2.1 x 100 mm, 5 micron particle size) column. The operation of the LC gradient involved the following two eluent components: A: 10 % methanol in water + 0.1 % formic acid + 50 mM ammonium acetate; B: 10 % water in methanol + 0.1 % formic acid +50 mM ammonium acetate. The gradient elution was as follows: 0 minutes isocratic 5 % B, 0.0–3.0 minutes linear from 5 % to 80 % B, 3.0–4.0 minutes linear from 80 % to 100 % B, 4–6.5.0 minutes linear from 100 % to 50 % B, with 6.5–8.0 minutes for initial conditions of 5 % B. The flow rate remained constant at 0.8 mL min^{-1} and injection volume was 10µL. The column temperature was kept at 40°C. The effluent from the LC system was introduced into Triple quadrapole API 3200 MS/MS system equipped with an electrospray ionization interface (ESI), operating in the negative ion mode. The source parameters were temperature 550^0C; ion gas (GS1) 50 psi, ion gas (GS2) 50 psi, ion spray voltage 5500 V, curtain gas 13 psi and compound dependent parameters are Declustering potential-35 V -Entrance potential-6 V, CEP-Collision cell

entrance potential-28 V, CE-Collision energy-16 V, CXP-Collision cell exit potential-3.0 V. Under these operating conditions the retention time of spiromesifen was found to be 4.31. The MRM transitions used for the quantitative estimation of spiromesifen was m/z 371.2→273.2 and for qualitative estimation m/z 371.2→255.1 (Fig. 1).

Field experiment

Chilli crop was raised in a field located at Kalliyoor, Thiruvanthapuram, Kerala and maintained as per the Package of Practices Recommendations of Kerala Agricultural University to conduct the study. The trial was laid out in randomized block design replicated thrice with a plot size of 2 x 2 m^2. Single spray of spiromesifen at the rate of 96 and 192 g ai ha^{-1} was given during the fruiting stage.

Tender fruits (500 g) were collected randomly on 0, 1, 3, 5, 7, 10, 15, 21 and 27 days after insecticide spray, pooled, extracted, and cleaned of spiromesifen as per the modified QuEChERS method (Xavier *et al*.,2014) and quantification was done by UPLC-MS/MS analysis for estimating the residues of spiromesifen present.

The fresh sample of the chilli fruits were sun dried and crushed well using a blender from which five replicates of three gram representative samples of the fruits were taken in 50 ml centrifuge tubes and spiked with 0.01 ml, 0.05, 0.1 ml and 0.5 ml of 10 µg ml^{-1} working standard mixtures of the insecticides.. After mixing of the sample with 10 ml distilled water, the samples were extracted using 20 ml acetonitrile. The extraction and clean up was done as described above and quantified using UPLC-MS/MS.

Soil samples were collected from 0 to 10 cm depth using a stainless steel soil tube drill. The samples collected from 3 randomly selected spots in each treatment were pooled, air dried, grinded and then passed through 2 mm sieve. The extraction and clean up was done as described by Asensio-Ramos *et al*., (2010) and quantified using UPLC-MS/MS.

The residue data obtained at different intervals were subjected to statistical analysis to determine the half-life values on the treated substrate as per the procedure outlined by Hoskins (1961) which was done using Micrsoft Excel spread sheet 2007.

Decontamination studies

The effect of different household practices on removal of residues of spiromesifen from chilli fruits was assessed by dipping the fruits collected from sprayed fields at two hours and on the third day after application in solutions of common salt 2% (20 g of common salt dissolved in one liter of water), tamarind 2% (20 g of preserved tamarind pulp extracted in one liter of water), vinegar 2% (20 ml of vinegar diluted in one liter of water), slaked lime 2% (20 g of hydrated lime dissolved in one liter of water), turmeric 1% (10 g of turmeric powder dissolved in one liter of water) for 20 minutes followed by washing for 2 minutes in running water. Scrubbing for 2 minutes followed by washing for 2 minutes in running water was also inclued as a treatment. The estimation of residues in the processed chilli samples were done as described above. The per cent reduction of residues was worked out by comparing the residue data in processed fruits with unprocessed fruits.

RESULTS AND DISCUSSION

Efficiency of the method

The method validation studies of spiromesifen on fresh and dry chilli fruits indicated good recovery and high sensitivity. The response function was found to be linear with a good coefficient of determination (R^2) higher than 0.99 in chilli and soil matrix standard solutions for spiromesifen. The fortification at levels of 0.01, 0.05 0.10 and 0.5 µg g^{-1} gave a recovery of 84.7 to 95.1% for fresh chilli and 76.5 to 86.8 % for dried chilli (Table 1). The relative standard deviation ranged from 6.5 to 13.10 and 5.80 to 12.50 respectively for fresh and dried chilli satisfying the acceptance criteria of the method. In the case of soil, recovery ranged from 78.6 to 84.8% and relative standard deviation from 8.6 to 11.90. The limit of detection (LOD), determined with a signal to noise ratio (S/N) ≥ 3, was found to be 0.003 µg g^{-1}, 0.005 µg g^{-1} and 0.004

Table 1. Recovery of spiromesifen residues in soil, fresh and dried chilli fruits

No.of replications	Fortification Level (µg g-1)	Soil		Fresh chilli fruits		Dried chilli fruits	
		Mean % recovery	RSD	Mean % recovery	RSD	Mean % recovery	RSD
5	0.01	78.6	10.3	90.5	13.1	76.5	12.5
5	0.05	80.3	11.9	84.7	10.4	81.4	11.2
5	0.1	84.8	9.2	95.1	6.5	83.2	9.50
5	0.5	81.5	8.60	85.2	7.9	86.8	5.80

Table 2.Dissipation of spiromesifen residues in/on chilli fruits

Days after spraying (DAS)	96 g a.i.ha^{-1}				Fresh chilli (192 g a.i.ha^{-1})	
	Fresh chilli		Dried chilli			
	Mean residue ± SD(µg g^{-1})	Dissipation %	Mean residue ± SD(µg g^{-1})	Processing factor	Mean residue ± SD(µg g^{-1})	Dissipation %
0 (2 hr after spraying)	0.62 ± 0.052		0.9 ± 0.07	1.45	1.20 ± 0.104	
1	0.43 ± 0.032	30.65	0.71± 0.06	1.65	0.91 ± 0.069	24.17
3	0.31 ± 0.025	50.00	0.66 ± 0.07	2.12	0.71 ± 0.051	40.83
5	0.23 ± 0.018	62.90	0.45 ± 0.04	1.95	0.55 ± 0.043	54.17
7	0.135 ± 0.01	78.23	0.24 ±0.02	1.77	0.26 ± 0.020	78.33
10	0.11 ± 0.015	82.26	0.18 ±0.013	1.64	0.15 ± 0.013	87.50
15	0.03 ± 0.004	95.16	0.04 ±0.03	1.33	0.08 ± 0.009	93.33
21	BDL		BDL		0.01±0.001	99.17
28	BDL		BDL		BDL	
$T_{½}$(days)	3.65				3.19	
$T_{tol\ /\ WP}$(days)	0.74				4.49	
Integrated first order rate equation	LogCt=1.75-0.189t/2.303				LogCt = 2.12 -0.217t/2.303	

BDL – Below detectable limit, SD – Standard deviation

$T_{1/2}$ -half life , T_{tol} /(WP)-Waiting period
LogCt –Logarithm of concentration of the residue at time (t), t-time in days

µg g^{-1} for fresh, dry chilli and soil respectively. The limit of quantitation (LOQ), determined with a signal to noise ratio (S/N) ≥ 10, was found to be 0.01 µg g^{-1} in fresh chilli, dry chilli and soil.

Persistence of spiromesifen in chilli and soil

The dissipation pattern of spiromesifen in chilli fruits, following application of spiromesifen (Oberon 240

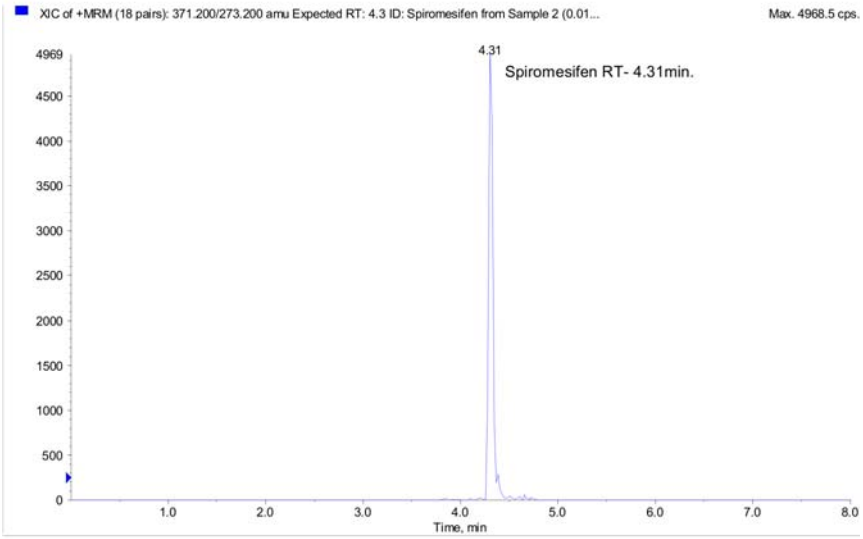

Fig. 1 The LC-MS/MS MRM chromatogram of spiromesifen in standard solution of 0.01 µg ml^{-1}

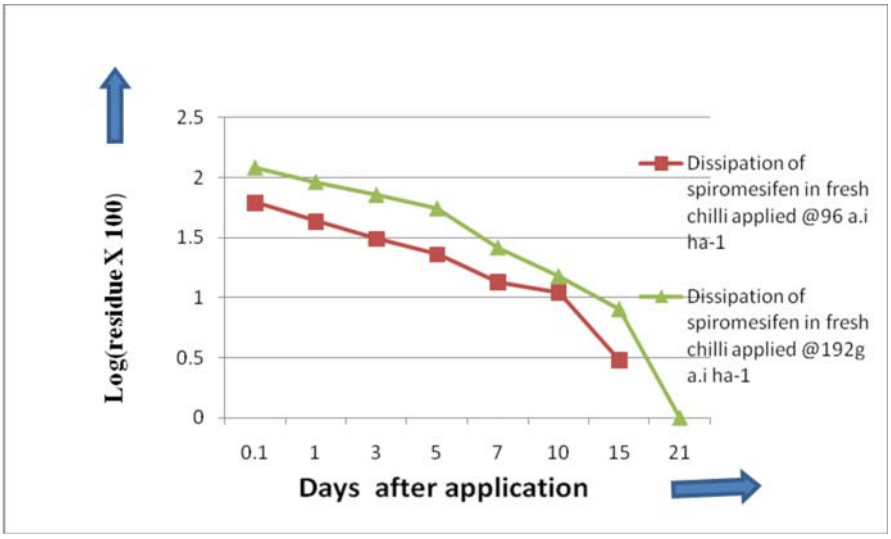

Fig. 2 Semi logarithm-graph showing dissipation kinetics of spiromesifen 240 SC in chilli

SC) at 96 and 192 g ai ha^{-1} are presented in Table 2 and Fig. 2. The mean initial deposit estimated in chilli fruits were found to be 0.62 and 1.20 µg g^{-1} respectively which reached below detectable level on the twenty first and twenty seventh day for single and double dose respectively. Conversely, the residue was found to be higher ie., 0.90 µg g^{-1} in dried chillies collected 2 h after the spray of spiromesifen @ 96 g a.i. ha^{-1} and the processing factor recorded was 1.45. On fifteenth day, the residue became 0.04 µg g^{-1} for dry chillies with the processing factor calculated being 1.33. Similarly, the initial residue was found to be 0.28 µg g^{-1} in the soil collected 2 h after the spray of spiromesifen @ 96 g a.i. ha^{-1} and the residues remained up to 10 days only (Table 3).The degradation half-life of spiromesifen, at single and double dose in the fresh fruits were 3.65 and 3.19 days respectively and the waiting period calculated was 0.74 and 4.49 days respectively which corroborated with the

Table 3. Persistence of spiromesifen residues in/on soil

Days after spraying (DAS)	Single dose	
	Mean residue± SD(μg g^{-1})	Dissipation %
0 (2 hr after spraying)	0.28 ± 0.03	
1	0.12 ± 0.015	57.14
3	0.06 ± 0.008	78.57
5	0.03 ± 0.004	89.28
7	0.01 ± 0.001	96.42
10	BDL	
Half life (days)	1.55	
Integrated first order rate equation	LogCt = 1.380-0.4470t/2.303	

BDL – Below detectable limit, SD – Standard deviation

findings of George *et al*., 2014 who recorded a waiting period of 3 days followed by application of spiromesifen at 150 g a.i. ha^{-1} in Tomato. Persistence data of spiromesifen was fitted into first-order dissipation kinetics with correlation coefficient value of greater than 0.99 for both doses. In the studies by Sharma *et al*., 2007 spiromesifen (Oberon 240 SC) when sprayed at 96 g a.i. ha^{-1} in chilli, the initial residues were in the range of 0.51-0.56 μg g^{-1} and the half life values of spiromesifen in chilies were calculated as 2-2.5 days. Varghese *et al*., 2011 also reported spraying of spiromesifen @ 100 g a.i ha^{-1} on chilli fruits deposited an initial average residue of 0.609 μg g^{-1} and the residues reached below detectable level on the tenth day.

Persistence of spiromesifen on chilli was low as the residues dissipated below EU MRL value of 0.5 μg g^{-1} on 0.74 days for the single dose application whereas it took 4.49 days in the case of double dose. Although adoption of safe waiting period is a means of reducing the risk of the residue problems, methodical processing with simple household practices further ensures safety of the vegetable to the consumers.

Effect of decontamination techniques

All the decontaminating treatments were found effective in removing spiromesifen residues substantially from chilli fruits. The initial deposits of 0.62 μg g^{-1} of spiromesifen on chilli samples were reduced appreciably to the range of 0.197 to 0.231 μg g^{-1} as a result of scrubbing, treatment with slaked lime 2 % and tamarind 2% solution, thus accounting a residue loss of more than 85 per cent, two hours after spraying. Same scenario was also found in case of samples treated with 2 % tamarind solution and scrubbing with more than 68 per cent reduction three days after spraying. Wisam *et al*., 2004 reported washing the cucumber fruit, Oberon residue obtained in first and second spray after 3 days was 0.21 μg g^{-1} and 4 days was 0.2 μg g^{-1}. Also, treatment of peeling, saline solution and pickling reduced spiromesifen residue in first spray to MRL after two days [0.21, 0.23 and 0.22] μg g^{-1} respectively, while in second spray, residues dropped to MRL after 2 days [0.2, 0.17 and 0.18] μg g^{-1} respectively for previous treatment. It is clear from the data that various processing methods are very useful for dislodging of spiromesifen residues. High amount of pesticide residues in spices especially chilli strengthens the need for simple domestic practices that will eliminate harmful pesticide residues in these fruits (Table 4).

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Table 4. Extent of removal of spiromesifen residues from chilli fruits in the treatments

Treatments	0 day		3 day	
	Mean residue ± SD (îg g^{-1})	% residue removal	Mean residue ± SD (îg g^{-1})	% residue removal
Unprocessed	0.62 ±0.041	-	0.31 ±0.028	-
Scrubbing	0.073 ±0.008	88.18	0.099 ±0.001	68.04
Tamarind	0.086 ±0.001	86.14	0.090 ±0.0016	71.00
Vinegar	0.138 ±0.001	77.68	0.138±0.010	55.46
Slaked lime	0.076 ±0.005	87.76	0.124 ±0.004	59.87
Turmeric	0.083 ±0.004	86.61	0.121 ±0.008	60.96

SD – Standard deviation

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First report of *Lema* sp nr *pectoralis* Baly, 1865 (Coleoptera: Chrysomelidae) on the green bay orchid *Eulophia andamanensis* Rchb.f (Orchidaceae: Epidendroideae)

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ABSTRACT: Incidence of *Lema* sp nr *pectoralis* Baly (Coleoptera: Chrysomelidae) on *Eulophia andamanensis* Rchb.f (Orchidaceae: Epidendroideae) is reported for the first time.

Key words: First report of *Lema* sp, green bay orchid, India

Orchids are prone to attack by a number of pests including thrips, scales, aphids, beetles, caterpillars, wasps, ants, mites, molluscs and nematodes. The green bay orchid, *Eulophia andamanensis* Rchb.f, indigenous to the islands of Andaman and Nicobar in India and the Langkawi Island in Malaysia, is known for the longest spike. *Lema pectoralis* described by Baly (1865) from Singapore is a serious pest of orchids (Mohammedsaid, 2004). The typical subspecies, *Lema pectoralis pectoralis* Baly occurs in Peninsular Malaysia and Singapore (Mohammedsaid, 2004) while *L. pectoralis unicolor* Clark occurs in Nepal, Thailand, Vietnam, Hainan, south China, and Taiwan (Kimoto and Gressitt, 1979).

Massive infestation of *Lema* sp nr *pectoralis* was observed on *E. andamanensis* in green house as well as under open conditions at Garacharma, south Andamans from December 2015 till April 2016. Grubs and adults feed on the raceme and adults did not spare any arial part Persistent feeding by adults and grubs resulted in drying up of racemes, yellowing and browning of leaves. Buds turned brown and necrotic. Flowers and buds were eaten up wholly or irregularly bitten and only green immature ones were spared. The plant as a whole presented a sickly appearance with clusters of pupae stuck in linear rows around the raceme.

The specimens collected were identified as *Lema* sp nr *pectoralis.* During January, 2016, an average of 0.5-1.0 grub, 2-3 live pupae, 2 pupal cases and 2.7 adults per plant were recorded. In February 2016, adult population was visibly high and as many as 12 beetles could be collected from a single plant. About 0.73, 3.60, 4.26, 2.0 grubs, pupae, pupal cases and adults respectively were noticed during the month.

Adult is a medium sized yellow beetle of about 0.9 - 1 cm length. They fed by scraping out the chlorophyll from raceme and leaf lamina and also bit through buds and flowers. Gregarious habit was common. The symptoms were noticed as linear, oblong or irregular circular transparent patches with excretory pellets strewn over. Being weak fliers, they could be easily handpicked. Several mating

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pairs were noticed during the afternoon. Amber coloured, cylindrical, slightly oblong eggs were singly laid, mostly on buds and partially eaten flowers. They resembled resin drops and were tightly glued to the substratum. The newly hatched larva carried the empty egg shell on its back for one day and was hardly distinguishable from the egg. The grubs were honey yellow, wrinkled with black prognathous head. They were also slightly humped. A pair of linear black spots appeared just beneath the head on the dorsum of thorax. They were covered by slimy greenish excreta. The larvae resembled bird excreta. Grubs were preferential over stalks of racemes and never fed on leaves or flowers. Pupa was bright yellow, enclosed within a whitish frothy, powdery, glutinous covering that stuck to the hands when touched. The pupal covering contained the head capsule of the last moult and also retained the sticky powdery coating for a few days. The adult emerged by biting a clear circular hole through it. As many as 10 pupae per raceme were found, leaving only a dried twig in the distal end.

Lema sp nr *pectoralis* differ from *L. pectoralis* Baly in having yellow antennae and tibiae (antennae and tibiae are black in both the subspecies of *P. pectoralis*). This species infests orchids throughout southern India and the *Lema* sp. reported on orchids by Kumari and Lyla (2001) is probably the same. In Kerala, adults of *Lema* sp. colonized on orchids soon after the rains. They were pale flavous and measured 9 mm. The yellowish, swollen grubs were not easily recognized in the field as they carried faecal matter on their back (Kumari and Lyla, 2001). *Lema pectoralis* is an oligophagous feeder on orchids. Grubs and adults cause heavy damage to flowers of *Spathoglottis* spp., *Epidendrum* spp., vanda and dendrobium (Joilvet, 1971; Hirao *et.al.* 2001). The larva of *L. pectoralis* passes through four stages and towards pupation excretes a meringue-like substance. Adults tend to remain in cocoon for few hours before they bite a circular exit hole and emerge out. They become sexually mature in 14-21 days and multiple mating is common. All the stages of the pest are yellow coloured and the total development period lasts for 24 days (Hirao et al., 2001).

Voucher specimens are deposited in the Central Island Agricultural Research Institute, Port Blair. This is the first report of *Lema* sp nr *pectoralis* on *E. andamanensis*.

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