Bioefficacy of *Rhynocoris fuscipes* (Fab.) (Hemiptera: Reduviidae) and *Beauveria bassiana* (Bals.) (Ascomycota: Hypocreales) toxic protein and its silver nanoparticles against cotton pests

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DOCTOR OF PHILOSOPHY IN ZOOLOGY

By

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CERTIFICATE

I certify that the thesis entitled **"Bioefficacy of *Rhynocoris fuscipes* (Fab.) (Hemiptera: Reduviidae) and *Beauveria bassiana* (Bals.) (Ascomycota: Hypocreales) toxic protein and its silver nanoparticles against cotton pests"** submitted by **Mr. Majesh Tomson (Reg. No. 4343)** for the award of the **Degree of Doctor of Philosophy in Zoology** at Manonmaniam Sundaranar University is a bonafide record of research work done by him independently in the Crop Protection Research Centre, Department of Zoology, **St. Xavier's College (Autonomous), Palayamkottai** under my guidance and supervision. The details furnished in the thesis are the original work of the candidate and have not been submitted elsewhere in part or full for the award of any other degree, diploma, associateship or other similar titles. It is not plagiarism of any other work either published or unpublished without acknowledgement.

Place: Palayamkottai

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DECLARATION

I do hereby declare that the thesis entitled **"Bioefficacy of *Rhynocoris fuscipes* (Fab.) (Hemiptera: Reduviidae) and *Beauveria bassiana* (Bals.) (Ascomycota: Hypocreales) toxic protein and its silver nanoparticles against cotton pests"** is the result of the original study carried out by me under the guidance of **Dr. K. Sahayaraj,** Associate Professor, Department of Zoology, St. Xavier's College (Autonomous), Palayamkottai for the award of the degree of **Doctor of Philosophy in Zoology**. This work has not been submitted elsewhere in part or in full for the award of any other degree, diploma, associateship or other similar title. It is not plagiarism of any other work either published or unpublished without acknowledgement.

Place: Palayamkottai Signature of the candidate

Date:

(MAJESH TOMSON)

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CONTENTS

Annexure (Papers published)

1. Sahayaraj, K. and Majesh Tomson. 2010. Impact of two pathogenic fungal crude metabolites on mortality, biology and enzymes of *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae). *Journal of Biopesticides*, 3 (1): 163 – 167.
2. Sahayaraj K., Majesh Tomson and Kalidas S. 2011. Artificial rearing of red cotton bug, *Dysdercus cingulatus* (Fab.) using cotton seed-based artificial diet. *Entomologia Generalis,* 33 (4): 283-288.
3. Sahayaraj, K. Kalidas, S. and Majesh, T. 2012. Stage preference and functional response of *Rhynocoris longifrons* (Stål) (Hemiptera: Reduviidae) on three hemipteran cotton pests. *Brazilian Archives of Biology and Technology* 55(5):733-740.

ABBREVIATIONS AND SYMBOLS USED

^{o}	-	Degree
^{o}C	-	Degree centigrade
λ	-	Lambda
%	-	Per cent
θ	-	Theta
µg	-	Microgram
µL	-	microlitre
µmole	-	Micro moles
$AgNO_3$	-	Silver nitrate
AgNP's	-	Silver nanoparticles
amu	-	Atomic mass unit
ANOVA	-	Analysis of variance
CBR	-	Cost benefit ratio
cm	-	Centimeter
Da	-	Dalton
FTIR	-	Fourier transform infrared spectroscopy
gm	-	Gram
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
hrs	-	Hours
kDa	-	Kilo Dalton
kV	-	Kilovolt
M	-	Molar
mM	-	Milli molar
MCU	-	Madras, Cambodia, Uganda
MEC	-	Micro Environmental Cage
mg	-	Milligram
mm	-	Millimeter
min	-	Minute
mL	-	Millilitre
m/z	-	Mass-to-charge ratio
N	-	Normality

nm	-	Nano meter
OD	-	Optical density
PAL	-	Percent Avoidable Loss
PDA	-	Potato Dextrose Agar
PDB	-	Potato Dextrose Broth
RH	-	Relative humidity
rpm	-	revolutions per minute
RT	-	Retention time
SDS PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	-	Seconds
SEM	-	Scanning electron microscope
TEM	-	Transmission electron microscope
UV-vis	-	Ultra violet-visible
XRD	-	X – Ray diffraction

CHAPTER 1

Biology, prey preference and bioefficacy of *Rhynocoris fuscipes* (Fab.) (Heteroptera: Reduviidae) against two cotton pests and a factitious host

1.1. ABSTRACT

The reduviid bug, *Rhynocoris fuscipes* Fab. (Heteroptera: Reduviidae) has tremendous potential as a biological control agent against many economically important pests. The relationships between the *R. fuscipes* and its prey organisms, such as *Dysdercus cingulatus* Fab. (red cotton bug), (Hemiptera: Pyrrhocoridae), *Phenacoccus solenopsis* Tinsely (cotton mealy bug) (Hemiptera: Pseudococcidae) and *Corcyra cephalonica* Stain. (rice flour moth) (Lepidoptera: Pyralidae), were studied to determine the influence of these hosts on biology and bioefficacy of the predator. The experiments were carried out when the reduviid predator fed, larvae of the factitious prey *C. cephalonica* (control) compared with data obtained when *R. fuscipes* fed on nymphs of red cotton bug and cotton mealy bug. In addition, choice tests were conducted to determine if the predators had a specific preference on stage and prey type. *Rhynocoris fuscipes* completed nymphal period in 41 days on *C. cephalonica*, 45 and 50 days on *D. cingulatus* and *P. solenopsis,* respectively. Adult longevity, fecundity, hatchability, nymphal survival rate and life table parameters were in favour of control category and least against *P. solenopsis.* Third instar *D. cingulatus* was preferred by both third and fourth instars predators. Fifth and adult stage predators preferred fourth and fifth instars of *D. cingulatus,* respectively. Invariably nymphs and adult predators preferred *P. solenopsis* adults. The adult predator voraciously fed *D. cingulatus* and *P. solenopsis* and life stages of the reduviid responded positively with type II functional response curve. The results obtained from the study signified that the high biocontrol potentiality of *R. fuscipes* against cotton pest management.

Key words: Biology, bioefficacy, functional response, life table, preference

1.2. INTRODUCTION

The control and restraint of natural herbivore populations are imperative to ecology. A variety of biotic and abiotic factors govern herbivore populations. Hairston *et al.* (1960) proposed a hypothesis that the world is green because natural enemies manage and limit herbivores, preventing them from depleting green plants completely. Consequently, it has been shown that natural enemies limit herbivore profusion (Schmitz *et al*., 2000; Terborgh *et al*., 2001), resulting in increased productivity of primary producers *ie,* plants (Pace *et al.,* 1999). There is now consensus that both resources and natural enemies interplay in herbivore population control (Rogers, 1972; James *et al*., 1994; Leibold, 1996; Denno *et al*., 2003; Sinclair *et al*., 2003). There is a range of possible interactions between resources and natural enemies (says Helen and Ted, 2008).

One of the major obstacles hindering cotton cultivation is insect pests infestation. The red cotton bug *Dysdercus cingulatus* Fab. (Hemiptera: Pyrrhocoridae) and mealy bug *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) causing serious damage by feeding on developing cotton bolls and ripe cotton seeds. Further more, *D. cingulatus* transmitting fungi, *Nematospora gossypii* Kurtzman that grows on the immature lint and seeds (Ahmed and Schaefer, 1987; Yasuda, 1992). These pests are difficult to control by insecticides either because of the high mobility of *D. cingulatus* (Kohno and Ngan, 2004) or *P. solenopsis* having a white waxy coat (Patel *et al.,* 2010) as adaptative mechanisms. Therefore, the use of natural enemies to control these pests can be considered.

The significance of reduviid predators as suppressors of herbivorous insect populations on crops has long been recognized (Schafer and Ahmed, 1987; Aldrich *et al.,* 1991; James, 1994; Schafer, 1998; Ambrose, 2000; James, 2000; Alan *et al.*, 2003; Grundy, 2004; Sahayaraj, 2007a; Sahayaraj and Balasubramanian, 2009; Alexandre *et al.,*

2009; El-Sebaey and El-Wahab, 2011; Freddy *et al.,*2011; Sahayaraj *et al.,* 2012; Guanyang and Christiane, 2013).

Cotton field offers a predominantly good example of such biological control by predators. Although about 250 insect species occur in the cotton ecosystem, only about 27 of these are well thought out prone to cause damage, of which only 5-6 species get to population levels beyond the economic threshold (Hossain *et al*., 2012; Ramesh Kumar *et al*., 2013). However, when profusion of naturally occurring predators decreases, the cotton pest populations increases (Hagerty *et al*., 2005; Vahid, 2013; Pickett *et al*., 2013). Predatory hemipterans are commonly found in several crop ecosystems, where they feed primarily on larval stages of various herbivore pests and non-pests (De Clercq, 2000; Torres *et al.,* 2006; Vahid, 2013).

The anticipation in any augmentative release programme is that predator population increase in the targeted area and its ambience. However, release of predatory reduviid bugs in to row crops ecosystems features different conditions than those in bordering ecosystems (Hossain *et al*., 2012). Several insect predator species including hunter reduviids have been observed feeding on *D. cingulatus* (Ambrose, 1999; Sahayaraj and Asha, 2010; Sahayaraj *et al*., 2012) and *P. solenopsis* (Tanwar *et al.,* 2007, David *et al.,* 2009; Anita Singh and Dolly Kumar, 2012; Asifa Hameed *et al*., 2013) in India.

1.2.1. *Rhynocoris fuscipes* (Fab.)

Rhynocoris fuscipes (Fab.) a crepuscular, fiercely coloured (black and red), entomophagous, harpactorine reduviid found in concealed habitats such as underneath the leaves, pebbles and crevices and common in agro ecosystems (Ambrose, 1988; George, 2000; George *et al.,* 2000; Sahayaraj and Selvaraj, 2003; Sahayaraj, 2007b; Sahayaraj and Sujatha, 2012). Bioecology of this predator has been studied by Ambrose and Livingstone (1986). Ambrose (1997) first described the mating behaviour of this bug; later, the impact

of mating on oviposition pattern and hatchability (Vennison and Ambrose, 1986). Previously, impact of sex, starvation, antennectomy, eye blinding and tibial comb coating on the predatory behaviour of this reduviid was studied (Ambrose and Mayamuthu, 1994). It is a predator of 44 insect pests (see Table 1). Moreover, food requirement has also been known for this predator (Ambrose and Kumaraswami, 1993). Both biotic and abiotic factors influence the distribution and diversity of reduviid predators. In agroecosystems, abundance of this predator has been artificially enhanced and makes them sustain; for instance, the influence of mulching and intercropping on the abundance of the Reduviid predator, *Rhynocoris fuscipes* (Claver and Ambrose, 2003a).

Many authors studied the pest suppression efficacy of *R. fuscipes* on various crop pests (Singh and Gangrade, 1975; Ponnamma *et al.,* 1979; Singh, 1985; Ambrose and Livingstone, 1986; Singh and Singh 1987; Ambrose, 1995), particularly *Riptortus clavatus* Thunberg (Heteroptera: Alydidae) and *Spodoptera lilura* F. (Ambrose, and Claver, 1995), three pests of pigeonpea *(Cajanus cajan)* (Ambrose and Claver, 2001), *Helicoverpa armigera* (Hubner), *Nezara viridula* (L.) and *Riptortus clavatus* Thunberg (Claver and Ambrose, 2003a) and reproductive performance on three lepidopteran pests (Babu *et al.,* 1995; Ambrose and Claver, 1995; George and Ambrose, 1998; George *et al.,* 2002) has also been studied. Claver and Ambrose (2001b) evaluated suitability of various substrata for the mass rearing of this reduviid. Moreover, field release studies showed its efficacy in pest management programme (Claver and Ambrose, 2003a,b).

The *R. fuscipes* nymphs are not found along with their parents or in groups. Adults are having nodding behaviour as defensive mechanism. Nymphal instars are orange red with black eyes, light yellowish green legs with brown spots. Black lateral and median abdominal spots and wing rudiments are seen in older instars. Rao (1974) first reported its development and breeding techniques. But he failed to provide the details of its biology.

Table 1. *Rhynocoris fuscipes* preys list with citation

1.	Pest	Order: Family	Lab/Field	Author
1.	*Achaea janata* (Linn.)	Lepidoptera: Noctuidae	Laboratory	Singh, 1985
2.	*Aphids gossypii* (Glover)	Hemiptera: Aphididae	Laboratory	Majesh *et al*., 2011
3.	*Aphis craccivora* (Kouch.)	Hemiptera: Aphididae	Laboratory	Sahayaraj *et al*., 2002
4.	*Aulacophora foveicollis* (Lucas.)	Coleoptera: Chrysomelidae	Laboratory	Ambrose, 1995
5.	*Calocoris angustatus* (Leth.)	Hemiptera: Miridae	Laboratory	Ambrose, 1980; 1985
6.	*Catopsilia pyranthe* (Linn.)	Lepidoptera: Pieridae	Laboratory	Hiremath and Thondarya, 1983
7.	*Chilo partellus* (Swinh.)	Lepidoptera: Pyralidae	Laboratory	Rao *et al.,* 1981
8.	*Clavigralla gibbosa* (Spinola)	Hemiptera: Coreidae	Laboratory	Das, 1996
9.	*Clavigralla horrens* (Dist.)	Hemiptera: Coreidae	Laboratory	Das, 1996
10.	*Corcyra cephalonica* (Stain.)	Lepidoptera: Pyralidae	Laboratory	Singh, 1985
11.	*Cyrtacanthacris succincta* (Kirby.)	Orthoptera: Acrididae	Laboratory	Ambrose, 1985
12.	*Diacrisia deligna* (Fab.)	Lepidoptera: Arctidae	Laboratory	Singh and Gangrade, 1976; Singh, 1985
13.	*Diacrisia oblique* (Walk.)	Lepidoptera: Arctidae	Laboratory	Singh and Gangrade, 1975
14.	*Diaphania indicus* (Saun.)	Lepidoptera: Pyraustidae	Laboratory	Nagarajan and Ambrose, 2010
15.	*Dicladispa armigera* (Oliver)	Lepidoptera: Pterophoridae	Laboratory	Singh, 1985
16.	*Dolycoris indicus* (Stat.)	Hemiptera: Pentatomidae	Laboratory	Das, 1996
17.	*Dysdercus cingulatus* (Fab.)	Hemiptera: Pyrrhocoridae	Laboratory	Ambrose, 1985; Singh and Singh, 1987
18.	*Earias insulana* (Boisd.)	Lepidoptera: Noctuidae	Laboratory	Ambrose, 1999
19.	*Earias vitella* (Fab.)	Lepidoptera: Noctuidae	Laboratory	Singh and Singh, 1987
20.	*Epilachna 12-stigma* (Muls.)	Coleoptera: Coccinellidae	Laboratory	Nayar *et al.,* 1976
21.	*Epilachna vigintioctopunctata* (Fab.)	Coleoptera: Coccinellidae	Laboratory	Nayar *et al.,* 1976
22.	*Epilacrisia stigma* (Muls.)	Coleoptera: Coccinellidae	Laboratory	David and Natrajan, 1989
23.	*Eutectona machaeralis* (Walker)	Lepidoptera: Pyralidae	Laboratory	Nagarajan and Ambrose, 2010
24.	*Exelastis atomosa* (Wlsm.)	Lepidoptera: Pterophoridae	Laboratory	Singh and Singh, 1987
25.	*Falanga succinct* (Linn.)	Lepidoptera: Pyraustidae	Laboratory	Singh and Singh, 1987

26.	*Foveicollis lucas* (Dist.)	Coleoptera: Chrysomelidae	Laboratory	Singh, 1985
27.	*Helicoverpa armigera* (Hub.)	Lepidoptera: Noctuidae	Laboratory	Singh, 1985; Claver and Ambrose, 2003
28.	*Henosepilachna vigintioctopunctata* (Fab.)	Coleoptera: Coccinellidae	Laboratory	Patalappa and ChannaBasavanna 1979,
29.	*Leptocorisa acuta* (Fab.)	Hemiptera: Coreidae	Laboratory	David and Ananthakrishnan, 2009
30.	*Lygus hesperus* (Fab.)	Hemiptera: Miridae	Laboratory	Singh, 1985
31.	*Myllocoris curvicomis* (Fab.)	Coleoptera: Chrysomelidae	Laboratory	Cherian and Brahmachari, 1941; Ponnamma *et al.,* 1979
32.	*Myzus persica* (Sulz.)	Hemiptera: Aphididae	Laboratory	Singh, 1985
33.	*Nezara viridula* (Linn.)	Heteroptera: Pentatomidae	Laboratory	Singh and Gangrade, 1975; Claver and Ambrose, 2003
34.	*Pelopidas mathias* (Fab.)	Lepidoptera: Hesperiidae	Laboratory	Das, 1996
35.	*Peregrinus maidis* (Ashm.)	Homoptera: Delphacidae	Laboratory	Ponnamma *et al.,* 1919
36.	*Plutella xylostella* (Linn.)	Lepidoptera: Plutellidae	Laboratory	Singh, 1985
37.	*Pterophorus lienigianus* (Linn.)	Lepidoptera: Pterophoridae	Field	Anand *et al.,* 2010
38.	*Raphidopalpa foveicollis* (Lucas)	Coleoptera: Chrysomelidae	Laboratory	Singh, 1985
39.	*Rhaphid opaipa* (Thunb.)	Coleoptera: Chrysomelidae	Laboratory	Singh, 1985
40.	*Riptortus clavatus* (Thunb.)	Heteroptera: Alydidae	Laboratory	Ambrose, and Claver, 1995
41.	*Semiethisa pervolagata* (Walker.)	Homoptera: Delphacidae	Laboratory	Singh, 1985
42.	*Spilosoma oblique* (Walker.)	Coleoptera: Chrysomelidae	Laboratory	Cherian and Kylasam, 1939
43.	*Spodoptera lilura* (Fab.)	Lepidoptera: Noctuidae	Laboratory	Singh, 1985; Ambrose, and Claver, 1995
44.	*Phenacoccus solenopsis* (Tinsely)	Hemiptera: Pseudococcidae	Laboratory/Field	Present observation

But in 1986, Ambrose and Livingstone described the life history of this bug on houseflies, ants and caterpillars. The nymphs took 58.15 day and consumed 60.99 second/third instar *Neara viridula* Linn (Singh and Singh, 1987). Earlier, Singh and Gangrade (1976) observed this predator feeding on the *Diacriia oblique* Walker (Lepidoptera: Arctiidae). They reported that nymphal instars needed 102 to 110 days for completing the lifetime and consumed 96 to 101 larvae. In Madhya Pradesh, Ponnamma *et al.* (1979) observed total nymphal period range from 33 to 44 days on *Myllocerus curvicornis* (Fab.), a pest of coconut palm in Kerala. In Tamil Nadu, *R. fuscipes* completed its lifetime within 41.22 days on houseflies, ants and lepidopteran caterpillars (Ambrose and Livingstone, 1986). Recently, George *et al.* (2000) found that it needs only 34.21 days to complete its nymphal stage. They used *C. cephalonica* as a prey and further reported that it laid 58.37 eggs.

The growth, development and reproduction of the reduviid predators vary in relation to hosts (Bass and Shepard, 1974; Sahayaraj *et al.,* 2004; Sahayaraj, 2007b; Sahayaraj, 2012). However, no information is available about the influence of *D. cingulatus* and *P. solenopsis* on the life history and bioefficacy of *R. fuscipes* moreover; the predator has not been released augmentatively under cotton field to manage the pests and previous reports confirmed that *R. fuscipes* can suppress the pest population. Hence the biology, life table parameter, and bioefficacy were recorded for this predator under laboratory, pot and field conditions with the following objectives,

Objectives

1. To record the biology and life table parameters of *Rhynocoris fuscipes* on *Dysdercus cingulatus*, *Phenacoccus solenopsis* and a factitious host *Corcyra cephalonica.*
2. To record the prey preference, stage preference and functional response of *R. fuscipes* against *D. cingulatus*, and *P. solenopsis*.

1.3. MATERIALS AND METHODS

1.3.1. Cotton plant cultivation and pest rearing

Cotton seedlings (SVPR variety 2) were individually maintained in 25 cm diameter pots kept inside the screen house (36 x 21.5 sq. ft.) until they were grown approximately 25 cm height (~30 day old) and suitable for rearing *Dysdercus cingulatus* and *Phenacoccus solenopsis*. The seedlings were watered twice in a week, and grown in composts. Separate cultures of *D. cingulatus*, and *P. solenopsis* were maintained on cotton plant for more than five generations. Insects reared on the cotton plants were used for the study. *Dysdercus cingulatus* and *P. solenopsis* nymphs and adults were collected on cotton at Munanjipatty ($N8^{0}52'11"$ $E77^{0}77'614"$), Panagudi ($N8^{0}48'00"$ $E77^{0}67'00"$), and Pavoorchathram ($N8^{0}69'33"$ $E78^{0}04'706"$), Tirunelveli district, Tamil Nadu, India. The laboratory stock culture of *D. cingulatus* and *P. solenopsis* life stages were maintained by using artificial diet (Sahayaraj *et al.,* 2011) and surface of cleaned pumpkin (Patel, 2010) respectively.

1.3.2. Maintenance of Reduviid predator

Individuals of reduviid predator were collected from cotton agro-ecosystems in and around Tirunelveli and Kanyakumari districts, Tamil Nadu, India. They were maintained room at a constant temperature of 28 ± 2^{o}C, 60-10% RH and photoperiod of 11: 13 (L:D) hrss in transparent plastic containers (15 cm diameter x 8 cm height) with fourth (15.3±0.05 mg) and fifth (19.6±0.02 mg) instar larvae of *C. cephalonica* and also *D. cingulatus* third (43.7±0.3mg), fourth (49.6±0.03mg) and fifth nymphal instars (78.8±0.05) and adult of *P. solenopsis* (17.4±0.3mg).

1.3.3. Biology and life table of predator

The study was performed in three experimental levels.

1.3.3.1. Experiments:

1) The first evaluated the effect of natural and factitious hosts on the biological characteristic features. For this experiment, freshly hatched *R. fuscipes* nymphs (≤ 24 hrs old) were used. *Dysdercus cingulatus* third (43.7±0.3mg), fourth (49.6±0.03mg) and fifth nymphal instars (78.8±0.05) and adults (17.4±0.3mg) of *P. solenopsis*, from colonies maintained at Crop Protection Research Centre (CPRC) were offered (1 pest/3 predators at first and second instars and 1 pest/1 predator at remaining stadia) to each nymph as per the method described by Sahayaraj *et al*. (2007a). The randomized complete block design with three treatments was maintained for the study. Reduviids provided with nymphs of *D. cingulatus.*

2) Reduviids provided with adults of *P. solenopsis* and

3) Reduviids provided with fourth and fifth instar larvae of *C. cephalonica* and maintained for three generations continuously. The predators were maintained in 300-ml transparent plastic containers (12 cm diameter x 5 cm height). One newly emerged nymph was placed in each container. For treatments with prey, two preys (nymphs or larvae or adult) per day were supplied to each of the predator nymphs throughout the experiment. After every 24 hrs, the consumed prey was removed from the experimental arena. Similarly, molted exuviae's and dead predators (if any) were removed every 24 hrs using a fine Camlin brush (2 mm). Total nymphal development, nymph mortality, adult longevity and adult weight (male and female separately of the predator on each pest was recorded separately.

After the emergence of adult, sex ratio (number of females/number of males + number of females) was calculated and each *R. fuscipes* pair was released into 500 ml (17 cm diameter x 7 cm height) plastic container for mating. Adults were also maintained on the same food regimen as that of the nymphs from whom they had originated. All *R. fuscipes* adults received three to four red cotton bug or cotton mealy bug or rice flour

moth larvae per day. The reduviid was maintained for three generations with the same food regime, space provision and environmental conditions over eight months. Following biological parameters were recorded during the experiment: duration of the pre-oviposition period, number of eggs per dropt, total number of eggs laid over the life-time, oviposition time (time from the mating to the last egg batch), oviposition index (number of egg laying days/female life time) and the post-oviposition time (after the last egg batch to death of the insects). Lower fitness index was calculated for each category to record the females' fitness to produce the progeny.

Using the data, life table was constructed (Southwood, 1978). Construction of a life table for a predator species is an important component for understanding its dynamics. Laboratory reared *R. fuscipes* on *D. cingulatus* and *P. solenopsis* were maintained and used in the study for life table parameters. Different batches of eggs were allowed to hatch separately in 1 L capacity plastic containers covered with netted lids for free aeration and also to avoid the nymphal cannibalism. The newly hatched nymphs were reared in laboratory condition on the above mentioned pests. To construct the life table, the rate of increase was determined by using the following formula:

$$\Sigma\, e^{-rmx}\, lxmx = 1 \text{ (or) } \Sigma\, e^{-rmx}\, lxmx = 1096.6$$

where e^{-rmx} = rate of increase

lxmx = net reproductive rate

Net reproductive rate, (R_0=lxmx), the rate of multiplication of the population in each generation was measured in terms of female produced per generation. The approximate value of cohort (Tc) was calculated as:

$$Tc = (\Sigma\, lxmx)x/Ro$$

where Σ lxmx =sum of net reproductive rate; Ro = net reproductive rate

The arbitrary value of innate capacity (rc) was calculated using the formula: Rc= log e Ro/ Tc. It was the arbitrary value of 'rm'. Since the value of $e^{-r}mx$ obtained from experiments often lay outsides the range, both sides equation were multiplied by a factor of $\Sigma\ e^{-rmx}$ lxmx = 1096.6. The innate rate of increase (λ) was calculated as λ=antilog e^{rm}/female/day. The weekly multiplication of the predator was calculated as antilog e^{rm}. The doubling time (DT) was calculated as DT= In^2/rm.

1.3.4. Macromolecular profile of preys

The macromolecules like total body carbohydrate, total body protein and total body lipid (Sadasivam and Manikam, 1997; Lowry *et al.,* 1951; Bragdon, 1951) contents of *D. cingulatus, A. gossypii, P. solenopsis* and *H. armigera* were quantified using standard procedure.

1.3.4.1. Total body carbohydrate (Sadasivam and Manikam, 1997)

Preparation of Anthrone reagent: Take 200 mg of anthrone (0.2%) added with 5 ml of ethanol solution and to this solution add 95 ml of 75 % Con H_2SO_4.

Standard glucose solution: Take 10 mg of glucose to be added with 100 ml of distilled water and it was considered as a standard glucose solution.

Extraction of carbohydrate from insect prey: 100mg weight of live animal was taken in a homogenizer tube with phosphate buffer and homogenised. Centrifuge it at 3000 rpm for 30 minutes. Collect the supernatant (1 ml) and take particular volume of aliquots for analysis of carbohydrate.

Procedure for carbohydrate estimation

Prepare the 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of standard glucose in a series of test tubes. '0' serves as blank. Make the volume to 1 ml in all the test tubes including the sample test tube by distilled water. Then add 4 ml of anthrone reagent. Heat them for 10 minutes in a boiling water bath. Cool rapidly and read the green colour at 630

nm. Standard graph was drawn according to the optical density (OD) of the standard. From the graph and also using formula the amount of carbohydrate present in the sample was calculated:

$$\text{Carbohydrate content in sample mg/100 mg} = \frac{\text{Con. of standard} \times \text{OD of the sample}}{\text{OD of the standard}}$$

1.3.4.2. Estimation of prey whole body protein (Lowry *et al.*, 1951)

Preparation of Reagents

Reagent A: 2% sodium carbonate (Na_2CO_3) in 0.1N sodium hydroxide (NaOH) (prepare freshly).

Reagent B: 0.5 % copper sulphate ($CuSO_4$. $5H_2O$) in 1% potassium sodium tartrate (PST) (prepare freshly).

Reagent C: Mix 50 ml of Reagent A and 1 ml of B (prepare prior to use).

Reagent D: Folin-ciocaltteau reagent dilutes the commercially available reagent with an equal volume of distilled water on the day of use.

Preparation of standard protein: Take 10 mg Bovine Serum Albumin (BSA). Add 50 ml of distilled water and it is considered as a standard.

Working standard: Dilute 10 ml of protein solution to 5 ml of distilled water in a standard flask.

Extraction of protein from insect prey: Take 100 mg of alive animal and homogenise with the 1 ml of phosphate buffer (pH 7.2) and centrifuge it at 3000 rpm for 30 minutes. Supernatant (1 ml) was used for estimating the protein.

Procedure for protein estimation

Pipette out 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of the working standard protein solution into a series of test tubes. Pipette out a known volume of sample into a test tube. The volumes of the sample as well as standard were made up to 1 ml by

distilled water. A test tube with 1 ml of water was serves as the blank. Add 5 ml of reagent (to each test tube including blank). Mix well and allow it to stand for 10 minutes, then add 0.5 ml of reagent D mixed well and incubate at room temperature for about 30 minutes. Blue colour was developed; the intensity of the colour was measured at 650nm. The optical density (OD) was compared with standard graph to estimate protein in mg/100g.

$$\text{Protein content in sample mg/100 mg} = \frac{\text{Con. of standard} \times \text{OD of the sample}}{\text{OD of the standard}}$$

1.3.4.3. Estimation of whole body lipid (Bragdon, 1951)

Preparation of Acetic anhydride solution: About 50 ml of acetic anhydride solution was taken in a beaker which was kept in an ice bucket. To this, 2 ml of concentrated H_2SO_4 acid was added carefully and gently stirred and kept in a cool place for some time. The mixture should be colourless otherwise it should be discarded and prepared freshly.

Preparation of Potassium dichromate: Potassium dichromate ($K_2Cr_2O_7$) (2%) was mixed with con. H_2SO_4. This reagent was prepared freshly.

Standard cholesterol solution: Take 10 mg of cholesterol; make it up to 100 ml of chloroform.

Preparation of standard graph

Standard cholesterol was pipetted out into a series of test tubes in different volumes from 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml. All the test tubes were made up to 1 ml with chloroform. A test tube with 1 ml of distilled water was served as blank. Then to each test tube, 5 ml of acetic anhydride was carefully added and mixed well. The test tubes were covered with dark black colour cloth and kept for about 15 minutes without any disturbance. In concentrated solution one can notice the colour becoming rosy red, to blue and greenish blue. The developed colour was measured at 640nm in a spectrometer and

optical density (OD) value was recorded. Standard graph was drawn by applying concentrations of cholesterol on X axis and optical density (OD) on Y.

Extraction of lipid from insect prey

Take 100 mg of animal was taken in a homogenizer tube and homoginize it with 2 ml of chloroform. This was centrifuged at 3000 rpm for 30 minutes. The supernatant (1 ml) was transferred to a test tube and evaporated to dryness. This was kept at room temperature for 2 days. Three ml of distilled water and an equal amount of freshly prepared potassium dichromate solution was added. The intensity of the colour developed was measured at 640nm in spectrophotometer. The optical density (OD) of the sample was compared with standard graph to estimate lipid in mg/100g.

$$\text{Lipid content in sample mg/100 mg} = \frac{\text{Con. of standard} \times \text{OD of the sample}}{\text{OD of the standard}}$$

1.3.5. Stage preference (Joseph, 1959)

To evaluate whether *R. fuscipes* life stages exhibited a preference over various life stages of *D. cingulatus* and *P. solenopsis* or preference differed with predator life stage, separate experiments were conducted. Choice test was carried out and it consisted of exposing a reduviid nymph from one of the three instars (all three tested separately) and adults to four prey of each of the following pest life stages: first, second, and third (08.5±0.2 mg) nymphal instar and adult of *P. solenopsis* (17.4±0.3mg) or all life stages of *D. cingulatus*. This resulted in their exposure to 16 and 24 total preys per test for the former and the latter test, respectively into a Petri dish (16 cm diameter x 2.0 cm height) containing fresh cotton leaf (SVPR II). The pests were allowed to move undisturbed for 10 minutes, then a third (28.7±0.08 mg), fourth (40.5±0.06 mg), fifth (64.8±0.02) nymphal instars and adults (male=78.6±0.04, female=96.9±0.07) of *R. fuscipes* were released into

each Petri dish separately and the successfully captured stage was recorded. The experiments were replicated 10 times for each instars with separate individuals.

Reduviid feeding behavior was observed visually. During observations, the number of each prey life stage encountered and consumed by the reduviids was recorded. Each test was terminated when a predator had consumed three preys, the point after which reduviid predator would become satiated. For this reason, we used three preys as the standard in all tests. Individual tests were mostly completed within 60 min for adults and within 90 min for the third, fourth and fifth instar predators. Tests with each reduviid predator stage against each host species were replicated 10 times. Prey acceptability for each prey life stage was measured as the percentage of encountered prey of each individual life stage that was consumed (i.e. for each individual host life stage, the number consumed over the number encountered x 100).

1.3.6. Host preference (Ambrose, 1999)

Once the stage preference was recorded, then host preference studies were conducted by introducing four numbers the preferred stages of *D. cingulatus* [third nymphal instar (43.7±0.3mg), for *R. fuscipes* third and fourth instars; fourth nymphal instar (49.6±0.03mg) for *R. fuscipes* fifth instars and fifth nymphal instars (78.8±0.05) for *R. fuscipes* adult) and *P. solenopsis* adult (17.4±0.3mg) (for all the tested stages of the predator) in to Petri dish (9 cm diameter x 1.5 cm height) containing fresh cotton leaf (SVPR II) to mimic the field condition. The pests were allowed to move undisturbed condition for 10 minutes, then a third (28.7±0.08 mg), fourth (40.5±0.06 mg), fifth (64.8±0.02) nymphal instars and adults (male=78.6±0.04, female=96.9±0.07) of *R. fuscipes* were released into each Petri dishes separately and the successfully captured prey was recorded visually for a period of 60 minutes. The experiments were replicated for 10 times for each instar with separate individuals.

1.3.7. Functional response (Holling, 1959)

Functional response is an appropriate way to characterize the interaction of *R. fuscipes* with two different hemipteran prey species in a highly simplified environment. Experimental arena is glass Petri dishes (9.0 cm diameter x 1.5 cm height). A cotton leaf was placed at the base of the arena to mimic the field condition during the experiments. To evaluate the functional response, 24 hrss pre-starved predators of third (28.7±0.08 mg), fourth (40.5±0.06 mg), fifth (64.8±0.02) and adult (male and female) (85.2±0.03) stages were used in each one of the densities of (1, 2, 4, 8, and 10) *D. cingulatus* third instars nymphs and *P. solenopsis* adults. The prey was first introduced in to the Petri dish and was allowed to settle for 15 min. Then the predator was introduced and the prey consumed or killed within 24 hrs was recorded. To understand the impact of prey and prey size on the functional response, all the functional response parameters were analyzed separately for *D. cingulatus and P. solenopsis.* The experiments were replicated 10 times with separate and healthy predators.

1.3.7.1. Data analysis

The modified "Holling disc equation" of Holling (1959) *ie* 'random predator equation' (Rogers 1972) was used to find out the functional response of *R. fuscipes* against *D. cingulatus* third instar and *P. solenopsis* adult. Prey density (No), total number of prey killed (Ne) in a given period of time, and the maximum prey consumed (k) were recorded. Prey attack ratio (E=Ne/No), rate of discovery per unit of searching time [a = (Ne/No)/Ts], time spent by the predator in searching for prey (Ts), and time spent for handling each prey by the predator (b = Th/k) were calculated. Data of prey attack ratio (E) was converted into prey attacking efficiency (PAE) by multiplying with 100 (PAE =Ne/No X 100).

1.3.8. Statistical analysis

Individual data of developmental time, adult longevity, fecundity, oviposition periods and adult weight of the reduviid reared on mealy bug and red cotton bugs were compared using one-way analysis of variance (ANOVAs) with factitious host insect species as fixed variables. The influence of feeding regime on nymphal survival was analyzed using one-way ANOVA and post ANOVA, Tukey test. Correlation was recorded between the attack ratios (Ne/No) and predicted attack ratio (Y'/No). Mean number of prey attacked by a predator as a function of prey density was subjected to ANOVA and significance was expressed at 5% level. All analyses were performed using the SPSS statistical software (Version 20.0) and all statistical tests were carried out at 5% level of significance ($\alpha = 0.05$).

1.4. RESULTS

1.4.1. Incubation

The mean duration of incubation period was significantly prolonged in both *D. cingulatus* (6.8±0.1 days; F= 3.399, df= 4, 176, P=0.01) and and *P. solenopsis* (6.5±0.1 days; F= 3.432, df= 4, 155, P= 0.01) when comparing with factitious host *C. cephalonica* (6.3±0.1 days; F= 3.009, df= 6, 174, P= 0.008) (Table 2, 3, 4, 5).

1.4.2. Nymphal developmental period

The total mean nymphal period of *R. fuscipes* was increased when reared on *D. cingulatus* (F= 2.550, df= 14, 48, P= 0.008) or *P. solenopsis* (F= 4.125, df= 11, 51, P=0.005). The same trend was noted in *D. cingulatus* reared predator's first instar (F=3.536, df=6,160, P=0.003); second instar (F=3.033 df=5,140, P=0.012) and fifth instar (F=4.007, df=3,88, P=0.010) and also in *P. solenopsis* reared predator's first instar (F=6.440, df=4,132, P=0.005); second instar (F=2.329, df=5,116, P=0.047); third instar

Table 2. Individual and total nymphal developmental period (days), oviposition periods (days ± SE), fecundity (eggs/female) and hatchability (%) of *R. fuscipes* on two cotton pests and a factitious hosts during first generation.

Life stages	Prey		
	C. cephalonica	***D. cingulatus***	***P. solenopsis***
Incubation	6.6±0.1 ^{abc}	6.8±0.1 ^{abc}	7.8±0.1 ^{abc}
First instar	7.9±0.1 ^{abc}	7.9±0.1 ^{abc}	8.1±0.1 ^{abc}
Second instar	6.7±0.1 ^{ab}	7.1±0.1 ^{abc}	7.4±0.1 ^{abc}
Third instar	8.0±0.1 ^{abc}	8.3±0.2 ^{abc}	8.8±0.1 ^{c}
Fourth instar	6.7±0.1 ^{abc}	7.4±0.1 ^{abc}	7.8±0.2 ^{abc}
Fifth instar	7.6±0.1 ^{ac}	8.3±0.2 ^{bc}	9.8±0.2 ^{bc}
Total	42.5±0.3 ^{abc}	45.6±0.4 ^{abc}	49.5±0.5 ^{abc}
Pre-oviposition	10.9±0.2 ^{abc}	09.1±0.4 ^{ab}	12.8±0.4 ^{abc}
Oviposition	18.9±0.7 ^{abc}	12.7±0.7 ^{abc}	10.9±0.2 ^{ac}
Post-oviposition	9.8±0.5 ^{abc}	10.2±0.8 ^{abc}	9.9±0.6 ^{bc}
Fecundity	44.5±0.8 ^{abc}	27.4±0.8 ^{abc}	21.6±0.8 ^{abc}
Hatchability	97.7±1.5 ^{abc}	97.1±1.4 ^{ab}	95.4±2.8 ^{bc}
Oviposition index	0.086	0.073	0.064
Minimum no. of eggs	4.6±0.2 ^{ab}	4.1±0.2 ^{abc}	3.1±0.4 ^{bc}
Maximum no. of eggs	24.8±0.2 ^{ab}	18.6±0.1 ^{ab}	13.2±0.1 ^{bc}

Mean followed by the same letter do not differ significantly by DMRT at 5% level. Comparison was made between *C. cephalonica, D. cingulatus* and *P. solenopsis.*

Table 3. Individual and total nymphal developmental period (days), oviposition periods (days ± SE), fecundity (eggs/female) and hatchability (%) of *R. fuscipes* on two cotton pests and a factitious hosts during second generation.

Life stages	Prey		
	C. cephalonica	***D. cingulatus***	***P. solenopsis***
Incubation	6.8 ± 0.1^{abc}	7.9 ± 0.2^{abc}	7.2 ± 0.2^{abc}
First instar	8.1 ± 0.1^{ac}	8.2 ± 0.1^{b}	9.4 ± 0.2^{ac}
Second instar	6.8 ± 0.2^{ac}	7.4 ± 0.1^{bc}	9.2 ± 0.2^{bc}
Third instar	8.1 ± 0.2^{abc}	8.1 ± 0.1^{ab}	9.4 ± 0.2^{bc}
Fourth instar	7.1 ± 0.1^{a}	8.1 ± 0.1^{abc}	9.9 ± 0.2^{ac}
Fifth instar	7.8 ± 0.2^{a}	8.7 ± 0.2^{bc}	10.7 ± 0.2^{c}
Total	44.1 ± 0.4^{ab}	48.6 ± 0.3^{abc}	56.3 ± 0.4^{abc}
Pre-oviposition	11.7 ± 0.3^{abc}	11.7 ± 0.4^{bc}	14.0 ± 0.6^{abc}
Oviposition	21.5 ± 0.8^{abc}	21.1 ± 0.5^{bc}	6.3 ± 0.4^{ac}
Post-oviposition	9.8 ± 0.4^{abc}	9.6 ± 0.5^{abc}	7.1 ± 0.5^{abc}
Fecundity	41.5 ± 0.3^{abc}	31.5 ± 0.2^{abc}	20.4 ± 0.2^{bc}
Hatchability	96.3 ± 0.1^{abc}	97.1 ± 0.2^{bc}	93.6 ± 0.3^{bc}
Oviposition index	0.080	0.065	0.045
Minimum no. of eggs	3.8 ± 0.02^{ab}	3.5 ± 0.1^{abc}	2.2 ± 0.2^{bc}
Maximum no. of eggs	20.1 ± 0.1^{ab}	13.8 ± 0.1^{ab}	09.8 ± 0.01^{bc}

Mean followed by the same letter do not differ significantly by DMRT at 5% level. Comparison was made between *C. cephalonica, D. cingulatus* and *P. solenopsis.*

Table 4. Individual and total nymphal developmental period (days), oviposition periods (days ± SE), fecundity (eggs/female) and hatchability (%) of *R. fuscipes* on two cotton pests and a factitious hosts during third generation.

Life stages	Prey		
	C. cephalonica	***D. cingulatus***	***P. solenopsis***
Incubation	6.8 ± 0.2^{abc}	7.3 ± 0.1^{abc}	7.8 ± 0.1^{abc}
First instar	7.9 ± 0.2^{a}	8.3 ± 0.1^{abc}	9.5 ± 0.2^{ac}
Second instar	7.3 ± 0.2^{abc}	7.5 ± 0.2^{ab}	8.9 ± 0.2^{abc}
Third instar	8.1 ± 0.2^{ab}	8.0 ± 0.1^{ab}	9.4 ± 0.2^{abc}
Fourth instar	6.7 ± 0.2^{abc}	7.9 ± 0.1^{abc}	10.0 ± 0.2^{abc}
Fifth instar	7.9 ± 0.2^{abc}	8.5 ± 0.2^{bc}	9.8 ± 0.3^{bc}
Total	44.1 ± 0.9^{abc}	47.9 ± 0.6^{abc}	56.8 ± 0.6^{bc}
Pre-oviposition	15.4 ± 0.3^{abc}	12.9 ± 0.7^{abc}	18.7 ± 0.3^{abc}
Oviposition	18.3 ± 0.2^{ab}	21.9 ± 0.5^{ab}	-
Post-oviposition	14.9 ± 0.5^{ab}	8.9 ± 0.8^{ab}	-
Fecundity	21.2 ± 0.04^{ab}	14.1 ± 0.0^{ab}	-
Hatchability	94.1 ± 0.04^{ab}	93.4 ± 0.2^{ab}	-
Oviposition index	0.080	0.061	-
Minimum no. of eggs	3.1 ± 0.1^{ab}	2.6 ± 0.1^{ab}	-
Maximum no. of eggs	16.6 ± 0.1^{ab}	14.8 ± 0.1^{ab}	-

Mean followed by the same letter do not differ significantly by DMRT at 5% level. Comparison was made between *C. cephalonica, D. cingulatus* and *P. solenopsis.*

Table 5. Nymphal developmental period ($\overline{X}$ ± SE) of *R. fuscipes* cumulative data of three generations on *C. cephalonica, D. cingulatus* and *P. solenopsis*

Life stage	Prey		
	C. cephalonica (N=186)	*D. cingulatus* (N=180)	*P. solenopsis* (N=159)
First instar	7.7 ± 0.1^{ab}	7.9 ± 0.1^{b}	8.4 ± 0.1^{c}
Second instar	6.3 ± 0.1^{ab}	7.1 ± 0.1^{bc}	7.4 ± 0.1^{bc}
Third instar	7.9 ± 0.1^{ab}	8.3 ± 0.2^{ab}	8.8 ± 0.1^{ac}
Fourth instar	6.2 ± 0.2^{ac}	7.4 ± 0.1^{b}	7.8 ± 0.2^{ac}
Fifth instar	7.4 ± 0.2^{a}	8.3 ± 0.2^{bc}	9.8 ± 0.2^{bc}
Total	41.1 ± 0.4^{ab}	45.6 ± 0.4^{ab}	48.9 ± 0.3^{c}

Mean followed by the same letter do not differ significantly by DMRT at 5% level. Comparison was made between *C. cephalonica, D. cingulatus* and *P. solenopsis.*

(F=4.095, df=4,98, P=0.004); fourth instar (F=2.644, df=3,77, P=0.055) and fifth instar (F=2.778, df=3,59, P=0.049) (Table 2,3,4,5).

1.4.3. Survival rate

The nymphal survival rate decreased while the predator grew older. The total nymphal survival per cent was significantly lower in *D. cingulatus* (F= 3.721, df= 10, 52, P= 0.03) and *P. solenopsis* (F= 4.964, df= 9, 53, P= 0.02) when compared to the *C. cephalonica* (58.8%). Higher survival was observed in the first instar predator invariably provided with *C. cephalonica* (F=0.898, df=4,5, P=0.528), *D. cingulatus* (F=0.484, df=4,5, P=0.749), and *P. solenopsis* (F=0.279, df=4,5, P=0.880). For a while, least survival rate was observed in fifth instars reduviid reared on *C. cephalonica* (F=0.051, df=5,4, P=0.997), *D. cingulatus* (F=0.807, df=5,4, P=0.599), and *P. solenopsis*. Invariably in three generations, total nymphal survival percent of *R. fuscipes* was lower in both *D. cingulatus* (F=3.721, df=10, 52, P=0.03) and *P. solenopsis* (F=4.964, df=9, 53, P=0.02) than *C. cephalonica* (F=0.754, df=9, 53, P=0.241) (Fig. 1).

1.4.4. Adult longevity

Female predator lived longer than the male predator (Fig. 2). The adult longevity was shorter for *D. cngulatus* (F=1.987, df=11, 48, P=0.075; F=1.087, df=12, 47, P=0.037 for male and female, respectively) and *P. solenopsis* (F=0.637, df=9, 27, P=0.05 for male; F=3.738, df=9, 10, P=0.026 for female) offered predators when compared to the factitious host, *C. cephalonica* (F=1.004, df=12, 47, P=0.460 for male; F=0.869, df=11, 48, P=0.324 for female).

1.4.5 Adult weight and sex ratio

When compared to *C. cephalonica* fed category, *D. cingulatus* fed animals were significantly heavier (F=5.624, df= 2, 7, P=0.026) than the *P. solenopsis* (F= 3.470, df=1, 8, P=0.054) fed male and female of *R. fuscipes* adults (Fig. 3). Invariably, male biased sex

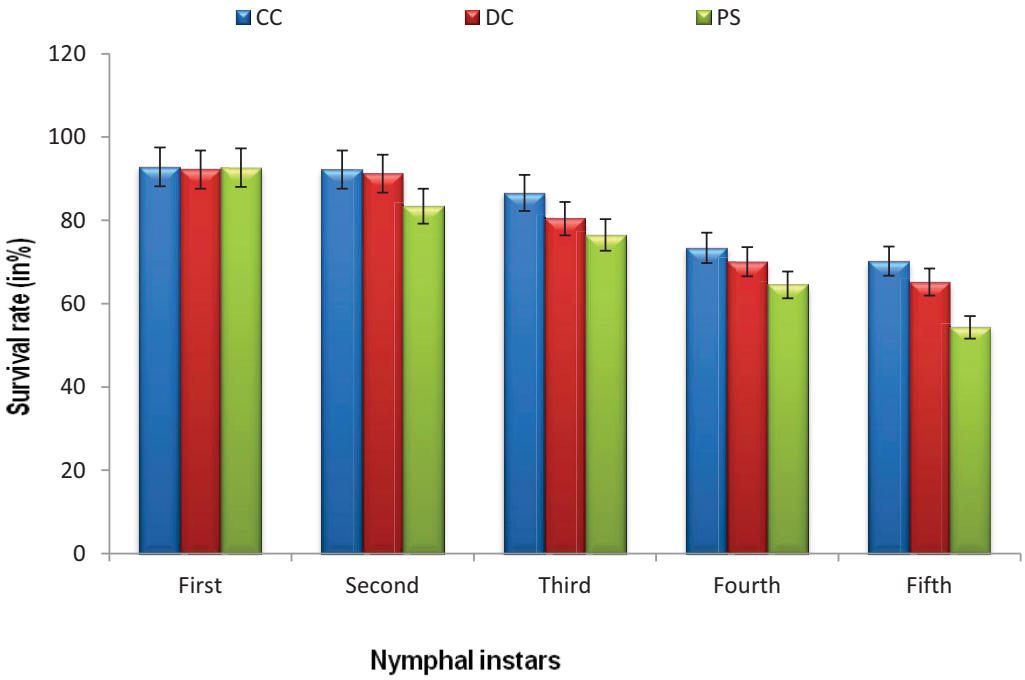


Figure 1. Mean nymphal survival rate (%) of *R. fuscipes* nymphal stages reared on host *C. cephalonica* (CC), *D. cingulatus* (DC) and *P. solenopsis* (PS).

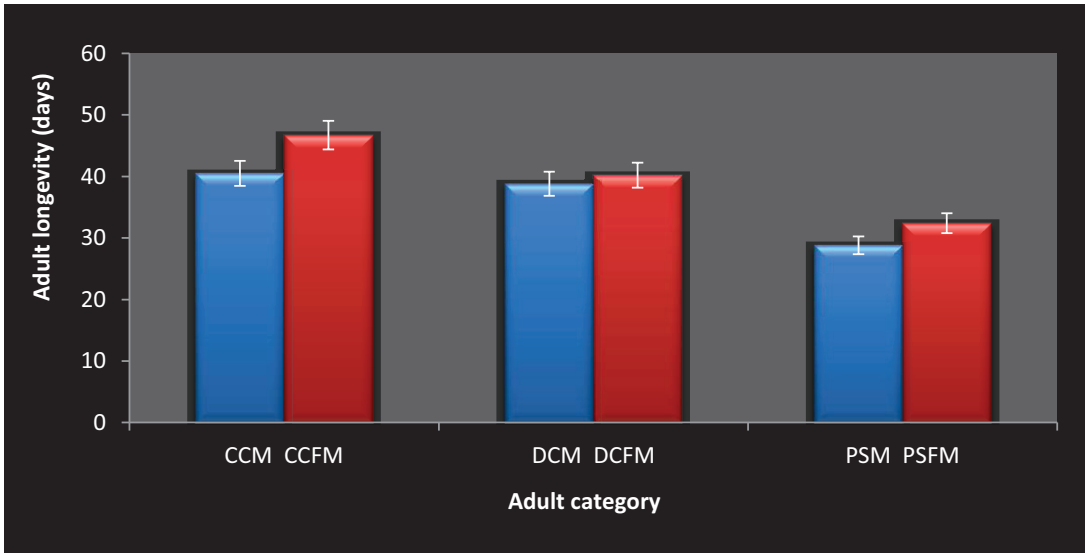


Figure 2. Mean adult longevity (days) of *R. fuscipes* male (suffix - M) and female (suffix - FM) reared on (CCM - *C. cephalonica* (CC) reared male; CCFM – *C. cephalonica* reared female; DCM – *D. cingulatus* (DC) reared male; DCFM–*D. cingulatus* reared female; PSM – *P. solenopsis* (PS) reared male; PSFM – *P. solenopsis* reared female).

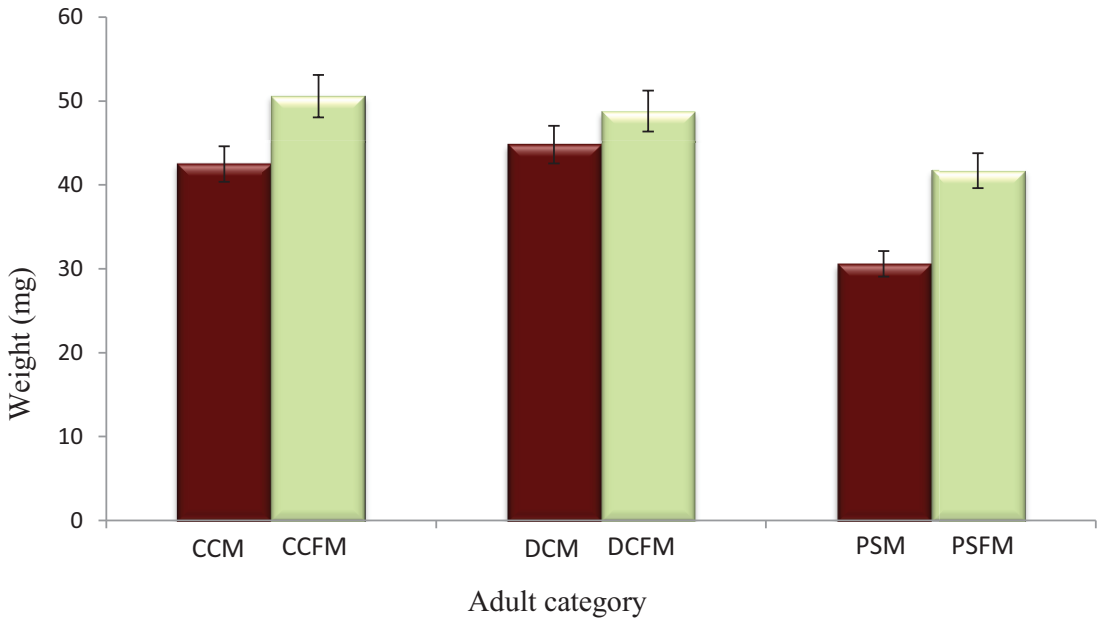


Figure 3. Adult weight of *R. fuscipes* reared on *C. cephalonica, D. cinguatus* and *P. solenopsis* (CCM - *C. cephalonica* reared male; CCFM - *C. cephalonica* reared female; DCM – *D. cingulatus* reared male; DCFM – *D. cingulatus* reared female; PSM – *P. solenopsis* reared male; PSFM – *P. solenopsis* reared female).

Table 6. Oviposition periods (days ± SE), fecundity (eggs/female) and hatchability (%) of *R. fuscipes* reared with *C. cephalonica, D. cingulatus* and *P. solenopsis*

Life stages of predator	Prey		
	C. cephalonica	***D. cingulatus***	***P. solenopsis***
Preoviposition (days)	12.7 ± 0.1^{abc}	15.2 ± 0.2^{bc}	16.3 ± 0.2^{bc}
Oviposition (days)	20.4 ± 0.2^{ac}	14.7 ± 0.17^{bc}	10.6 ± 0.2^{abc}
Post-oviposition (days)	11.8 ± 0.3^{abc}	9.4 ± 0.13^{abc}	5.6 ± 0.3^{abc}
Fecundity (no./female)	32.6 ± 0.3^{ac}	27.4 ± 0.1^{bc}	21.6 ± 0.2^{bc}
Hatchability (%)	97.7 ± 1.5^{ab}	97.6 ± 1.4^{ab}	94.4 ± 2.8^{bc}
Oviposition index	0.082	0.068	0.053
Minimum number of eggs/batch/female	4.3 ± 1.2^{ab}	3.8 ± 1.8^{abc}	3.96 ± 1.3^{bc}
Maximum number of eggs/batch/female	33.6 ± 1.2^{ab}	28.3 ± 1.68^{ab}	22.4 ± 1.3^{bc}

Mean followed by the same letter do not differ significantly by TMRT at 5% level. Comparison was made among *C. cephalonica, D. cingulatus* and *P. solenopsis.*

ratio was observed in all the three tested preys [1:0.43, 1:0.37 and 1:0.37 (Female: Male) for *C. cephalonica, D. cingulatus* and *P. solenopsis* respectively].

1.4.6. Fecundity and hatchability

The duration of the preoviposition period got reduced (F=0.696, df=5, 26, P=0.638), whereas both oviposition (F=8.342, df=7, 24, P=0.004) and post-oviposition (F=2.688, df=4, 27, P=0.221) periods were extended when *C. cephalonica* was provided to immature stages and adults of *R. fuscipes*. Longer pre-oviposition (F=4.000, df=7, 24, P=0.092) and shorter oviposition (F=0.602, df=7, 24, P=0.647) periods were observed in *P. solenopsis* fed predators. The oviposition index was higher in *C. cephalonica* reared predator than in *D. cingulatus* and also in *P. solenopsis* offered predators. The predator laid eggs as single as well as in batches, having a maximum of 34 and minimum of 4 eggs. Significantly higher fecundity was observed in *D. cingulatus* (F=2.643, df=7, 31, P=0.036) than in *P. solenopsis* (F=1.396, df=6, 10, P=0.244) fed group. These differences translated into a significantly lower fitness index for *P. solenopsis* (r= 0.028), *D. cingulatus* (r= 0.031) and *C. cephalonica* (r = 0.037). The hatchability of predators varied with pests. Significant higher percent hatchability was recorded in *C. cephalonica* fed predator (F=0.845, df=7, 38, P=0.238) rather than in *D. cingulatus* (F=3.144, df=7, 31, P=0.043) and also in *P. solenopsis* (F=1.434, df=7, 9, P=0.134) fed groups (Table 6).

1.4.7. Life table

The prepared life table showed that the gross reproductive rate (GRR) was higher in all the categories than the net reproductive rate (Ro). *Corcyra cephalonica* fed predator showed maximum GRR (94.32) and Ro (72.03). The mean length of generation (Tc) was higher in *P. solenopsis* (57 days) category than in *D. cingulatus* (55 days) and also in *C. cephalonica* (53 days) fed groups. Higher innate capacity was recorded in *C. cephalonica* (0.080) fed category followed by *D. cingulatus* (0.074). Finite rate of

increase was higher in *C. cephalonica* fed predator (1.08) than *D. cingulatus* (1.075) and *P. solenopsis* (1.07). Shorter weekly multiplication was observed in *P. solenopsis* category (1.56) followed by *D. cingulatus* (1.66). Shorter doubling time was observed in *C. cephalonica* fed predator (8.25) and longer in *P. solenopsis* (10.83). Maximum hypothetical female in F2 generation was in *C. cephalonica* fed predator rather than *D. cingulatus* (3360.52) and *P. solenopsis* (1120.9) fed predators. In general, all the life table parameters were in favour of the *C. cephalonica* fed *R. fuscipes* (Table 7).

1.4.8. Macro molecular profile of cotton pests

Among the three pests analysed, the larvae of *C. cephalonica* contain more amount of total body protein (14.7/100 mg) followed by nymphs of *D. cingulatus* (11.5 mg/100 mg) and *P. solenopsis* (4.50 mg/100 mg) adult (Fig. 4). The total body carbohydrates content was higher in the larvae of *C. cephalonica* (22.8 mg/100 mg) followed by nymphs of *D. cingulatus* (13.6 mg/100 mg) and adults of *P. solenopsis* (10.8 mg/100 mg). The lipid content was higher amount in nymphs of *D. cingulatus* (16.6 mg/100 mg).

1.4.9. Stage preference

Binomial distribution value was calculated for the preference of *R. fuscipes* on cotton pests. The third (0.9989), fourth (0.9999) nymphal instars of *R. fuscipes* preferred third nymphal instar of *D. cingulatus.* Fifth nymphal instars *R. fuscipes* preferred (0.9989) fourth nymphal instar of *D. cingulatus* and adult *R. fuscipes* preferred (0.9989) fifth nymphal instar of *D. cingulatus* (Fig. 5a). However, invariably all the tested life stages of reduviid preferred *P. solenopsis* adults (0.9999, 1.0, 1.0 and 0.9989 for third, fourth, fifth instars and adult respectively) (Fig. 5b).

1.4.10. Host preference

When the predator were released in to the Petri dish, *R. fuscipes* showed more positive response to *D. cingulatus* ($P<0.05$) by Chi-square as compared to that of

Table 7. Mean life table parameters of *Rhynocoris fuscipes* reared on caterpillars of *C. cephalonica* (fourth and fifth instar), nymphs of *D. cingulatus* (third, fourth and fifth instars) and *P. solenopsis* (third instar and adult) continuously for three generations.

Life table parameter	Host insect		
	C. cephalonica	*D. cingulatus*	*P. solenopsis*
Gross reproductive rate (GRR)	94.32	85.24	67.02
Net reproductive rate (NRR) Ro	72.03	57.97	33.48
Length of generation (Tc)	53	55	57
Innate capacity for increase (r_c)	0.080	0.074	0.063
Corrected (r_m)	0.084	0.073	0.064
Finite rate of increase (λ)	1.08	1.075	1.07
Weekly multiplication	1.71	1.66	1.56
Doubling time (in days)	8.25	9.49	10.83
Hypothetical female in F2 generation	5188.32	3360.52	1120.9

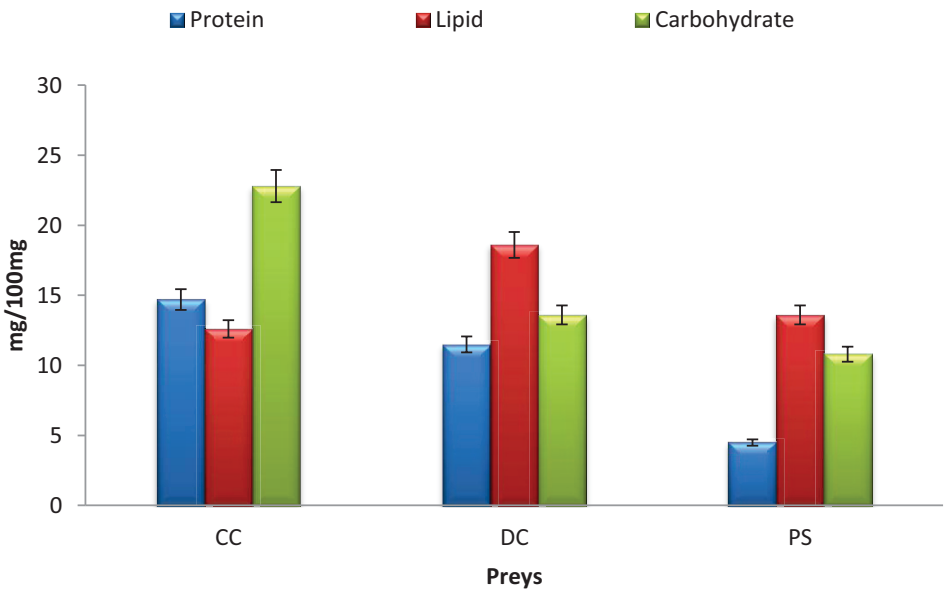


Figure 4. Biochemical analysis of total body protein, lipid and carbohydrate (mg/100mg) in *C. cephalonica* (CC), *D. cingulatus* (DC) and *P. solenopsis* (PS).

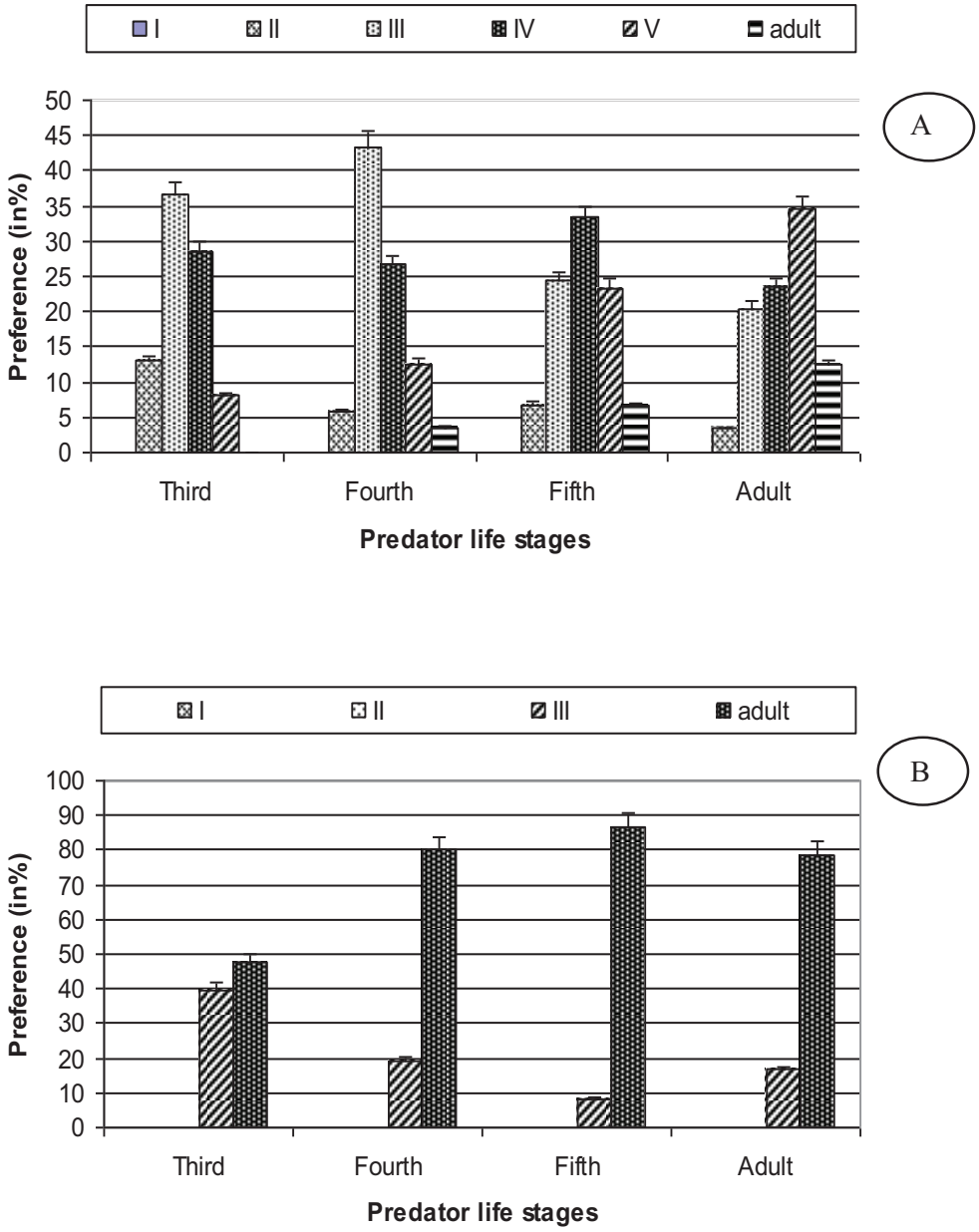


Figure 5. Prey stage preference (%) of *R. fuscipes* life stages against first, second, third, fourth and fifth nymphal instars and adults (both male and female) life stages of *D. cingulatus* (A) and *P. solenopsis* (B).

P. solenopsis (P<0.05). The adult *R. fuscipes* showed higher preference against *D. cingulatus* (χ^2=0.05) than third instar (χ^2=0.400), fourth instar (χ^2=1.60) and fifth instar (χ^2=0.400). The adult predator showed more positive choice (r=0.9999) on *D. cingulatus* than other nymphal instars. Invariably all the life stages of the predator showed equal choice (χ^2=6.40) against *P. solenopsis* (r=0.9318) (Table 8).

1.4.11. Functional response

It is a typical density dependant function of the tested predators responding to the increasing prey density by killing more number of prey than they killed at lower prey densities *ie,* its predatory rate increased with increasing of preys density (Fig. 6). The functional responses of third, fourth, fifth and adult of *R. fuscipes* were recorded, and the results revealed that the attack ratio (E) decreased with increase of *D. cingulatus* (Table 9) and *P. solenopsis* densities (Table 10). The predators exhibited Holling's Disc equation type II functional curve. The maximum and minimum attack ratio was observed in prey 1 and 10 prey/predator respectively (F=4.324, df=2, 7, P=0.039 and F=6.546, df=1, 8, P=0.046 for fifth instar nymphs and adult predator respectively). Similar trends were observed for *D. cingulatus* (F=4.258, df=3, 6, P=0.046 and F=7.526, df=1, 8, P=0.054 for fourth instar nymphs and adult predator respectively). The attack ratio 'E' and the predicted attack ratio (Y^1/No) were positively correlated for the third, fourth, fifth and adult stages of the predator (r= 0.99) and two types of the preys encountered (r= 0.99).

The rate of discover 'a' values were decreased while the prey density increased both for *P. solenopsis* (F=5.160, df=2, 7, P=0.042; F=6.241, df=2, 7, P=0.043; F=5.580, df=2, 7, P=0.027 and F=4.359, df=2, 7, P=0.038 for third, fourth, fifth instars and adult predator, respectively) and *D. cingulatus* (F=6.562, df=4, 5, P=0.055 and F=5.364, df=4, 5, P=0.015 for fourth instar nymphs and adult predator respectively) (Table 9). The correlation data was observed between preys offered and prey consumption, and it was

Table 8. Host preference (%) of *R. fuscipes* third, fourth and fifth nymphal instars and adults (male and female) stages against *D. cingulatus* and *P. solenopsis*

Parameter	Predator life stages			
	III	IV	V	Adult
	D. cingulatus			
Positive choice	8	6	8	10
Negative choice	12	14	12	10
χ^2	0.400	1.60	0.400	0.00
Significance	P<0.05	P<0.05	P<0.05	P<0.05
Preference (%)	40	30	40	50
Binomial distribution value	0.9999	0.9989	0.9999	0.9999
	P. solenopsis			
Positive choice	2	2	2	2
Negative choice	18	18	18	18
χ^2	6.40	6.40	6.40	6.40
Significance	P<0.05	P<0.05	P<0.05	P<0.05
Preference (%)	10	10	10	10
Binomial distribution value	0.9138	0.9138	0.9138	0.9138

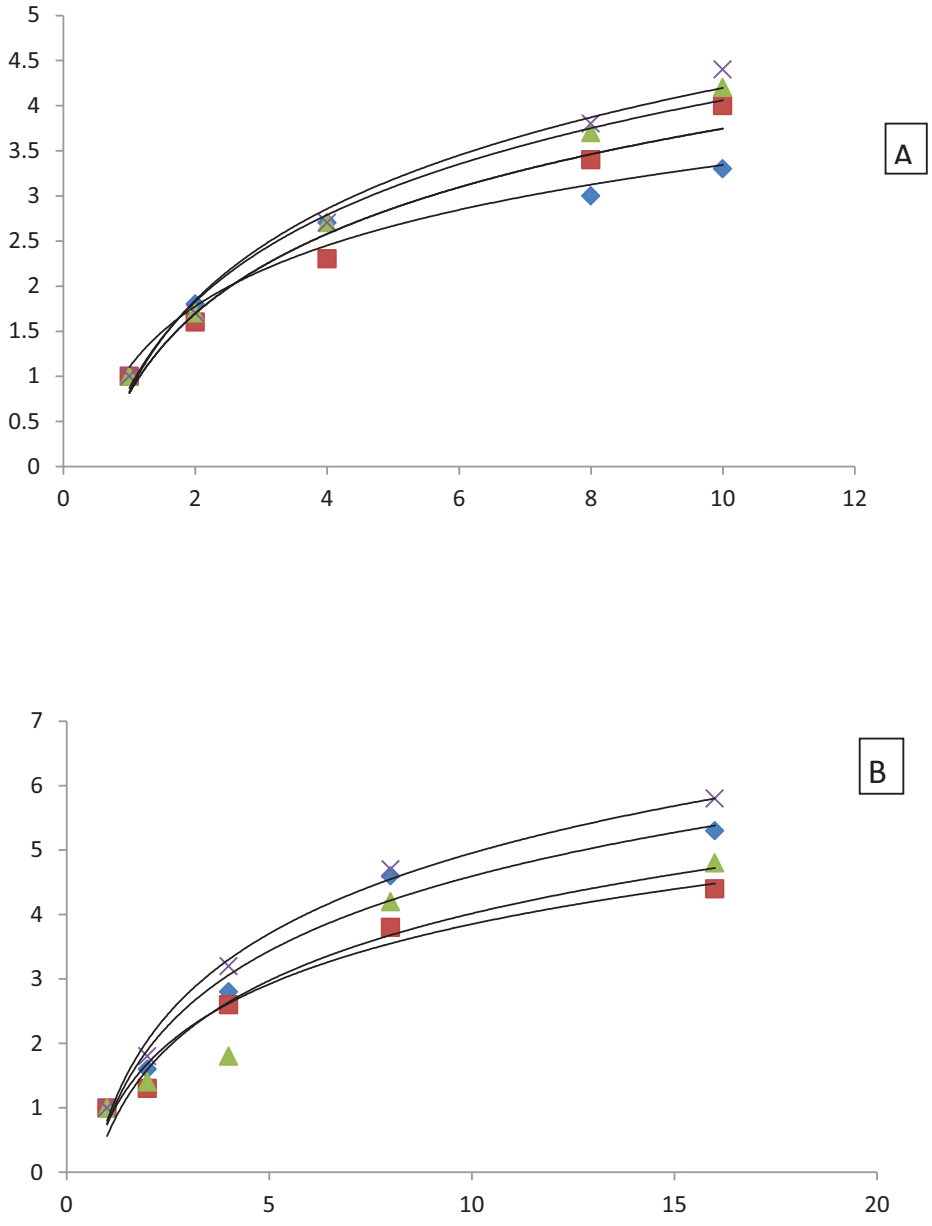


Figure 6. ***Rhynocoris fuscipes*** **third, fourth, fifth nymphal instars and adult functional response curve when provided with** ***D. cingulatus*** **(A) and** ***P. solenopsis*** **(B).**

highly significant in both the pests. For instance third instar (0.915, P=0.029), fourth instar (0.994, P=0.001), fifth instar (0.981, 0.003) and adult (0.987, 0.002) predator against *D. cingulatus* and third instar (0.929, P=0.023), fourth instar (0.921, P=0.026), fifth instar (0.935, 0.020) and also adult (0.940, 0.018) predators against *P. solenopsis*.

The third, fourth and fifth nymphal instars and adult of *R. fuscipes* on *D. cingulatus* showed maximum Na values (3.33, 3.96, 4.23 and 4.41) at 10 prey densities respectively (y=1.24+0.22x, r=0.921; y=0.89+0.32x; r=0.99; y=0.96+0.34x; r=0.98; y=0.92+0.36x; r=0.98). The same result was also observed in *P. solenopsis* at the prey densities of 1, 2, 4, 8 and 16 for preys per predator recorded high biocontrol potential (y=1.30+0.28x; r=0.93; y=1.22+0.23x; r=0.92; y=0.99+0.26x; r=0.93 and y=1.40+0.31x; r=0.94 for third, fourth, fifth and adults stages of the predator respectively) (Fig. 7). Maximum number of preys eaten (Ne) was higher for adult predator at 24 hrs when *D. cingulatus* and *P. solenopsis* were offered as a prey. Prey attacking efficiency (PAE) was high in the prey density of 1(100) for both pests, and it decreased when the prey density was increased (Table 9, 10, 11). Lower PAE was observed in third instar of *R. fuscipes* for *D. cingulatus* (330) and fourth instar of *R. fuscipes* for *P. solenopsis* (280).

1.5. DISCUSSION

The biology of the predators were affected by a variety of external and internal factors and among them, nutrition seems to be the most crucial single factor affecting the development and reproduction in many ways in reduviid predators (Ambrose *et al.*, 1990; Sahayaraj, 1993; Claver, 1998).

1.5.1. Development

For a prey species to be suitable, it must provide all nutritionally important factors, such as proteins, carbohydrates, lipids, vitamins and minerals in balanced proportion and concentration to meet a predator's metabolic requirements (House, 1977; Cohen and Tang,

Table 9. Functional response parameters (No. of prey eaten, attack ratio, handling time, rate of discover) of third, fourth and fifth nymphal instars and adults of the *R. fuscipes* exposed to different densities (1, 2, 4, 8, 10 preys/predator) of *D. cingulatus* for 24 hrs in Petri dish.

Predator life stage	Prey densities (No)	No. of prey eaten (Ne)	Attack ratio (E)	Handling time (Th)	Days searching (Ts)	Predicted Ne (Y')	Predicted attack ratio	Handling time/Prey (b)	Rate of Discover (a)	Slope (r2)
III	1	1.0	1.00	0.16	0.84	0.99	0.99	0.04	1.19	0.837
	2	1.8	0.92	0.08	0.92	1.84	0.92		1.00	
	4	2.7	0.66	0.17	0.83	2.66	0.67		0.80	
	8	3.0	0.38	0.16	0.84	3.02	0.38		0.45	
	10	3.3	0.33	0.12	0.88	3.34	0.33		0.38	
IV	1	1.0	1.00	0.15	0.85	1.00	1.0	0.04	1.18	0.988
	2	1.6	0.82	0.25	0.75	1.65	0.83		1.10	
	4	2.3	0.57	0.13	0.87	2.3	0.58		0.66	
	8	3.4	0.43	0.18	0.82	3.4	0.43		0.52	
	10	4.0	0.40	0.16	0.84	4.0	0.4		0.48	
V	1	1.0	1.00	0.18	0.82	0.98	0.98	0.03	1.20	0.963
	2	1.7	0.84	0.15	0.85	1.66	0.83		0.98	
	4	2.7	0.68	0.16	0.84	2.72	0.68		0.81	
	8	3.7	0.46	0.13	0.87	3.69	0.46		0.53	
	10	4.2	0.42	0.12	0.88	4.22	0.42		0.48	
Adult	1	1.0	1.00	0.24	0.76	0.99	0.99	0.03	1.30	0.973
	2	1.7	0.87	0.18	0.82	1.74	0.87		1.06	
	4	2.7	0.67	0.20	0.80	2.69	0.67		0.84	
	8	3.8	0.48	0.16	0.84	3.83	0.48		0.57	
	10	4.4	0.44	0.14	0.86	4.39	0.44		0.51	

Table 10. Functional response parameters (No. of prey eaten, attack ratio, handling time, rate of discover) of third, fourth and fifth nymphal instars and adults of the *R. fuscipes* exposed to different densities (1 , 2, 4, 8, 16 preys/predator) of *P. solenopsis* for 24 hrs in Petri dish.

Stage of predator	Prey densities (No)	No. of prey eaten (Ne)	Attack ratio (E)	Handling time (Th)	Days searching (Ts)	Predicted Ne (Y')	Predicted attack ratio	Handling time/Prey (b)	Rate of discover (a)	Slope (r^2)
III	1	1.0	1.00	0.05	0.95	1.05	1.05	0.03	1.10	0.848
	2	1.6	0.80	0.10	0.90	1.60	0.80		0.89	
	4	2.8	0.70	0.12	0.88	2.78	0.69		0.79	
	8	4.6	0.58	0.17	0.83	4.65	0.58		0.70	
	16	5.3	0.33	0.14	0.86	5.23	0.32		0.38	
IV	1	1.0	1.00	0.09	0.91	0.99	0.99	0.03	1.09	0.874
	2	1.3	0.65	0.25	0.75	1.31	0.66		0.87	
	4	2.6	0.65	0.13	0.87	2.61	0.65		0.75	
	8	3.8	0.48	0.21	0.79	3.86	0.48		0.61	
	16	4.4	0.28	0.13	0.87	4.45	0.28		0.32	
V	1	1.0	1.00	0.06	0.94	0.99	0.99	0.03	1.06	0.862
	2	1.4	0.70	0.15	0.85	1.40	0.70		0.82	
	4	1.8	0.45	0.18	0.82	1.80	0.45		0.55	
	8	4.2	0.53	0.15	0.85	4.20	0.53		0.62	
	16	4.8	0.30	0.13	0.87	4.70	0.29		0.34	
Adult	1	1.0	1.0	0.17	0.83	0.99	0.99	0.03	1.20	0.882
	2	1.8	0.90	0.18	0.82	1.80	0.90		1.10	
	4	3.2	0.80	0.26	0.74	3.20	0.80		1.08	
	8	4.7	0.58	0.32	0.68	4.60	0.60		0.85	
	16	5.8	0.36	0.18	0.82	5.10	0.32		0.39	

Table 11. Proportion (%) of *D. cingulatus* and *P. solenopsis* attacked by *R. fuscipes* third, fourth and fifth nymphal instars and adult (male and female) in laboratory conditions.

Prey density	Predatory rate			
	Third instar	Fourth instar	Fifth instar	Adult
D. cingulatus				
1	100.0	100.0	100.0	100.0
2	90.0	80.0	85.0	85.0
4	67.5	57.5	67.5	67.5
8	37.5	42.5	46.3	47.5
10	33.0	40.0	42.0	44.0
P. solenopsis				
1	100.0	100.0	100.0	100.0
2	80.0	65.0	70.0	90.0
4	70.0	65.0	52.5	80.0
8	57.5	47.5	45.0	58.7
16	33.13	27.5	30.0	36.2

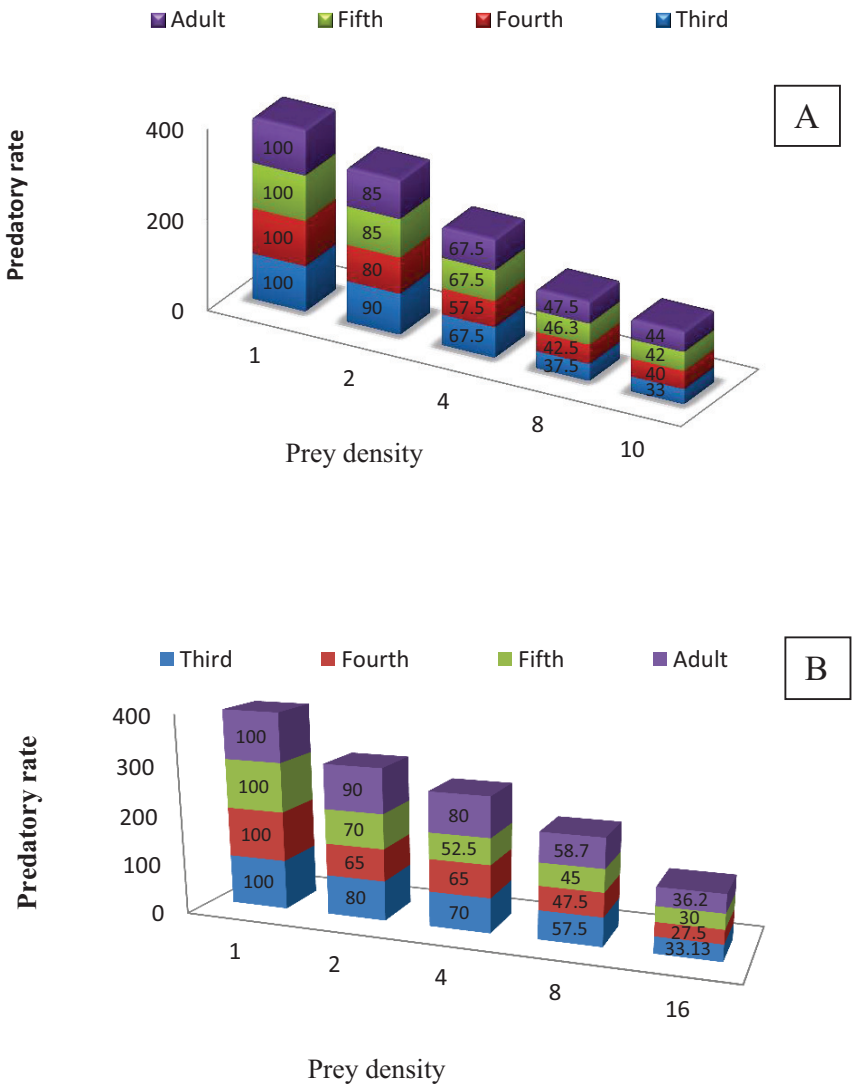


Figure 7. Cumulative predatory rate of *R. fuscipes* against *D. cingulatus* (A) and *P. solenopsis* (B) at different densities.

1997). For example, George (2000) and Sahayaraj *et al.* (2004) found that among several species of lepidopteran prey for a reduviid, *R. marginatus*, *S. litura* and *Helicoverpa armigera* were the most suitable preys tested for its development. From our study, we observed that *C. cephalonica* has higher primary nutrients for development of reduviids than *D. cingulatus* and *P. solenopsis.* Non-nutritional factors such as sequestered secondary plant metabolites (Bruno *et al.,* 2001), odor, texture and mobility of prey (Chandral and Retna Latha Sinazer, 2011) also play a large role in prey selection. For example, nymphal instars of *R. marginatus* are likely to have difficulty in feeding on prey having well developed or strong mandibles (Ambrose *et al.,* 1990), or hairs (Sahayaraj, 1999), or very large and active preys (elusive behaviour) (Sahayaraj 1995a) and prey with impenetrable integuments (Ambrose, 1999; Sahayaraj, 1999b) etc..

1.5.2. Survival of reduviids

The present results showed that all *R. fuscipes* nymphs and adults used larvae of *C. cephalonica* as suitable prey, when compared with *D. cingulatus* and *P. solenopsis*. The survival rate was in each stadium was also higher in *C. cephalonica* fed predators than *D. cingulatus* and *P. solenopsis* fed predators. Same kind of results were observed by George (2000) and Claver and Ambrose (2002). This was reflected that *C. cephalonica* larvae, reduced predator total nymphal developmental period (4-7 days) and nymphal mortality (10-30%), shorter pre-oviposition period (3-4 days), high fecundity (24-30%), hatchability, and oviposition index. The most direct way in which nutrition can influence the predator biology is on its nymphal developmental period and nymphal mortality (Ambrose, 1999). The lepidopteron pests are the suitable preys for the growth of reduviid predators (Claver and Ambrose, 2001a, b, George, 2000). In the present study the total nymphal development was prolonged by *P. solenopsis* followed by *D. cingulatus* and *C. cephalonica.* This indicates that the latter factitious host is the suitable prey for

reduviids. This, in a broad sense, agrees with the concept of Slansky (1982), who establishes a correlation between prey preference and accelerated developmental rate in predators. Previously, studies on *Cydnocoris gilvus* (Venkatesan *et al*., 1997), *Acanthaspis siva* Fab. and *R. fuscipes* Fab. (George, 2000), *R. marginatus* Fab. (Sahayaraj *et al*., 2004, 2006a; Sahayaraj, 2007a, b; 2012) proved the above statement along with the results of present study. All the reports mentioned confirm the higher nutritional contents of lepidopteran larvae. The results of this study indicated that when fed on *C. cephalonica,* the development of *R. fuscipes* into adult was a little faster than feeding on *D. cingulatus* and *P. solenopsis.*

1.5.3. Sex ratio

Sex ratio is the key factor for the development of an insect population (Ambrose, 1999). Male based sex ratio was observed in the present study, but the increased number of females gives the potential development of insect population, especially the predatory insects. Sahayaraj and Selvaraj (2003) reported that the life table of *R. fuscipes* was in accordance with sex ratio, They found that 2:1 (Male: Female) was the best fit for the population dynamics of *R. fuscipes.*

1.5.4. Body weight and Reproduction

Furthermore, in most cases the adults gained more weight (51.4 mg) when maintained on this prey. But the lower female weight was observed in *P. solenopsis* fed insects (41.2 mg) than *D. cingulatus* (48.7 mg). The adult female body size is often influenced by the nymphal nutrition, which in turn affects the fecundity of the insect (Sahayaraj and Ambrose, 1994; Sadeghi and Gilbert, 1999; George, 2000; Sahayaraj, 2003; Claver and Ambrose, 2003a; Sahayaraj and Balasubramanian, 2009). Stewart *et al.* (1991) reported the diet relationship between female body size and fecundity.

The results of Phoofolo and Obrycki (1997), confirm the fact that female body size may be a good indicator of potential fecundity. This generalization is supported by the results of the present study, wherein the fecundity was higher in *R. fuscipes* reared on *C. cephalonica*, which were heavier as compared to the smaller *R. fuscipes* females reared on *P. solenopsis* (41.2 mg) and *D. cingulatus* (48.7 mg). The nature of the prey species consumed by the predator has a major impact on the fecundity of the predators as stated by Enkegaard *et al.* (1997), Hansen *et al.* (1999), Sahayaraj and Selvaraj (2003), Sahayaraj *et al.* 2004). Our results have a resemblance with the observations of previously mentioned studies. Higher fecundity was observed in weighty females. Thus the higher fecundity of *R. fuscipes* females reared on *C. cephalonica* may be due to the higher primary nutrients in the latter.

Also higher fecundity and higher adult longevity were observed in *C. cephalonica* provided *R. fuscipes* than *D. cingulatus* and *P. solenopsis.* These differences translated into a significantly lower fitness index (r= 0.028) for *D. cingulatus* (r= 0.031) and for *C. cephalonica* (r = 0.037). This also seems to be a reflection of the superior nutritional quality of the larvae. This conclusion concurs with that of Sahayaraj *et al.* (2006b, 2012), who observed high fecundity and higher adult longevity in reduviid predator when reared on lepidopteran larvae. The fact that in the present study larvae of *C. cephalonica* as prey for *R. fuscipes* were found to favor for faster development, longer survival, high fecundity, higher net reproductive rate and shorter population doubling time than *D. cingulatus* and *P. solenopsis* offered predator. This result might make the *C. cephalonica* larvae an ideal candidate for the laboratory rearing of *R. fuscipes* for use in biological control programs. The hatchability of the reduviids eggs were not differentially varied according to the prey offered (Sahayaraj and Paulraj, 2001). Percent hatchability was similar in three categories.

1.5.5. Adult longevity

The longevity of females fed at different prey densities as nymphs and adults did vary. The longest nymphal time observed among the individuals fed with *P. solenopsis* might be due to the feeding stress developed by the size of the prey. Due to its smaller size and its waxy coating on the surfaces the predator was compelled to capture a higher number of prey to satiate itself which in turn developed a feeding stress. Similar type of result was reported by Ambrose and Livingston (1986), Ambrose and Claver (1999), Cohen and Tang (1997). The present study indicates that *C. cephalonica* is the most suitable prey for rearing predator. Because all *R. fuscipes* nymphs and adults used *C. cephalonica* and *D. cingulatus* larvae as efficient prey, indicating that they provide all necessary nutrients in adequate concentration and proportion and have a penetrable integument, texture and *C. cephalonica* odor does not present a barrier against larval feeding (George, 2000). The prey type couldn't affect the hatchability of *R. fuscipes* eggs. Higher percent hatchability was observed in *C. cephalonica* and *D. cingulatus* fed category and a slightly decreased hatchability in *P. solenopsis* reared predator. The same result observed by Sahayaraj *et al.* (2012) revealed that the reduviid predator, *R. longefrons* make characteristic growth and development on *C. cephalonica* larvae, and show maximum fecundity and hatchability.

The life table result of *R. fuscipes* varied with *P. solenopsis* and *D. cingulatus* compared to *C. cephalonica*. The net reproductive rate was higher for *C. cephalonica* reared *R. fuscipes* as compared to *P. solenopsis* and *D. cingulatus* fed *R. fuscipes*. This is in accordance with the results of George *et al.* (2000), who have attributed such finding to the sharp decline in the survivorship value of parent females. However, the net reproductive rate, innate capacity for increase, intrinsic rate of increase and weekly multiplication declined in the case of *Cydnacoris gilvus* (Venkatesan *et al.,* 1997), *R. kumarii* (Claver, 1998), *Cotesia glomeratus* (Linn.) (Singh *et al.,* 2002), *R. marginatus* (Sahayaraj *et al.,*

2004) and *R. fuscipes* (George *et al.,* 2000; Shayaraj *et al.,* 2006a). The values of the intrinsic rate of increase (r), the finite rate of increase (λ) and the population doubling time (DT), which reflect the overall effect of prey type on immature development, survival and fecundity were greater for individuals of *R. fuscipes* fed on larvae of *C. cephalonica*. Higher values for these parameters were due to faster immature development time, lower immature mortality, higher daily rate of progeny production (24/female) and an earlier peak in oviposition (12 days). Results obtained in this study can be useful for further study of population dynamics and the development of management tactics for control of aphid and mealy bug pests, as controlled laboratory studies provide insights into the development and population dynamics of insects.

1.5.6. Bioefficay

The present finding shows that the size of the *R. fuscipes* nymphal instar affects prey selection. For instance, third instar reduviid did not accept adults *D. cingulatus*; this tendency diminished and was lost as predators increased in age. It has also been reported that mean prey preference increases with body size of the predator (Cisneros and Rosenheim, 1997). The result revealed that the particular stage of the predator preferred the desired stage of the pests. Moreover, younger reduviid preferred younger prey and vice versa. Stage preference exhibited by the life stages of *R. fuscipes* could be attributed to the dynamics of prey – predator interaction which is governed by the size of both predator and prey. Our results were similar to the studies carried out with different reduviids predators on cotton stainer, *D. cingulatus* such as *Rhynocoris marginatus* (Ambrose and Kumaraswami, 1990), *Allaeocranum quadrisignatum* (Ambrose and Sahayaraj, 1993), *Ectomocoris tibialis* (Sahayaraj, 1995a; 1997). Reduviids are non-specific predators, but some species are known to exhibit preferences for particular prey when they are simultaneously offered a number of different species. Changes in prey preference

associated with the age of the predator have been well documented among invertebrates, including reduviid (Ambrose, 1999, Sahayaraj, 2007a). The biological control potential of *R. fuscipes* was previously studied against *Heliothis armigera* (Hub.) (Lepidoptera: Noctuidae) (Rao, 1974), Nagarkatti (1982); *Corcyra cephalonica* (Stain.) (Lepidoptera: Pyralidae), *Chilo partellus* (Swinh.), *Achaea janata* L. (Lepidoptera: Noctuidae), *Plutella xylostella* F. (Lepidoptera: Plutellidae), *Spodoptera litura* F. (Lepidoptera: Noctuidae), *Myzus persicae* S. (Hemiptera: Aphididae) (Rao *et al.,* 1981); *Dicladispa armigera* (Oliver) (Lepidoptera: Pterophoridae) (Singh, 1985) ; *Epilachna 12-stigma* Muls. (Coleoptera: Coccinellidae), *E. vigintioctopunctata* Fab. (Coleoptera: Coccinellidae) (Nayar *et al.,* 1976); *Raphidopalpa foveicollis* Lucas (Coleoptera: Chrysomelidae), *Semiothisa pervolagata* Walker, *Diacrisia oblique* Walk. (Lepidoptera: Arctidae) (Singh, 1985); *Terias hecab* L., *Coptosilla pyranthe* (Linn.) (Lepidoptera: Pieridae) (Hiremath and Thontadarya, 1983); *Calocoris angustatus* Leth. (Hemiptera: Miridae), *Cyrtacanthacris succincta* Kirby (Orthoptera: Acrididae), *Dysdercus cingulatus* Dist. (Hemiptera: Pyrrhocoridae), *Earias vittella* F. (Lepidoptera: Noctuidae) (Ambrose, 1988); *E. insulana* Bios. (Lepidoptera: Noctuidae) (Ambrose, 1999). *Rhynocoris fuscipes* is the most abundant reduviid predator in cotton ecosystems (Hafeez *et al.,* 2006; Sahayaraj, 2007; Nurindah, 2008; Kalidas and Sahayaraj, 2012) and reported to feed on 44 insect pests of economical importance (Ambrose and Claver, 1999; Sahayaraj, 2007a). The biology of this predator was carried out by George (2000) on *C. cephalonica* and the life table was constructed by Sahayaraj and Selvaraj (2003). The bioefficacy of this reduviid was evident under laboratory (Ambrose and Livingstone 1986; Ambrose and Livingstone, 1993; Ambrose and Claver, 1995, 1997, Claver and Ambrose 2002, Sahayaraj *et al.,* 2002; Ambrose and Nagarajan, 2010; Nagarajan and Ambrose, 2010) and field conditions (Anand *et al*., 2012), *Diaphania indicus* (Nagarajan and Ambrose, 2012) and *Eutectona machaeralis* (Ambrose *et al.,* 2010).

The results of previous studies revealed that the *R. fuscipes* has high biocontrol potentiality against wide ranges of insect pest. Similarly, in the present study a new emerging pest *P. solenopsis* is also suppressed by the predator. The results obtained from the present study showed that the reduviid predator, *R. fuscipes* has high ability to control the cotton sucking pests such as *D. cingulatus* and *P. solenopsis.*

In this study, *R. fuscipes* presented a Type II functional response (Holling, 1959), similar to several reports on the functional response in *Rhynocoris marginatus* (Ambrose and Kumaraswami 1990, 1993), *Allaeocranum quadrisignatum* (Ambrose and Sahayaraj, 1993), *Ectomocoris tibialis* (Sahayaraj, 1995a, 1997), *Coranus nodulus* (Sahayaraj, 1993), *Neohamatorhhophus therasii* (Sahayaraj, 1996), *Antilochus coqueberti* (Kohno *et al.,* 2004) and *R. fuscipes* (Ambrose and Nagarajan, 2010). The proportion of red cotton bug and cotton mealy bug preyed by the reduviid life stages increased at a decreasing rate in case of *D. cingulatus* and *P. solenopsis* densities, and the proportion of preys fed was not constant (Table 9 and 10). The Type II functional response was more evident at 24 hrs (Fig. 6), possibly because they are voracious feeders, a plateau or predator satiation was not reached at 24 hr with 10 and 16 red cotton bug and cotton mealy bug density respectively.

The present study showed that the type of functional response shown and the magnitude of predation by *R. fuscipes* against *D. cingulatus* and *P. solenopsis* could be adversely affected by ageing. The maximum prey consumption was observed in adult reduviids rather than nymphal instars evaluated. *Rhynocoris fuscipes* exhibited Type II responses at various ages tested, and the magnitudes of functional response in adults were significantly greater than nymphal life stages, because the adult predator consumed higher number of preys in each prey density, so the predatory rate was higher in adult *R. fuscipes*. This could be attributed to the fact that reproductive preparation started at adult stage and their ability to respond to increasing prey density indicated that physiological senescence in

adults was faster than in nymphs (Ambrose, 1999; Rocha and Redaelli, 2004; Sahayaraj, 2007a). Accelerating functional response, which are more typical of generalist than specialist predator, can result in density dependent predation (Murdoch and Qaten, 1975). The statement was directly related to the results obtained from the present study. The predatory rate of *R. fuscipes* on cotton pests was directly proportional to the density of *D. cingulatus* and *P. solenopsis*. The predation increased, when the prey density increased.

The handling time is a feature of predatory behavior, and influences the functional response by decreasing the search-and-attack rates as prey density increases (Nordlund and Morrison, 1990). The handling time of *R. fuscipes* against both prey species decreased over time and the attack coefficient increased at 24 hrs (Table 9 and 10). It is important to observe the handling time of the predator up on a prey, because it gives the time taken to paralyze or kill and utilize of the prey. Nordlund and Morrison (1990) observed that the handling time affect the type of functional response: the shorter it is, the faster the curve reaches the asymptote. Our results are in agreement with the observations of Ambrose and Claver (1997). They observed the efficacy of *R. fuscipes* against *S. litura* and reported that handling time decreased while prey density increased. Besides, handling time can influence other components such as attack ratio and search efficiency (Beddington, 1975).

Another important component of functional response is the attack ratio. The maximum attack ratio was observed in prey density 1 and minimum at the higher density might be due to lesser time required to find each prey and more time spent in non-searching activities at higher densities (Ambrose *et al.,* 1990; Claver and Ambrose, 2002). The results obtained from the present study is similar to the observations of Nagarajan and Ambrose (2010). They recorded the functional response of *R. fuscipes* against *Diaphania indicus* (Saun.) and *Eutectona machaeralis* (Walker). The reduviid showed maximum prey

consumption at higher density. These results might be due to the predators taking time to acclimate to the new experimental arena.

Rhynocoris fuscipes consumed more number of *P. solenopsis* (k) than *D. cingulatus,* although the calculated handling time was almost the same (b= 0.03). However, laboratory measure of the functional response provides a biased perspective on the effectiveness of predators in the field because, in laboratory trials, the prey density is artificially high with low search requirements and an absence of alternative prey (O'Neil, 1997). Field trials of functional response are necessary to understand the predatory level of *R. fuscipes* and its effectiveness as a biological control agent.

1.6. CONCLUSION

Corcyra cephalonica larvae can be used for the laboratory rearing of *R. fuscipes* in a small scale. The results confirmed that *P. solenopsis* is a lower quality feed than *D. cingulatus* and *C. cephalonica*. The nymphs fed with the former prey took significantly longer period to develop into adults (50 days), body weight was less (40.4 mg) and laid minimum eggs (22 eggs/female). These differences translated into a significantly lower fitness index (r= 0.028) for *D. cingulatus* (r= 0.031) and for *C. cephalonica* (r = 0.037). In general, *R. fuscipes* fed on *P. solenopsis* had extended developmental time but with low reproductive performance. The life stages of *R. fuscipes* preferred different stages of the pests tested. The results also suggest that both fifth instar and adult predators were more successful in encountering the large sized *D. cingulatus* and *P. solenopsis*. In the order of the stage of the predator, they invariably preferred *D. cingulatus* than *P. solenopsis.* The feeding efficacy of the predators was increased while the prey density increased. This exhibits a typical type II model holling's disc equation. The feeding efficacy was positively correlated with prey density but the attack ratio was higher in low prey densities. This experiment clearly revealed that different stages of the predator can be utilized in pest

management program. The laboratory results presented in this study suggest that *R. fuscipes* could be considered as a prospective candidate for use as a commercial biological control agent for cotton mealy bug and red cotton bug in India.

CHAPTER 2

Adult group rearing and augmentative release of *Rhynocoris fuscipes*

2.1. ABSTRACT

Adult group rearing in Micro Environmental Cage (MEC) and biocontrol potential of *Rhynocoris fuscipes* Fab. (Hemiptera: Reduviidae) was carried out under pot and field conditions. MEC was prepared and the natural conditions provided to the predator with natural food such as lepidopteran larvae and hemipteran nymphs along with artificial diet. Higher fecundity and adult longevity were recorded when the predator was reared in MEC. The reduviid predators preferred stones and fallen leaves as their hiding places than other objectives in the natural microplot condition. During morning hrs, the predator showed higher biocontrol potential against *Dysdercus cingulatus* Fab. (Hemiptera: Pyrrhocoridae) and *Phenacoccus solenopsis* Tinsely (Hemiptera: Pseudococcidae) under pot condition. Adult predator consumed more number of *P. solenopsis* (2.8) than *D. cingulatus* (1.5) during morning hrs. Under field condition, *R. fuscipes* also highly reduced *D. cingulatus* (53.2%) population rather than *P. solenopsis* (51.2%), *Helicoverpa armigera* (50.0%) and *Aphis gossypii* (4.3%). However, the reduviid predator did not affect the other natural enemies' dwelling in the cotton agrosystem. Cost Benefit Ratio (CBR) was higher in chemical pesticides sprayed fields (1.72) than *R. fuscipes* released field (1.32) and control field (1.17). The Percent Avoidable Loss (PAL) was higher in chemical pesticide sprayed field (0.276) than reduviid released field (0.185). The results obtained from the present study reveals that *R. fuscipes* can mass produce under laboratory condition in MEC.

Key words: Augmentative release, biocontrol potential, field evaluation, mass production, Micro Environmental Cage, pot study.

2.2. INTRODUCTION

Relying on insects that prey on crop pests has a drawback. By the time the predator numbers become large enough to do a high control potential, the pests have already been started taking an economic toll on the crop. But it has been expensive to rear massive number of predators in captivity for release into field at the most invented time (Grundy, 2007). Mass production and rearing of biocontrol agents have been the current topic of research for applied entomologists, due to its application in the field of agriculture and plant protection; switching focus on the mass production of insects needs pointed interests. De Bach and Rosen (1991) described that the biological control is the action of parasitoids, predators and pathogens in maintaining other organisms' density at a lower level than would occur in the absence. The use of predatory insects in the field of agriculture has been studied for more than six decades, but there is no possible way to compete with pesticides and other pest control strategies. For successful introduction of the predator as a component of Biointensive Integrated Pest Management (BIPM) is the lack of mass rearing of the insects at the much needed time. Lakkundi (1989), states that non-availability of reduviid predators in large numbers is the main impediment for the practical use of reduviid predators in augmentative biological control program of the insect pests. Hence, previously "larval card method" was developed for the mass production of reduviid predators (Lakkundi, 1989; Sahayaraj, 2002), but the method needs high cost, more man power, additional rearing of factitious host, etc.. Later another technique namely, "artificial diet" was introduced diet for the mass production of reduviid predators by Sahayaraj *et al.* (2006b, 2007a), Sahayaraj and Balasubramanian (2009), this method also having the constraints as high cost, more man power and the diet should keep in aseptic condition and it can be used only a few days after preparation. So this

technique is applicable for limited rearing. Previously Ambrose and Claver (1999) studied the impact of different substrates such as untreated plastic, tissue and glutting papers, sand and stone, litter and strip and green leaves and stem for the mass production of *R. marginatus.* The stadial periods of nymphal instars of *R. marginatus* reared on dry litter with strip and green leaves with stem were significantly reduced over plastic, tissue and glutting papers, and sand with stone substrata. It helps in mass production of reduviids with a low cost, but with difficulty to provide such provisions in the entire field where the augmentation occurred. General statement of Grundy (2007) is that the biological control agents should be produced in cost effective manner and should have the ability to suppress a wide range of pests. Only then could it be utilized for the augmentative release in various crops.

Biological control by predators such as assassin bugs helps in the regulation of insect pest population in Biointensive Integrated Pest Management (BIPM). Reduviids are zoophagous predator of insect pests (Sahayaraj, 2002; Grundy, 2004; Kalsi and Seal, 2011; Sahayaraj *et al.,* 2012; Alain *et al.,* 2012; Nagarajan and Ambrose, 2010; Hwang and Weirauch, 2012) and played a major role in suppressing the pest population in India (Sahayaraj, 2007a, 2012; Nagarajan and Ambrose, 2012; Kalidas and Sahayaraj, 2012; Kailash Chandra *et al.,* 2012; Sujatha *et al.,* 2012; Gopinathan, 2012) and they could be utilized as a biological control agent, where a variety of pests occured (Schaefer, 1988; Lakkundi, 1989; Ambrose, 1999; Sahayaraj, 2002; Sahayaraj and Martin, 2003; Sahayaraj, 2007a; Sahayaraj, 2012; Kailash Chandra *et al.,* 2012).

Rhynocoris fuscipes Fab. (Hemiptera: Reduviidae) is an effective biological control agent of cotton pests (Ambrose and Claver, 1995, 1997; George *et al*., 2000; Sahayaraj and Selvaraj, 2003, Sahayaraj, 2007a; Ambrose and Nagarajan, 2010; Sujatha *et al.,* 2012) and

groundnut pests (Prasanna and Shirely, 2002; Sahayaraj *et al*., 2004; Claver and Ambrose, 2003a). *Rhynocoris fuscipes* is an entomophagous insect scattered in many agroecosystems and feeds on more than 44 economically important insect pests. (Sahayaraj, 2007a; Ambrose, 1999; Nagarajan and Ambrose, 2010; Sahayaraj and Sujatha, 2012). The biocontrol potential of *R. fuscipes* as a biological control agent under laboratory (Ambrose and Claver, 1997; Sahayaraj *et al.,* 2006a; Ambrose and Nagarajan, 2010; Sujatha *et al.,* 2012) and field cage (Claver and Ambrose, 2002; Claver and Ambrose, 2003b; Anand *et al*., 2012) conditions has been described previously.

2.2.1. Hiding behavior

Under field condition the predatory insect exhibits a variety of adaptations. One of the important adaptations is the hiding behavior either to escape from the natural enemies or searching the prey or sheltering purpose. Generally the hiding behavior is referred to as antipredator behavior (say Isabelle Coolen *et al*., 2005; Sahayaraj, 2007a). Despite the commonness of hiding as an antipredator tactic, it has not been studied extensively by behavioral ecologists on any predatory insects.

2.2.2. Biocontrol Potential

2.2.2.1. Pot condition

Biological control through predators and other natural enemies are gaining interest as a suit of alternative control measures of pesticides. Reduviids are profuse, they occur world wide, and they are voracious predators and most of them are generalist predators (Ambrose, 1999; Sahayaraj, 2012). Being larger than many other predacious land bugs and encompassing in their development a great range of size, reduviid predators consume not only more prey but also a wide array of preys (Schaefer, 1988; Ambrose, 2003; Sahayaraj, 2007a;

Michael and Christiane, 2012; Sujatha *et al.,* 2012). Because they are polyphagous, reduviids may not be useful as predators on specific pests, but they are valuable predators in situations where a variety of insect pests occur. It is important to study the biocontrol potential of predatory insects under pot condition, because it is the preliminary assessment about the biocontrol potential of the predator for the utilization in augmentation in field conditions. Much less is known about the biocontrol potential of generalist predators under pot condition.

2.2.2.2. Field evaluation

It is a popular biological control approach amongst professional and progressive farmers, and has been stimulated by the current international attitudes regarding reduction of pesticide use. Augmentative biological control practice has been in vogue worldwide and more than 150 species of natural enemies are now commercially available (Ricardo and Pratissoli, 2009). However, generalist predators, particularly predatory bugs, have been largely ignored for augmentation in cotton pest management (King and Powell, 1992; Goldstein and Whalen, 1993; Kehrli and Wyss, 2001; Prabhakar and Roy, 2010; Bayoumy, 2011; Jalalizand *et al.,* 2011; Reddy and Rosalie, 2011; Sushilkumar and Ray, 2011; Venkatesha and Dinesh, 2012). Field testing is an important step in evaluating the use of natural enemies as augmented biological control agents (Cloutier and Bauduin, 1995; Grundy, 2004; Sahayaraj and Ravi, 2007).

Previously reduviids like *Platymeris longicollis* Distant (Antony *et al.,* 1979), *R. marginatus* Fab. (Ambrose and Claver, 1999; Sahayaraj, 1999; Sahayaraj and Martin, 2003; Sahayaraj and Ravi, 2007; Balasubramanian, 2008), *Pristhisancus plagipennis* Walker (Grundy and Maelzer, 2000a,b; Grundy, 2004), *R. kumarii* Ambrose and Livingstone (Claver and Ambrose, 2001a), were released augmentatively and their biological control potential in

various agro-ecosystems evaluated. There is no report available on the augmentative release of *R. fuscipes* in the field condition; hence we propose the study of biocontrol potential of *R. fuscipes* against the cotton pest under field condition with the following objectives,

Objectives

1. *Rhynocoris fuscipes* adults group rearing using Micro Environmental Cage (MEC) method and artificial diet.
2. To record the hiding behavior of *R. fuscipes* under field cage at different time intervals.
3. To evaluate the biocontrol potential of *R. fuscipes* against *Dysdercus cingulatus* and *Phenacoccus solenopsis* under pot condition.
4. To evaluate the biocontrol potential of *R. fuscipes* against cotton pests under field condition (@ 4500/Acre); calculate the cost benefit ratio (CBR) and percent avoidable loss (PAL).

2.3. MATERIALS AND METHODS

2.3.1. Adult group rearing

2.3.1.1. Micro Environmental Cage (MEC)

The reduviid predator, *Rhynocoris fuscipes* was mass reared under laboratory condition (27±2^{0}C, 66±4 RH (%), 11L: 13D) using a Micro Environmental Cage (MEC) method (Plate 1). The MEC was developed using card board box (24 cm height, 30 cm width, 44 cm length, 0.3 mm thickness). The cage window was covered with nylon net (1 mm) for easy aeration. Two pairs of windows (18 cm height x 16 cm width) were made on the lateral sides and one on the roof (20 cm length x 17 cm width) of the cage as shown in plate 1. Furthermore, an opening window of 10 cm length and 12 cm width was also made on the roof

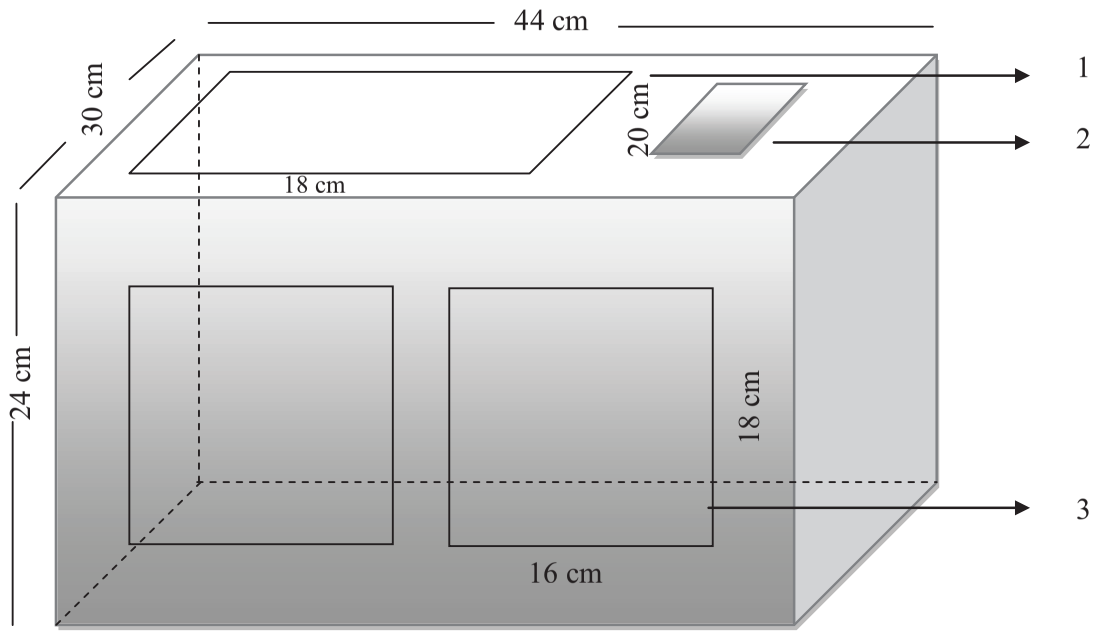


Plate 1. Micro Environmental Cage (MEC) for the rearing of reduviid predator

1. Upper window; 2. Opening; 3. Side window

with a lid. Provision was provided to lift and close the window. The edges of the MEC were tightly sealed with cello tap and /or paper. Three zig-zag folded thick board (10 cm length x 5 cm width x 0.1 mm thickness), five small stones (7 cm length x 5 cm width x 3 cm height) and 3 or 4 dry twigs of 20 cm with branches were placed over the floor of MEC. Care was taken for placing the concave side of the stone facing the earth to facilitate oviposition and hiding nature of reduviid. Natural prey such as *D. cingulatus* nymphs (20 number with a mixture of third, fourth and fifth instars) and five each fourth and fifth instars of *S. litura, A. janata, H. armigera* (once in a time) along with 10 ml of meat based artificial diet provided through small cotton balls (20 mg) (Sahayaraj *et al*., 2007) were supplied to the reduviid predator once in five days. Previously the nymphs of *R. fuscipes* were reared in plastic containers (15 cm diameter X 8 cm height) (10 number per container). Thirty newly hatched predators were maintained in each replication with the sex ratio of 0.45:1 – 0.5:1 (female: male) and three replications were maintained. After the preoviposition period, the observations were made every day for fecundity on the cage sides and under the pebbles. The cage was cleaned and new preys were supplied twice a week. Adult longevity, fecundity, hatchability and oviposition periods of the reduviids were recorded.

2.3.1.2. Group rearing of *R. fuscipes* using artificial diet

In another experiment, adults of *R. fuscipes* were provided with an artificial diet prepared according to the method of Sahayaraj and Balasubramaniam (2009). Thirty insects with the sex ratio of 0.5: 1 (Female: Male) were maintained. The diet was supplied to the insects using small cotton bolls (20 mg). Every 24 hrs the dried cotton bolls were replaced with fresh diet swapped cotton bolls. Fecundity, hatchability, adult longevity, oviposition period and oviposition index were recorded.

2.3.2. Hiding behavior of the predator under Screen house

The relevance of this study was aimed at recording the hiding places of the predator when it was released to cotton field under augmentative pest management programme. The hiding behavior of *R. fuscipes* was carried out under screen house (36 x 21.5 sq. ft.) in 30 day old cotton plant (SVPR II). Two plots were maintained (10 x 4 m) with 52 plants each at spacing of 35 x 75 cm and oriented at east and west. Ten 24 hour pre-starved fourth stadium predators were released at 6.00 am, 8.30, 11.00 am and 1.30 pm separately using Camlin brush (2 mm) in the north east corner on to the ground level. After every two hrs (8 am, 10. 30 am, 1 pm and 3. 30 pm), number of insects settled/ dwelt/ hid at the base of the cotton plant, below the fallen leaves, below the small pebbles naturally present in the micro-field and other objects (if any) were recorded. Similar procedure was followed for fifth stadium and adult (male and female) of *R. fuscipes.* Immediately after each experiment the insects were collected back.

2.3.3. Bioefficacy under pot condition

Bioefficacy of *R. fuscipes* [third, fourth and fifth stadium nymphs and adult (male and female)] reared with mixture of its factitious host *C. cephalonica* as well as natural hosts (*D. cingulatus*) were carried out under pot condition (Plate 2) against *P. solenopsis* and *D. cingulatus.* The experiments were carried out in the cement pots (36 cm upper diameter, 30 cm lower diameter, 22 cm height) using 30 day old healthy cotton plants (SVPR II) which was covered with nylon net as shown in plate 2. They were maintained inside the screen house (36 x 21.5 sq ft) of Crop Protection Research Centre, St. Xavier's College, Palayamkottai. Two sets of experiments were carried out; one in day hrs between 7 am and 6 pm and another during night hrs between 7 pm and 6 am. The preferred life stages of

Plate 2. Bioefficacy evaluation of *R. longifrons* under pot condition

D. cingulatus (third, fourth, and fifth nymphal instars) and *P. solenopsis* (adult) were released on to the tender parts of the plant (6 am) at optimum densities of 3preys/predator and 5 preys/predator respectively. The released predators were allowed to acclimatize for 1 hour. Then 24 hrs pre-starved predator life stages (2 predator/ plant) were released individually on to the tender leaves using Camlin brush (2 mm). After 12 hrs (6 pm) the number of preys consumed by two predators was recorded and the predatory rate (number of preys killed by a predator) was calculated. Ten replications were maintained for third, fourth and fifth instar nymphs and adult (male and female). In the second set of experiment, the pests and predators were released at 7 pm and the number of preys consumed was recorded after 12 hrs i.e., next morning at 6 am. The predatory rate was calculated as mentioned previously.

2.3.4. Augmentative release under cotton field

Rhynocoris fuscipes were augmentatively released to the farmers cotton (SVPR 4) field within a small plot size of 150 x 15 sq. ft. cultivated in irrigated condition at Kothankulam (N 9^0 47' 41" and E 77^0 57' 28"), Virudhunagar district, Tamil Nadu, India. The experiments were conducted during 2011 *Kharif* (July to September) season. The pesticides free cotton field was arranged in a randomized complete block design (RBD) and divided into five plots (15 x 30 sq. ft.). Likewise the control field and chemical pesticide (Monocrotophos, DDT) sprayed fields were divided in the same manner. A pre-count of the predominant pest and predator populations were recorded two days before the releasing of predators at 7 am from 10 randomly selected plants from each sub-plot. Uppermost 10–15 foliage was considered for counting. First, second, third, fourth and fifth nymphal instars of *R. fuscipes* (fifty numbers each) were released on to the cotton foliage on 40^{th}, 55^{th} and 70^{th} day after the seedling emergence (DASE). In addition, two egg masses, approximately with 50 eggs

(hereafter called egg card) were also tied on the twigs of the cotton plant. In all 900 (4500/Acre) *R. fuscipes* life stages were released including 150 eggs during the study period. The predator nymphs were transported to the cotton field within the plastic containers (15 cm diameter x 8 cm height) having three zig-zag folded papers (10 cm length x 5 cm width) inside. Nymphs for each replication were released separately on the terminal shoots of the cotton crop foliage using a Camel brush (2 mm) in the morning hrs (7.00 am). The post- count was made with the interval of three days after each predator was released. The growing points and squares of upper two-thirds of the growing ten randomly selected ten cotton plants were searched in each replication for larvae, nymphs and adult of most predominant pests like *D. cingulatus, P. solenopsis* and *A. gossypii.* Flowers and balls throughout the cotton were also inspected for larvae of *H. armigera*. The number of reduviid predators, big black ants, coccinellids, wasps and spiders were recorded two days before and after the release of *R. fuscipes* expressed in number of predator/ plant.

2.3.5. Cost Benefit Ratio (CBR) and Percent Avoidable Loss (PAL)

At the completion of the cotton growing season (September - October), the raw cotton in each treatment replicate was harvested periodically. The harvested cotton was cleaned, weighed and sold (kg/ha) in the local market. The Cost Benefit Ratio (CBR) was calculated according to Kallyanasundaram *et al.* (2004) methodology. Furthermore the Percent Avoidable Loss (PAL) was also calculated using the formula of Krishnaiah (1980),

Cost Benefit Ratio (**CBR**) = Total gain/Total cost of cultivation

Percent Avoidable Loss (**PAL**) = Mean yield from protected field - Mean yield from unprotected field/ Mean yield from protected field

2.3.6. Statistical analysis

Individual data of oviposition, oviposition periods, adult longevity and hatchability of *R. fuscipes* reared in micro environmental cage (MEC) were compared using ANOVA and post-ANOVA, Tukey test with factitious host as fixed variables. The prey consumption of *R. fuscipes* during morning and evening time in pot study and the pest and predator population in cotton field in augmentative release were analyzed using ANOVA and post ANOVA, Tukey test. All analyses were performed using the SPSS statistical software (Version 20.0) and all statistical tests were carried out at 5% level of significance ($\alpha = 0.05$).

2.4. RESULT

2.4.1. Group rearing of adult reduviid predator

2.4.1.1. Micro Environmental Cage

The reduviid predator can adapt and survive in the laboratory condition at an optimum level. The result from the present study reveals that the MEC method significantly affects the adult biology of *Rhynocoris fuscipes.* It provides sufficient prey and niche for the predator and also it increases the chances for copulation within the predator. The fecundity (F=1.249, df=14, 9, P=0.377) and hatchability (F=0.634, df=8, 15, P=0.738) of *R. fuscipes* were recorded higher in MEC maintained reduviid, but it is did not significantly differ from laboratory rearing method. *Rhynocoris fuscipes* adults lived longer in MEC method (df=9, 13, F=1.337, P=0.047 and df=8, 15, F=3.433, P=0.019 for male and female, respectively). The mean length of oviposition periods was moderately high in MEC reared *R. fuscipes* (df=12, 11, F=0.695, P=0.729) (Table 12).

Table 12. Group rearing of *R. fuscipes* adults under Micro Environmental Cage (MEC) method and with artificial diet.

Parameters	MEC	Artificial diet
Sex ratio	1:0.45	1:0.45
Fecundity (No./female)	46.9±0.05[ab]	38.2±0.24[b]
Hatchability (%)	98.8±0.03[ab]	93.7±0.06[ab]
Male longevity (days)	49.8±0.33[a]	34.5±0.21[b]
Female longevity (days)	53.7±0.40[a]	42.5±0.04[b]
Oviposition period (days)	22.3±0.43[ab]	16.5±0.04[ab]
Oviposition index	0.088[ab]	0.076[ab]

Mean followed by the same letters in column do not differ significantly by TMRT at 5% level

2.4.1.2. Artificial diet

Higher fecundity was recorded for *R. fuscipes* when rearing with artificial diet (df=9, 14, F=2.260, P=0.083) when comparing with predators reared on *C. cephalonica*. Hatchability was lower (F=1.502, df=11, 12, P=0.247) and both the male (F=2.873, df=9, 14, P=0.041) and the female (df=11, 12, F=4.092, P=0.011) longevity were significantly reduced (for male and for female). Oviposition period was shorter (df=2, 21, F=0.445, P=0.895) compared with MEC method (Table 12).

2.4.2. Hiding behavior

The hiding behavior of *R. fuscipes* reveals that the percentage of animal hiding under pebbles was higher between 6.00 am and 10.00 am. When day time increases the predator moves under plants for hiding. Fourth and fifth instar predators preferred to hide under plants during late hrs (3.30 pm), but the adult predators hid under plants immediately after release and later moved towards the stones and fallen leaves for hiding. The selection of other objectives for hiding purpose by the predator was very much limited, because they were searching for the prey during morning hrs on the plants and later hid under the stones and fallen leaves. All the stages of the predator were found on plants almost throughout the experimental period (Fig. 8).

2.4.3. Bioefficacy evaluation under pot condition

Rhynocoris fuscipes adult consumed more number of *P. solenopsis* (F=2.551, df=2, 21, P=0.012) and *D. cingulatus* (F=2.296, df=2, 21, P=0.047) during morning hrs than in evening hrs (F=3.231, df=2, 21, P=0.043 for *P. solenopsis* and F=4.645, df=1, 22, P=0.042 for *D. cingulatus*). Between these two preys, *R. fuscipes* consumed more number of *P. solenopsis* than *D. cingulatus.* Higher *D. cingulatus* consumption was observed during morning hrs by

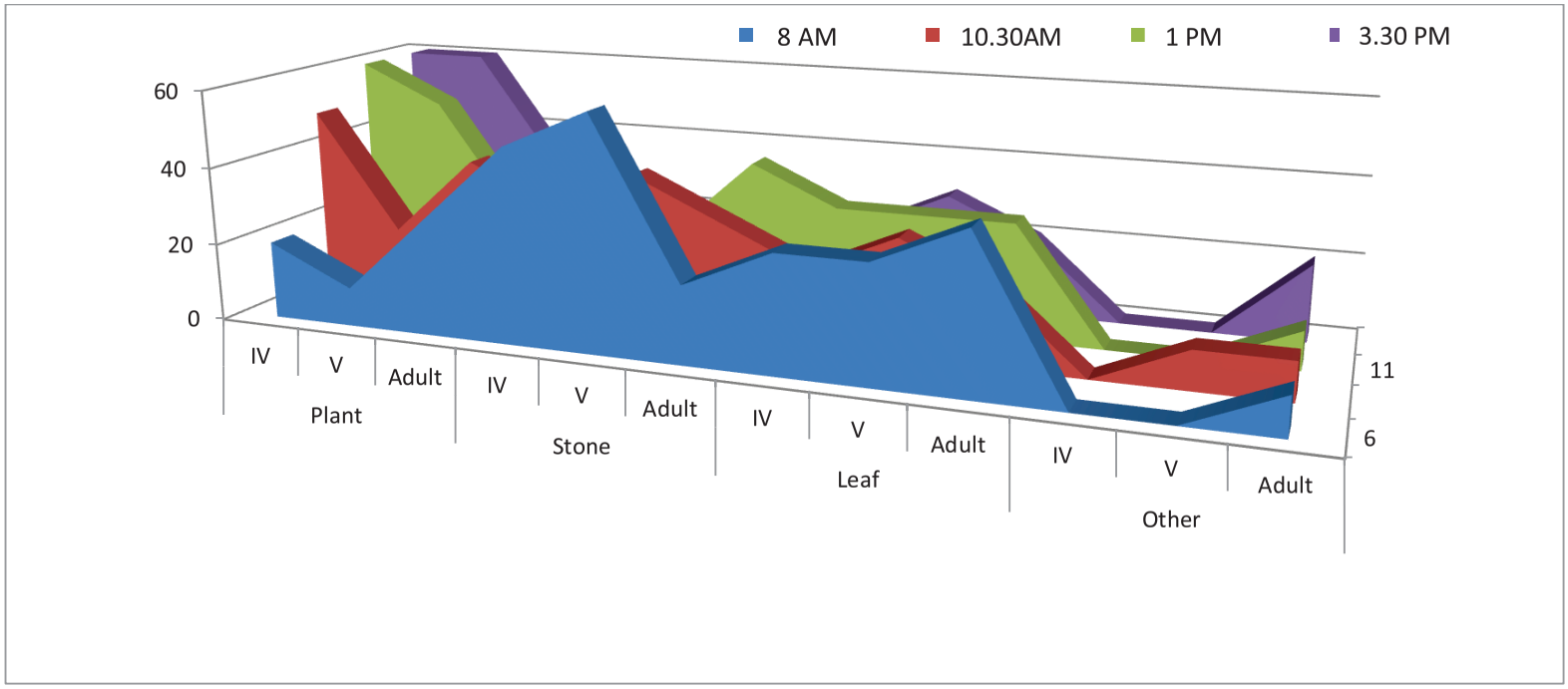


Figure 8. Hiding behavior (%) of *R. fuscipes* fourth and fifth nymphal instars and adult (male and female) released from morning 6.00 AM to evening 3.30 PM and observed their hiding after 2 hours interval under screen house condition.

third (F=6.667, df=1, 22, P=0.017), fourth (F=3.621, df=2, 21, P=0.045) and fifth (F=0.496, df=1, 22, P=0.489) instars of *R. fuscipes* than evening hrs, but statistically more significant consumption was observed during evening hrs (F=3.933, df=2, 21, P=0.035; F=8.229, df=1, 22, P=0.009 and F=3.184, df=2, 21, P=0.062 for third, fourth and fifth instars respectively). Similarly, *R. fuscipes* consumed more number of *P. solenopsis* during morning hrs (F=0.435, df=1, 22, P=0.517; F=1.771, df=2, 21, P=0.195 and F=0.522, df=2, 21, P=0.601) for third, fourth and fifth instars, respectively than evening hrs (F=1.955, df=2, 21, P=0.166; F=1.127, df=2, 21, P=0.343 and F=0.740, df=2, 21, P=0.740) (Table 13).

2.4.4. Augmentative release of *Rhynocoris fuscipes*

2.4.4.1. Pest population

Bioefficacy of *R. fuscipes* was evaluated in cotton field from May 2011 to August 2011 cultivated under irrigated condition. The most abundant pest insects were *A. gossypii* (96.3%), *P. solenopsis* (5.23%), *D. cingulatus* (0.30%), and *H. armigera* (0.02%). Hence we mainly concentrated on these pests throughout our observations. After third release of *R. fuscipes* the mean number of pest population reduction indicated that the chemical pesticide spray reduced the population of *A. gossypii* (F=1.013, df=167, 432, P=0.453) and it was minimum in *R. fuscipes* released field (F=1.137, df=167, 432, P=0.153) with respect to control field. During the first counting, the population of *A. gossypii* (F=1.746, df=118, 81, P=0.004), *D. cingulatus* (F=4.431, df=4, 195, P=0.002) and *P. solenopsis* (F=1.735, df=11, 188, P=0.069) was high. Later these pest population was reduced in second [*A. gossypii* (F=1.746, df=118, 81, P=0.004), *D. cingulatus* (F=4.431, df=4, 195, P=0.002) and *P. solenopsis* (F=1.735, df=11, 188, P=0.069)] and third [*A. gossypii* (F=1.746, df=118, 81, P=0.004), *D. cingulatus* (F=4.431, df=4, 195, P=0.002) and *P. solenopsis* (F=1.735, df=11,

Table 13. Total number of prey consumption and predatory rate (no. prey consumed/predator/day) of *R. fuscipes* against *D. cingulatus* third and fourth instar nymphs (in equal proportion) and *P. solenopsis* (adult) on cotton (SVPR II) cultured under pot condition and maintained in screen house.

Predator life stages	Predator released during evening hours		Predator released during morning hours	
	Total prey consumed	predatory rate	Total prey consumed	predatory rate
	D. cingulatus			
III	0.70±0.12 ^{acd}	0.11±0.02 ^{acd}	1.37±0.10 ^{acd}	0.20±0.04 ^{acd}
IV	0.66±0.09 ^{bcd}	0.11±0.01 ^{bcd}	1.25±0.12 ^{bcd}	0.20±0.03 ^{bcd}
V	0.66±0.09 ^{abcd}	0.12±0.01 ^{abcd}	1.50±0.12 ^{abcd}	0.20±0.02 ^{abcd}
Adult	1.04±0.12 ^{bcd}	0.12±0.01 ^{bcd}	1.54±0.12 ^{bcd}	0.23±0.04 ^{bcd}
	P. solenopsis			
III	2.30±0.33 ^{abcd}	0.75±0.12 ^{abcd}	2.60±0.41 ^{abcd}	1.00±0.09 ^{abcd}
IV	1.80±0.03 ^{abcd}	1.00±0.09 ^{abcd}	2.80±0.33 ^{abcd}	0.75±0.14 ^{abcd}
V	2.10±0.33 ^{abcd}	0.66±0.10 ^{abcd}	2.40±0.44 ^{abcd}	0.92±0.21 ^{abcd}
Adult	2.40±0.18 ^{bcd}	0.48±0.23 ^{bcd}	2.80±0.19 ^{bcd}	0.83±0.13 ^{bcd}

Mean followed by the same letters in column do not differ significantly by TMRT at 5% level

188, P=0.069)] counting. However *R. fuscipes* reduced *D. cingulatus* (F=5.297, df=9, 590, P=0.005) more than chemical pesticides spray did (F=0.246, df=9, 590, P=0.987). Similar observations were also recorded for *P. solenopsis* for pesticides (F=1.891, df=32, 567, P=0.003) and predator released (F=1.288, df=32, 567, P=0.136) fields (Table 14).

2.4.4.2. Pest population reduction

Chemical pesticides more effectively control *A. gossypii* population (F=1.013; df=167, 432; P = 0.453) than the *R. fuscipes* (F=1.137; df=167, 432; P=0.153). Moreover, the population reduction of *D. cingulatus* (F=0.246; df=9, 590; P = 0.987, F=5.297; df=5, 590; P = 0.005), *P. solenopsis* (F=1.891; df=32, 567; P = 0.003, F=3.351; df=44, 555; P = 0.005) and *H. armigera* (P > 0.005) was more or less similar in chemical pesticides sprayed field and *R. fuscipes* released field, respectively (Fig. 9).

2.4.4.3. Natural enemies population

Ants, coccinellids, reduviids, spiders and wasps were observed in the cotton fields. These natural enemies population slightly increases during the experimental periods in control field and also in *R. fuscipes* released field. The population of ants increased in reduviids released field (F=5.292; df=6, 593; P = 0.005) when compared to control field. The same result was observed for coccinellids in reduviid released field (F=7.191; df=3.596; P=0.005) and in chemical pesticide field (F=0.637; df=3, 596; P = 0.592) but in the chemical pesticide field the population of ants remained constant (F=0.782; df=6, 593; P = 0.584). Very low level of reduviid population was observed in chemical released field (F=29.776; df=1, 598; P = 0.005) rather than in the control field (F=29.776; df=1, 598; P = 0.005). More or less similar observations were made for the population of spider (F=2.962; df=3, 596; P = 0.032) and wasp (F=7.994; df=3, 596; P = 0.005) too (Table 15).

Table 14. Augmentative release of *R. fuscipes* life stages (egg to adult), pesticide spray (monocrotophos) and control field on pest population (number of pest/plant±S.E.) under irrigated cotton (SVPR IV) field condition during June to August 2011 (*Kharif*).

No. of release	Field category	Pest(s)			
		D. cingulatus	***A. gossypii***	***P. solenopsis***	***H. armigera***
First release	**Control**	0.90±0.14 ^{ac}	107.4±4.59 ^{abc}	5.0±0.64 ^{ac}	0.0±0.0 ^{abc}
	R. fuscipes	0.40±0.07 ^{abc}	86.3±8.94 ^{bc}	1.0±0.17 ^{abc}	0.0±0.0 ^{abc}
	Pesticide field	0.37±0.08 ^{abc}	80.8±3.75 ^{abc}	1.0±0.17 ^{abc}	0.0±0.0 ^{abc}
Second release	**Control**	0.14±0.03 ^{abc}	100.3±4.64 ^{abc}	0.92±0.18 ^{abc}	0.01±0.007 ^{abc}
	R. fuscipes	0.07±0.01 ^{bc}	67.8±2.88 ^{abc}	0.46±0.09 ^{ab}	0.0±0.0 ^{abc}
	Pesticide field	0.07±0.02 ^{abc}	78.8±2.71 ^{abc}	4.42±0.63 ^{c}	0.0±0.0 ^{abc}
Third release	**Control**	0.13±0.03 ^{abc}	82.5±2.68 ^{abc}	5.79±1.13 ^{ac}	0.0±0.0 ^{abc}
	R. fuscipes	0.35±0.01 ^{abc}	53.9±2.20 ^{abc}	1.33±0.28 ^{ab}	0.0±0.0 ^{abc}
	Pesticide field	0.27±0.08 ^{abc}	62.3±3.06 ^{abc}	0.98±0.16 ^{abc}	0.0±0.0 ^{abc}
Total mean population	**Control**	0.30±0.04 ^{ac}	96.3±2.36 ^{abc}	5.23±0.43 ^{ac}	0.003±0.002 ^{abc}
	R. fuscipes	0.24±0.04 ^{bc}	74.02±1.88 ^{abc}	3.20±0.42 ^{ab}	0.00±0.00 ^{abc}
	Pesticide field	0.17±0.03 ^{abc}	69.35±1.86 ^{abc}	0.96±0.12 ^{c}	0.00±0.00 ^{abc}

Mean followed by the same letters in column do not differ significantly by TMRT at 5% level

Figure 9. Augmentative release of *R. fuscipes* (RF) life stages (egg to adult) on pest population (%) reduction under irrigated cotton (SVPR IV) field condition during June to August 2011 (*Kharif*).

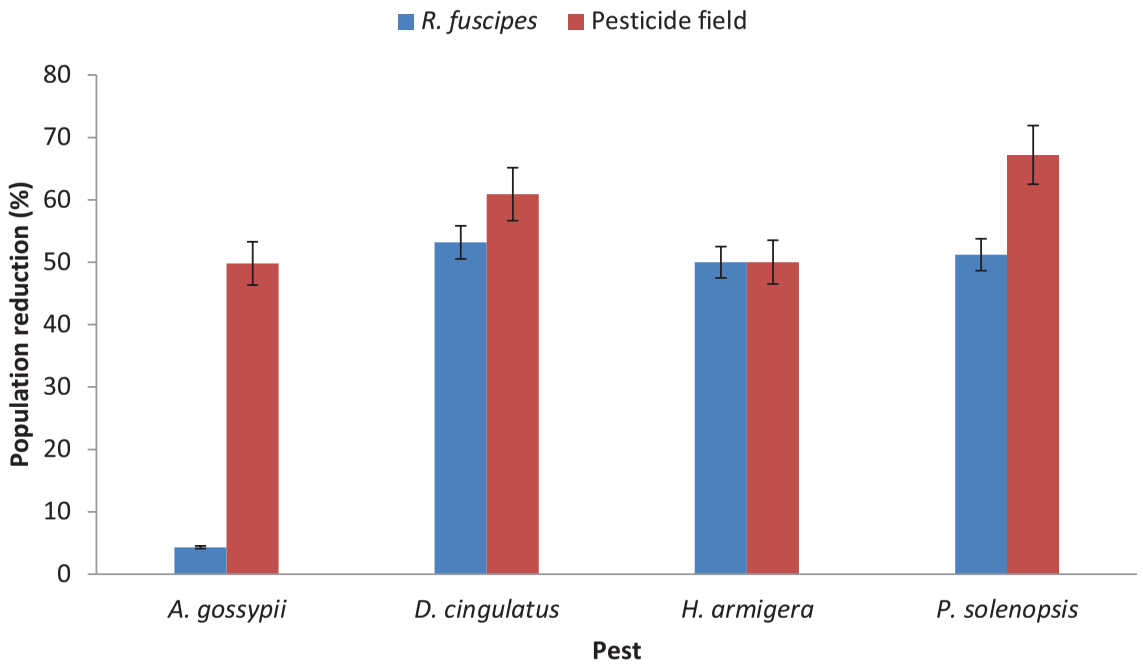


Table 15. Augmentative release of *R. fuscipes* (RF) life stages (egg to adult) on natural enemies population (number of predator/plant±S.E.) under irrigated cotton (SVPR IV) field condition during June to August 2011 (*Kharif*).

No. of release	Field category	Predator				
		Ants	Coccinellids	Reduviids	Spiders	Wasps
First release	Control	0.64±0.09 ^{ac}	0.23±0.04 ^{ac}	0.00±0.00 ^{ac}	0.12±0.03 ^{ac}	0.08±0.02 ^{abc}
	RF	0.51±0.09 ^{ab}	0.31±0.05 ^{b}	0.05±0.01 ^{bc}	0.18±0.03 ^{bc}	0.14±0.02 ^{abc}
	Pesticide field	0.95±0.08 ^{abc}	0.61±0.06 ^{abc}	0.005±0.005 ^{abc}	0.47±0.04 ^{abc}	0.29±0.03 ^{abc}
Second release	Control	0.35±0.04 ^{ac}	0.25±0.03 ^{abc}	0.01±0.007 ^{abc}	0.21±0.03 ^{abc}	0.17±0.03 ^{abc}
	RF	0.47±0.04 ^{bc}	0.61±0.05 ^{ab}	0.17±0.02 ^{abc}	0.42±0.03 ^{abc}	0.33±0.03 ^{abc}
	Pesticide field	0.46±0.04 ^{abc}	0.45±0.04 ^{abc}	0.005±0.005 ^{abc}	0.33±0.47 ^{abc}	0.20±0.02 ^{abc}
Third release	Control	0.64±0.04 ^{ab}	0.41±0.04 ^{abc}	0.01±0.007 ^{ab}	0.42±0.04 ^{abc}	0.28±0.03 ^{ac}
	RF	0.84±0.05 ^{b}	0.55±0.04 ^{b}	0.04±0.01 ^{abc}	0.55±0.04 ^{abc}	0.43±0.04 ^{bc}
	Pesticide field	0.69±0.07 ^{abc}	0.45±0.05 ^{abc}	0.01±0.007 ^{bc}	0.33±0.03 ^{abc}	0.28±0.03 ^{abc}
Total mean population	Control	0.70±0.04 ^{a}	0.50±0.03 ^{a}	0.01±0.003 ^{ab}	0.38±0.02 ^{ac}	0.26±0.02 ^{ac}
	RF	0.61±0.04 ^{b}	0.49±0.03 ^{bc}	0.09±0.01 ^{bc}	0.38±0.02 ^{bc}	0.30±0.02 ^{bc}
	Pesticide field	0.52±0.01 ^{abc}	0.29±0.012 ^{abc}	0.01±0.01 ^{abc}	0.26±0.02 ^{abc}	0.18±0.02 ^{abc}

Mean followed by the same letters in column do not differ significantly by TMRT at 5% level

2.4.5. Cost Benefit Ratio (CBR) and Percent Avoidable Loss (PAL)

Cost Benefit Ratio was higher in chemical pesticides sprayed field (1.72) than in *R. fuscipes* released field (1.32) and control field (1.17). Higher Percent Avoidable loss was recorded in chemical pesticides sprayed field (0.276) than in *R. fuscipes* released field (0.185) (Table 16).

2.5. DISCUSSION

Mass production of reduviids and their utilization in biological control programme has been recognized in recent years. Natural enemies are an important component of Biointensive Integrated Pest Management (BIPM) programme.

2.5.1. Group rearing of reduviid predator

The reduviid predator, *R. fuscipes* was reared with a suitable condition and an appropriate density; females are prolific and showed high fecundity. It was reported previously that 1:2 (female: male) is the suitable sex ratio (Sahayaraj and Selvaraj, 2003) and needs more comfort facility for prey searching, mating and oviposition. By providing all these facilities under laboratory, a 'Micro Environmental Cage' was designed in this study (Plate 1). The study reveals that *R. fuscipes* can be mass produced under laboratory condition. Grundy *et al.* (2000) developed a mass rearing method for *Pristhesancus plagipennis* (Walker) using the 'net lid method'. The lepidopteran larvae were supplied on the net lid and the reduviids were allowed to feed. They observed that different prey combinations were better than a single prey species for producing predators which were healthier (152 mg) and developed faster (43 days). Similar hypothesis was also proposed by Ambrose and Sahayarani (1991). In the same manner we supplied different types of preys along with artificial diet for the rearing of *R. fuscipes.* The laboratory observations from the prey selection by the predator led to the

Table 16. Cost Benefit Ratio analysis of *R. fuscipes* released cotton field (SVPR IV) under irrigated condition during June to August 2011 (*Kharif*)

Parameters	Cotton field condition		
	R. fuscipes	**Pesticide field**	**Control**
Cost of cultivation (Rs)	22943.00	25496.00	21242.00
Total income (Rs)	30504.00	43876.00	24872.00
Total production Kg/ha)	877.5	988.5	715.5
CBR	1.32	1.72	1.17
Percent Avoidable Loss	0.185	0.276	-

Values in parenthesis are indicating the control

conclusion that the slow moving larger lepidopteran larvae were the highly preferred feed of *R. fuscipes,* which can be utilized in future for large scale mass rearing as well as short term experiments. The results support the observations of George (2000) on *R. fuscipes* against *S. litura.* Grundy *et al.* (2000) which revealed that higher densities of adult reduviids reduced in both the total number of rafts laid and the mean number of eggs per raft. In the present study 30-35 predators were reared in a single cage. So the cage provided the best overall compromise between space utilization and maintenance of high level of fecundity (46.9±0.05) and adult longevity (49.8±0.33 for male and 53.7±0.40 for female).

When compared other techniques such as providing different substrata (Ambrose and Claver, 1999), net lid (Grundy, 2007), larval card method (Sahayaraj, 2002), artificial diet (Sahayaraj and Balasubramanian, 2009), MEC could easily be adapted by the predator and can be prepared at low cost (Rs. 3000/5000 predator). Also by providing seasonally available insect pests, the predator has a different choice to select their prey for sustenance. The space and substrata inside the cage were prolifering prey searching, mating, movements and oviposition of the reduviid. In this method nearly 2 males were maintained for a female as to have every female get the chance for mating; it leads to increase in fecundity. Finally the MEC has a compact shape and size, so it is handy and can be placed in a small area and can also be carried to the field providing different kinds of foods (insects).

Considering the potential efficacy of *R. fuscipes* against economically important insect pests, this biocontrol agent can be better directed towards high value crops such as cotton, groundnut and bhendi, where *R. fuscipes* was already recorded as a potential mortality agent of insect pests (Sahayaraj, 2007a; Sujatha *et al.,* 2012). Within such a perennial system of

cotton, a lower cost inoculative rather than inundative release strategy might be effective for increasing predators' numbers to gain effective biological control (says Grundy, 2007).

2.5.2. Hiding behavior

The study of hiding behavior was aimed at identifying and recording the hiding places of this predator when it was released in the field condition. It is also considered as a pre-requisite for BIPM too. From the results it was clear that the predator was hiding under different objects, which is naturally present in the agro-ecosystem at different time. The reduviid predator mostly preferred small pebbles to hide under followed by leaves (Ambrose, 1999). Sahayaraj (2007a) previously reported that the reduviid predator mostly preferred under shrubs, boulder, herbs, on the bark and foliage of trees. In the present study, the reduviid preferred fallen leaves and stones for their hiding purpose when the day time increased. Consequently, it is not advisable to provide any materials such as stones, banana leaves or palm leaves (Claver and Ambrose*,* 2003a, b; Ganesh Kumar, 2011) artificially for hiding. Introduction of these artificial provisions could alter the natural balance, or would lead to the entering of termite pest in the ecosystem, chemical cues may voluntarily invite quite a lot of pests which may act as serious pests of our sole crop in the course of time.

Hiding behavior is commonly an anti-predatory behavior; the prey is generally adapted for its security (Masatoshi *et al.,* 2011). The hiding behavior exhibited by the insects is not only for attacking – escaping mechanism but also for parental care (says Gustavo *et al*., 2010). Present results also reveals that at different time intervals the predator preferred different objects for hiding which may be due to searching for better adaptability and suitability for searching their prey and also for escaping from other enemies too, because in

the field condition, spiders (Kimberl *et al.,* 1987) and wasps (Timbilla and Braimah, 2002) were reported as the predators of reduviids.

Reduviid predators, *Zelus longipes* (Linn.) (Kalsi and Seal, 2011) and *Scipinnia repax* (Stal.) (Harland and Jackson, 2004; Jackson *et al.,* 2010) hide inside the foliage in order to find their prey, whereas, *Pergrinator biannulips* (Montrouzier *et* Signore), a predator of stored products pests hide under detritus present in the store room (Shin ichi *et al*., 2003) to find the prey. Similarly, praying mantids, hide behind the leaves and inflorescences (Sampaio *et al*., 2008) in search of preys as observed in the present study very often in field condition. The *R. fuscipes* hide under stones during late evening and prefer fallen leaves at morning. From the present study, we concluded that the reduviid can adapt themselves in the natural ecosystem wherever they released augmentatively present in a niche. Hence, it is not advisable or required any provision for their survival under natural ecosystem. Furthermore, survival of the fittest can also be considered, since it is a native predator they adapted in the agro-ecosystems as observed here.

2.5.3. Bioefficacy

Maximum predatory rate was recorded against *D. cingulatus* rather than *P. solenopsis.* Sahayaraj *et al.* (2012) also reported the efficacy of *R. longefrons* against the cotton pests such as *A. gossypii, D. cingulatus* and *P. solenopsis* and recorded similar observations. The population of *D. cingulatus* and *P. solenopsis* were reduced up to 50% by the predator. On the other hand, the pest incidence of *H. armigera* in the cotton field was very low during the experimental period. Although, the efficacy of *R. fuscipes* release insignificantly pronounced against *A. gossypii,* it was 4.30% lower than predator non-released and chemical pesticides sprayed field. Similarly, the field release of *Pristhisancus plagipennis* (Grundy, 2004; Grundy

and Maelzer, 2000a, b; Grundy and Maelzer, 2002a, b), *P. laevicollis* (Antony *et al*., 1979) and *R. marginatus* (Sahayaraj, 1999, 2003; Sahayaraj and Martin, 2003; Sahayaraj and Ravi, 2007) were successful in reducing various pests such as *S. litura* (Sahayaraj, 1999, 2003; Sahayaraj and Martin, 2003)*, H. armigera* (Grundy and Maelzer, 2000a; Grundy, 2004) and *A. craccivora* (Sahayaraj and Ravi, 2007) in cotton (Grundy and Maelzer, 2000b; Grundy, 2004) and groundnut field (Sahayaraj, 1999; Sahayaraj and Martin, 2003; Sahayaraj and Ravi, 2007). But there are no reports available about the augmentative release of *R. fuscipes* for cotton pests management.

In the present study *R. fuscipes* successfully control *D. cingulatus* and *P. solenopsis.* Similar results were observed in the cotton pests complex in the field by the augmentative release of *P. plagipennis* (Grundy and Maelzer, 2000a; Grundy, 2004). Sahayaraj and Martin (2003) reported that *R. marginatus* controlled *H. armigera* and aphids population more significantly than other pests of ground nut. But in the present study results revealed that *R. fuscipes* has no serious impact on the population reduction of *A. gossypii*, and also the *H. armigera* incidence was very low in the study field during experimental time. Present study results suggest that the release of *R. fuscipes* nymphs caused a subsequent reduction in the number of *D. cingulatus* and *P. solenopsis* in cotton field. The high level of reduction was observed in chemical pesticides sprayed field for all the pests.

Several studies have long evaluated the role of natural enemies in the control of cotton insect pests (Habibullah *et al.*, 2013; Trevor Williams *et al.*, 2013). However, very few of these studies have assessed the use of predatory reduviid bugs against insect pests in the field condition (Grundy and Maelzer, 2002a; Grundy, 2004; Balasubramanian, 2008). Though augmentative release of predators especially reduviids, is a major concept in Biointensive

Integrated Pest Management (BIPM) (Sahayaraj and Martin, 2003; Grundy and Maezler, 2002b; Grundy, 2004; Kalsi and Seal, 2011) and very limited information is available in the literature. Hence the present study is considered as an important one for "Classical Biological Control (CBC)". In the present study, the *P. solenopsis* population was highly reduced in chemical pesticides field (62.7%) (P=0.003) followed by reduviid released field (51.2%) (0.005). There is no significant difference in the aphid population between *R. fuscipes* released field (4.30%) (P=0.153) and chemical pesticide sprayed field (49.8) (P=0.453). But in *D. cingulatus,* population reduction is significant in *R. fuscipes* released field (P=0.005) but not significant in chemical sprayed field (P=0.987). Moreover, *P. solenopsis* reduction was significant in chemical pesticide sprayed field. In Australia, Grundy (2007) reported the efficacy of *P. plagipennis* as a biological control agent within an Integrated Pest Management program for *Helicoverpa* spp. and *Creontiades* spp. in cotton field along with compatible insecticides. Inundative releases of *P. plagipennis* integrated with insecticides identified as being of low toxicity were then tested and compared with treatments of *P. plagipennis* and the compatible insecticides used alone conventionally sprayed usage practice and an untreated control during two field experiments in cotton.

Present research together with earlier studies (Sahayaraj and Ravi, 2007; Grundy, 2007; Balasubramanian, 2008) suggests significant potential for the use of reduviid predators as a inudative biocontrol agent in cotton, and the adoption of reduviid predator as a part of an integrated pest control strategy. Most IPM systems aim at enhancing biological control through conservation of existing natural enemies, or introducing new ones through inoculation or inundation (Balakrishnan *et al.,* 2010). It is there for, important to minimize the non-target effects of other IPM components, such as pesticides, GM plants or habitat

manipulation. The future of biological control agents is bright but depends on technological advancements and market opportunities.

2.6. CONCLUSION

The use of reduviid predator for pest control is coming under the topic of Classical Biological Control; it could be an important part in Biointensive Integrated Pest Management (BIPM) Programme. The MEC provides good provisions for the mass production of *Rhynocoris fuscipes* under laboratory condition. The fecundity and adult longevity were high when the reduviids were reared in MEC. The adult *R. fuscipes* showed higher prey consumption than other instars; it consumed more number of preys during morning hrs (1.54) than evening hrs (1.04) under pot condition. *Rhynocoris fuscipes* is a suitable aspirant for augmentative release for the insect pest control in cotton field and it suppresses 50% pest population of *Dysdercus cingulatus, Phenacoccus solenopsis* and *Helicoverpa armigera.* Hence the reduviid predator, *R, fuscipes* can be recommended for Biointensive Integrated Pest Management (BIPM) Program.

CHAPTER 3

Isolation, purification, characterization and toxicity of entomotoxin of *Beauveria bassiana*

3.1. Abstarct

The entomotoxic protein of *Beauveria bassiana* (Bals.) (Ascomycota: Hypocreales) was precipitated with 90% saturated $(NH_4)_2SO_4$ and the precipitate was eluted in Sephadex G-25 column. The protein content was higher in fraction II (0.026 µg/ml) than fraction I (0.024 µg/ml) and III (0.020 µg/ml) supported by UV spectroscopy absorbance. The vibration frequency in FTIR appeared between the ranges of 1650–1580cm^{-1} due to N-H bond formation for amines. Bioassays of fractions (I, II and III) reveal that second fraction caused significantly higher insecticidal activity against *Dysdercus cingulatus* Fab. (Hemiptera: Pyrrhocoridae) third instar (LC_{50} = 800.2 ppm) and *Phenacoccus solenopsis* Tinsely (Hemiptera: Pseudococcidae) adult (LC_{50} = 713.3 ppm). Fraction II also alteredamylase, protease and lipase (digestive enzymes), esterase, glutathione s-tranferase and lactate dehydrogenase level/activity in insects. Hence, the fraction II was subjected to sodium dodesyl sulphate–poly-acrylamide gel electrophoresis (SDS-PAGE), high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/deionization – time of flight (MALDI-TOF) analyses. SDS-PAGE result revealed that fraction II contains two polypeptides. Two peaks were obtained when the sample was subjected to analytical HPLC at the retention time of 4.262 min^{-1} and 5.915 min^{-1}. The preparative HPLC fraction II was subjected to MALDI-TOF which showed a peak with174 Da. It was designated as FII.

Key words: *Beauveria bassiana,* characterization, entomotoxin, insecticidal activity.

3.2. INTRODUCTION

Entomopathogenic fungi are widely-available as biological control agents (BCAs) to control agricultural pests worldwide (Chutao *et al.,* 2007; Zimmermann, 2007; Inigo *et al.,* 2008; Safavi, 2010; Goettel *et al.,* 2010; Safavi, 2013).Several species from the genera *Beauveria, Metarhizium, Isaria*, and *Lecanicillium* and others too have been registered in the United States by the Environmental Protection Agency and commercialized (EPA) for various pest managements. *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) is an important natural pathogen of insects, able to cause epizootics among populations of invertebrates including insects. It has also been developed as a microbial insecticide for use against many major pests, including hemipteran (Uzma Mustafa and Gurvinder Kaur, 2010; Rohlfs and Churchill, 2011) lepidopteran (Fuguet *et al.,* 2004; Safavi, 2013); coleopteran (Óscar *et al.,* 2010; Yulin Gao *et al.,* 2012a, b; Meyers *et al.,* 2013) and orthopteran (Kaaya *et al*., 2002; Bidochka *et al.,* 2010; Yulin Gao *et al.,* 2012b; Pelizza *et al.,* 2013) pests. *Beauveria bassiana* is capable of penetrating through the insect cuticle, secreting hydrolytic enzymes such as chitinases, proteinases and lipases (cuticle-degrading enzymes) (Michael and George, 1987; St. Leger *et al*., 1996a; Fang *et al*., 2005; Yongjun *et al.,* 2010; Bidochka *et al.,* 2010; Youshan Li *et al.,* 2012; Ming *et al.,* 2013) being effective against insect pests.

3.2.1. Toxicity against pests

Control of *Dysdercus cingulatus* Fab. (Hemiptera: Pyrrhocoridae) and *Phenacoccus solenopsis* Tinsely (Hemiptera: Pseudococcidae) are preliminarily accomplished through the use of conventional insecticides. Chemical control has resulted in the development of resistant, increasing of *D. cingulatus* and *P. solenopsis* population. Its negative impact has encouraged the development of alternative pest management strategy in which fungal entomotoxins play an important role (Mullen and Goldsworthy,

2003; Fuguet *et al*., 2004; Pava-Ripoll *et al.,* 2008; Mouchet *et al.,* 2008; Molnar *et al.,* 2010). *Beauveria bassiana* produces several toxic compounds *in vitro* and *in vivo* (Strasser *et al*., 2000; Vey *et al.,* 2001) having low molecular weight secondary metabolites (Peczynska *et al*., 1991; Zimmermann, 2007). Cyclosporins A and C, (Zizka, and Weiser, 1993; Uzma Mustafa and Gurvinder Kaur, 2010), beauvericin, bassianin, bassianolide, beauverolides, beauveriolides, tenellin, oosporein (Strasser *et al*., 2000; Vey *et al*., 2001, Safavi, 2013), oxalic acid (Roberts, 1981), bassiacridin (Quesada-Moraga and Vey, 2004) are some of the important entomotoxins of *B. bassiana*. Among them, Beauvericin showed insecticidal activity against many insect pests (Suzuki *et al*., 1977; Kanaoka *et al*., 1978; Champlin and Grula, 1979; Roberts, 1981; Qadri *et al*., 1989; Zizika and Weiser, 1993; Gupta *et al*., 1995; Vey *et al.,* 2001). Nevertheless, there are some reports of no toxicity against certain insects (Champlin and Grula, 1979) too. There are no reports available about the *B. bassiana* entomotoxic protein against *D. cingulatus* and *P. solenopsis.* Hence we proposed the present study with the following objectives:

Objectives:

1. To precipitate, purify, fractionate and characterize with UV-visible spectroscopy, FTIR spectroscopy, SDS-PAGE, HPLC, MALDI the *B. bassiana* entomotoxic proteins.
2. To assess the virulence of *B. bassiana* entomotoxic protein fractions against *D. cingulatus* and *P. solenopsis*and their protein, digestive and detoxification enzymes.

3.3. MATERIALS AND METHODS

3.3.1. Fungal Strain

The *Beauveria bassiana* isolate was obtained from Crop Protection Research Center, St. Xavier's College (Autonomous) Palayamkottai. For inoculation, 10^3 *B. bassiana* conidia were inoculated into 100 mL of potato dextrose broth (Himedia

Mumbai) in a 250 mL Erlenmeyer flask. The culture was incubated on a rotator shaker (180 rpm) at 27^0C for 7 days and the spore concentrations recorded using a haemocytometer. Viability of conidia was checked before preparation of suspension by germinating test in liquid Czapek-Dox broth with 1% (w/v) yeast extract medium (CDBYEM) to harvest conidia. To inoculate secondary cultures for large scale growth of the fungus, 2 ml of primary culture was transferred into 250 ml of the same medium in the Erlenmeyer flask and cultured at 25^0 C for 20 days.

3.3.2. Isolation of culture filtrate

After 20 days of growth in CDBYE medium, semi-liquid culture was filtered by suction through no. 1 filter paper (Whatman Ltd. Kent, England). The filtrate was passed through 0.45 μm pore size filter (Millipore Corp), fungal matt was separated and freeze dried at -4^0 C (LG, Japan). This was used to produce entomotoxin and subsequently used to assay the virulence of the toxin against two cotton pests. Protein from the dried fungal matt was ground with 90% ice cold saturated $(NH_4)_2SO_4$ solution ($681gL^{-1}$) for protein precipitation (Quesada-Moraga and Vey, 2004). The precipitated protein was collected by centrifugation at 8000 rpm for 30 minutes. The crude protein was subjected to a gel filtration through a Sephadex G-25 (Sigma) column (1 x 10 cm) in 50 mM tris/HCl buffer at P^H 8. The column flow rate was adjusted at the rate of 1ml/hr. The aliquots were collected and subjected to UV, FTIR, HPLC and MALDI-TOF.

3.3.2.1. Determination of total protein concentration

Total protein concentration of *B. bassiana* entomotoxin was determined using the Lowry *et al.* (1959) method with Bovine Serum Albumin (BSA) (S.D. Fine-Chem Limited, Mumbai) as the standard.

3.3.2.2. UV–spectroscopy analysis

The fungal protein fractions collected through Sephadex G-25 column were analyzed using UV spectrometer (Schumadzu, Japan) to determine protein content. The samples were prepared by mixing 100 µl of aliquots with 2 ml of distilled water. The pre-run Tris HCl buffer through Sephadex G-25 column sample was used as the blank. The base line was corrected with the blank solution between 600 nm and 100 nm. The absorbance peak reading of the each fraction was recorded categorized in to fraction I (FI), fraction II (FII) and fraction III (FIII).

3.3.2.3. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

FT-IR spectra of FI, FII and FIII was determined on a FTIR spectrometer (Perkin Elmer, Spectrum RX I, Japan) in the range of 4000–400cm^{-1} with a resolution of 4 cm^{-1}. The FTIR was carried out for each sample separately for the identification of possible resonance vibration of chemical bonds.

3.3.2.4. Gel Electrophoresis of *Beauveria bassiana* entomotoxins

Tricine-SDS-PAGE was used to determine number and relative molecular weight of polypeptides/proteins found in the *B. bassiana* entomotoxins. All solutions and reagents were prepared according to Schagger and von Jagov (1987). A 16% separating gel (pH 8.2) was prepared from N, N'-methylene Bisacrylamide stock solution (49.5% T, 3% C). Similarly gel buffer stock solution (3.0M Tris and 0.3% SDS, pH 8.2); anode electrophoresis buffer (2M Tris pH 8.9) and cathode electrophoresis buffer (0.1M Tris, 0.1M Tricine, and 0.1% SDS pH 8.2) were prepared. Acrylamide stock solution **[**Acrylamide (49.5%), methylene bis acrylamide (3%C) and keep at 4^0C]; gel buffer (pH 8.4) [3.0M Tris and 0.3% SDS kept at room temperature], Glycerol 70%, cathode buffer (Upper buffer) pH 8.2 (1M Tris, 1M Tricine, 1% SDS and keep in RT), anode buffer

(lower buffer) pH 8.9 (2.1M Tris keep in RT), Ammonium per sulphate (APS) [APS 10% kept at 40C] and TEMED were prepared and used for this study.

Equal amount of 20 uL entomotoxin was denatured into the buffer solution of 100% Glycerol, 2-Mercaptoethanol, 20% SDS and 1M Tris at pH 6.8, and then loaded to gels. A constant voltage of 30 V was maintained until the samples concentrated on the stacking gel, before adjusting the voltage to 80 V for the remaining separation time. Electrophoretic separations were maintained for 7-8 hrs with 25 mA/gel stable current. The gel was stained overnight in 0.1% Coomassie Blue R-250 (in 30% methanol, 10% acetic acid) and then destained. After destaining, the gel was placed in 7.5% acetic acid for preservation and imaged with Olympus camera attached to Gel documentation unit (Biotech, Yerkard, India) with D-Gel Das software.

3.3.2.5. High Performance Liquid Chromatography (HPLC)

The *B. bassiana* entomotoxin (FII) was analyzed on a HPLC (Schimadzu LC/10AD, Japan) equipped with an injector (20 ul loop) and C-18 column (5um particle size)(250 mm x 4.6 mm I.D) using CH3OH/H2O (1:1 v/v) at a flow rate of 0.8 ml min-1with detection gradient from 3% MeOH (Himedia, India) and increased up to 70% MeOH by UV absorption at 280 nm using a detector (SPD-10A/UV-Vis). Purification was executed by analytical HPLC(Schimadzu) using a LC/10ADODS, 250 mm X 4.6 mm column, MeOH/H2O linear elution gradient starting from 3% MeOH during 5 min and increased up to 70% MeOH in 30 min with a flow rate of 1.0 mL min . The analytical chromatogram showed one major compound UV maximum at 3000 nm. The procedure yielded 5 mL of a pure white column solution. The compound was subjected to MALDI-TOF for identification of *B. bassiana* entomotoxin as described below.

3.3.2.6. Matrix-assisted laser desorption/ionization-TOF (MALDI-TOF)

Liquid fraction II obtained from the preparative HPLC was allowed to dry and then subjected to MALDI-TOFMS analyses (Voyager-DETM PRO Biospectrometry TM cspectrometer (Applied BioSystems, Framingham, MA, USA) equipped with a model VSL-337ND Nitrogen Laser (Laser Science, USA). The accelerating voltage was 20 KV. The matrix was a-Cyano-4 hydroxycinnamic acid (Sigma Aldrich, India), (10 mg/ml in 1:1 CH3CN/0.1% TFA). An equal amount of sample and matrix were dropped onto the MALDI sample plate and allowed to dry at room temperature. Time-to-mass conversion was achieved by external and/or internal calibration using standards of bovine pancreatic beta insulin (m/z 3496.9), bovine pancreatic insulin (m/z 5734.6), and apomyoglobin (m/z 16,952.6) (Sigma Aldrich, India). Experiments were facilitated by the Voyager version 5 with Data Explorer TM software.

3.3.3. Collection and rearing of cotton pests

Adults and nymphs of *Dysdercus cingulatus* were collected from cotton field in and around Tirunelveli District, Tamil Nadu, India. Collected insects were maintained under laboratory condition (28±2° C temperature, 70-75 RH, 11L: 13D photoperiod)in plastic container (13 cm height x 7 cm diameter) primarily provided with water soaked cotton seeds, later with artificial diet (Sahayaraj *et al.,* 2011). The newly hatched third instars were used for the experiment. The field collected *P. solenopsis* were cultured on clean pumpkin as described by Venkatesha and Dinesh(2012). The laboratory emerged insects were used for the study.

3.3.4. Bioassay

Bioassays were conducted as previously described (Gatehouse *et al.,* 1997; Cuthbertson, *et al.,* 2009). Adult *P. solenopsis* (0-1 day old) (17.4±0.3mg) and third instar *D. cingulatus* (0 day old) (43.7±0.3mg) were maintained as described by Venkatesha and

Dinesh(2012) and Sahayaraj *et al.* (2011) respectively. Then different concentrations of *B. bassiana* entomotoxic fractions (FI, FII and FIII) were prepared such as 100, 200, 400, 800 and 1600ppm by adding required amount of distilled water. For oral toxicity bioassay, different concentrations of FI, FII and FIII were mixed with 5 mL of artificial diet (Sahayaraj *et al*., 2011) and 50 µl of diet was poured into 10 mg of cotton ball and provided to *D. cingulatus* daily. Leaf dip method (Cuthbertson, *et al.,* 2009) was followed for *P. solenopsis* adult. For leaf-dip bioassay, 10 mL of each different fraction concentration was taken in a 100 mL beaker into which were added 50 µL (0.05%) Tween 80 and mixed well. Healthy cotton leaves were dipped separately in different concentrations for 10 min then air dried for 5 min. The air dried leaves of various concentrations were kept in a Petri dish (9 cm diameter x 1 cm height) and adults of *P. solenopsis* were introduced and allowed to feed the leaves. The mortality of insects was recorded every 24 hrs up to 96 hrs continuously. Each treatment was replicated 3 times with ten animals per replicate.

3.3.5. Enzyme quantification

Preparation of enzyme source: Enzyme samples were prepared by the method of Applebaum *et al.* (1961). After 96 hrs of exposure period, the live insects were supplied with normal feed (artificial diet for *D. cingulatus* and normal cotton leaves (SVPR IV for *P. solenopsis*) and maintained under laboratory condition mentioned above. After the adulteration of the insects, three live as well as healthy *D. cingulatus* and five *P. solenopsis* adults from each concentration (100, 200, 400, 800 and 1600 ppm) of fungal fraction II and control categories were starved for twelve hours before dissection for the accumulation of digestive enzymes. The insects were placed in the -4^0C (LG, Korea) for 5 minutes and then dissected in ice cold insect ringer's solution (Sodium chloride – 0.65g, Sodium carbonate – 0.02g, Calcium chloride – 0.03g, Potassium chloride – 0.025g and

100 mL of distilled water) under a dissection microscope. The digestive system/alimentary canal was exposed by holding the abdomen with fine forceps and the malphigian tubules and adhering tissues were gently removed with fine forceps. The separated alimentary canal was weighed (accuracy in mg) and separately homogenized for 5 minutes at -4^{o} C with 1 mL ice cold phosphate buffer (Disodium hydrogen orthophosphate - 1.6g + Sodium dihydrogen orthophosphate - 1.1 g in 100 mL distilled water) (pH - 6.8) using a tissue homogenizer with a Teflon homogenizer. The homogenate was centrifuged at 8000 rpm for 15 minutes and the supernatant was made up to 5 mL with phosphate buffer and used as the enzyme source (ES). The ES was kept under -20^{0}C for quantification studies. Digestive enzymes such as amylase (Ishaaya and Swirski, 1970), protease (Morihara and Tsuzuki, 1977), lipase (Cherry and Crandal, 1932) and detoxification enzymes like, esterase (Van Asperen, 1962), glutathione-S-transferase (Yu, 1982) and lactate dehydrogenase (King, 1965) levels were quantified using standard procedures as specified below.

3.3.5.1. Digestive enzymes

Amylase: Amylase activity in animal gut was determined as described by Ishaaya and Swirski (1970). The reaction mixture consisted of 1 mL of 0.01 M phosphate buffer (w/v), and 0.25 mL of enzyme extract. After incubating the reaction mixture for about 60 minutes at 37^{0}C, the enzyme activity was determined by adding 0.4 mL of 3, 5-dinitrosalicyclic acid. The reaction mixture was maintained at 100^{0}C for 5 minutes. Absorbance of the sample was measured in optical density (OD) units 575 nm against a blank in which the enzyme source was replaced with deionized water. The enzyme activity was expressed in terms of the weight of the reducing sugar, glucose (g) produced by the enzyme action per unit weight of gut, per unit time, using glucose as the standard.

Protease: Proteolytic activity was assayed spectrometrically according to the method of Morihara and Tsuzuki (1977). The reaction mixture of 1 mL (1 % w/v) casein solution and 0.5 mL of enzyme extract was incubated at 35^0C for 30 min in a water bath (Remi, India). The enzyme reaction was terminated by addition of 5 mL of 10 % (w/v) trichloro acetic acid (TCA) and kept at room temperature for 10 minutes. The reaction mixture was centrifuged at 3000 rpm for 10 minutes and the supernatant was collected. The concentration of the digested protein was determined by the Folin - Ciocalteu's phenol reagent method of Lowry *et al.* (1951). To 5 mL Lowry's reagent 1 mL of supernatant and 0.5 ml of three fold diluted Folin- Ciocalteu's phenol reagent was added, mixed thoroughly and incubated at room temperature for 30 minutes. The absorbence measured against blank (enzyme extract was replaced by double distilled water in a spectrophotometer) at 670 nm. Tyrosin was used as the standard. The protease activity was expressed as μmoles of tyrosine released per minute per mg of protein.

Lipase: Lipase activity was determined following the method of Cherry and Crandal (1932). The reaction mixture consisted of 1 mL of enzyme extract, 1 mL of olive oil emulsion and 0.5 mL phosphate buffer.Enzyme extract was replaced by distilled water in the control category. Both enzymes and non-enzyme categories were shaken well and incubated at 37^0C for 12 hrs. After the incubation period, 3 mL of 95 % ethanol (v/v) and 2 drops of 2 % phenolphthalein (v/v) was added and titrated against 0.05 N NaOH solution. The end point was the appearance of permanent pink colour. Titre value of the experiment mixture was compared with control. Lipase activity was calculated using the following formula:

$$\text{Lipase (meq/ min/g/sample)} = \frac{\text{Volume of NaOH consumed x Strength of alkali}}{\text{Sample weight in g x Time in minutes}}$$

3.3.5.2. Detoxification enzymes

Esterase: The rate of degradation of α - and β - Naphthyl acetate was measured by the method of Van Asperen (1962), with slight modifications. One mL of the enzyme extract was incubated for 15 min at 37^0C after addition of 0.5 mL of 0.5 mM α - and β - Naphthyl acetate in ethanol. The reaction was stopped and colour developed by adding 0.5 mL dye solution (2 parts of 1% diazoblue B salt: 5 parts of 5% sodium lauryl sulphate) for 20 min. The absorbence was read at 600 nm for α naphthol and at 550 nm for β - naphthol with spectrophotometer.

Glutathion S – transferase: Glutathion S - transferase activity was assayed with 1 – Chloro - 2, 4 - dinitrobenzene (CDNB) as substrate using the method of Yu (1982) with slight modification. The 1.98 mL reaction mixture containing 100 mM Tris HCl buffer (pH - 8.0 with 1mM EDTA), reduced glutathione (10 mM), and the supernatant of the homogenate (containing 50 µg total protein) was first incubated for 3 min at room temperature. Then the reaction was initiated by adding 20 µL of 20 mM CDNB and the absorbance was measured at 340 nm. The enzyme activity was expressed as nanomoles of CDNB conjugated per minute per milligram of protein using an extinction coefficient of 9.6 mM^{-1} cm^{-1} for S-(2–chloro–4-nitrophenyl) glutathione.

Lactate dehydrogenase: To standardize volumes 0.2 mL of NAD solution was added to the test and 0.2 mL of water was added to the control test tube, each containing 1 mL of the buffered substrate; 0.01 mL of the sample was also added to the test tubes. Samples were incubated for exactly 15 min at 37^oC and then arrested by adding 1 mL of colour reagent (2, 4 – dinitrophenyl hydrazine) to each tube and the incubation was continued for an additional 15 min. After the contents were cooled to room temperature, 10 mL of 0.4 N NaOH was added to each tube to make the solutions strongly alkaline to maximize development of hydrazine. At exactly 60 seconds after the addition of alkali to each

tube, the intensity of colour was measured at 440 nm. Replicated blanks with standards were run through the same procedure. Inclusion of the calculated amount of reduced co-enzyme in standard makes allowance for the chromogenicity of NADH2 formed in these tests. The enzyme activity is expressed as multi International Unit (mlU) per milligram protein per minute (King, 1965). An mlU is defined as the amount of enzyme that is required to catalyze the conversion of 1μm lactate to pyruvate or pyruvate to lactate per minute per milliliter of the sample under the prescribed assay conditions.

3.3.5.3. Gel Electrophoresis of Insects total body protein profile

The protein profiles of *D. cingulatus* and *P. solenopsis* treated with fungal fraction II, were analyzed by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) with 12% gels and the buffer system described by Laemmli (1970). Six each live *D. cingulatus* andfive *P. solenopsis* adults from each concentration of fraction II and control were homogenized in different test tubes containing 1 mL phosphate buffer (pH -7.2) (Disodium hydrogen orthophosphate - 1.6g + Sodium dihydrogen orthophosphate - 1.1 g in 100 mL distilled water) using homogenizer, transferred to different eppendorf tubes and centrifuged at 7000 rpm for 10 minutes. The supernatant was collected in different eppendorf tubes containing 75 JLLI of sample buffer (Stacking gel buffer - 1.25 mL (pH 6.8) + 10 % SDS - 2 mL + glycerol - 1 mL + bromophenol blue - 1 mg + mercapto ethanol - 500 μl and made up to 5 mL using distilled water). The sample was boiled at 100^{o} C for three minutes and then allowed to cool. The cooled sample was stored at -10^{0}C in a refrigerator (LG, Korea) whenever necessary and used as the protein sample for Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS - PAGE). A sandwich was made with two glass plates separated by spacer strips. The glass plates were kept vertically in electrophoresis stand. The resolving gel of 12 % was poured

into the space between the glass plates. The level should be about 2 cm below from the notch. It was kept for polymerization for about 30 minutes.

After polymerization, stacking gel was poured over the resolving gel and the Teflon comb with seven fingers (each fingers of 7 mm wide) inserted to form the wells, and allowed to polymerize for 30 minutes. After polymerization the glass plates were clipped out from the stand fit to the electrophoretic apparatus. The electrophoresis buffer was poured to the lower and upper chambers. Then Teflon comb was carefully removed from the gel. Supernatant of the previously prepared sample of the whole body of the cotton pestsand fungal protein fraction were added into each well separately for each experiment in a volume of about 35 µL with a microlitre pippete. High molecular-weight standards (125000 Da, 97000 Da, 66000 Da, 43000 Da) (Genei, Bangalore, India) were run in parallel. Initially, a current of 50 volts was supplied until the sample entered the separating gel and electrophoresis was continued at 100 volts till the marker dye reached the bottom of the separating gel (resolving gel). At the end of electrophoresis run, glass plates were gently moved apart with a spatula, by running a stream of buffer between them, transferring the gel into a solvent resistant plastic tray for staining. The gels were stained with 0.1% Coomassie Brillianr blue R-250 in methanol-acetic acid-water (30: 10: 60 V/V) at 29^0c for 12 hrs and destained in methanol-acetic acid-water (30: 20: 50). Gels were scanned with gel documentation system (Biotech - India) for analysis.

3.3.6. Statistical analysis

The LC_{50} (Protein concentration to kill 50% of insects) values and their fiducial limits were determined by Probit analysis. The data obtained from the enzyme level of insects were analyzed by MANOVA and Tukey's test. P values arrived at less than 0.05 were considered as significant. All the analyses were carried out using SPSS software version 20.0.

3.4. RESULT

3.4.1. Purification of entomotoxin

The toxic protein of *B. bassiana* was precipitated with 90% $(NH_4)_2SO_4$ and the precipitate was eluted from Sephadex G-25 column. Totally 12mL elutes (each 1 mL) were collected, separated primarily and analyzed by UV - visible spectroscopy (Fig. 10). It was observed that the total protein surface plasmon resonance band occurs initially at 210 nm. The proteins surface plasmon band recorded between 210-280 nm in an aqueous medium. Based on the UV absorption the eluents were categorized into three fractions such as fraction I (FI), fraction II (FII) and fraction III (FIII). The total protein content was higher in FII followed by FI and FIII (Fig. 11).

3.4.1.1. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

FTIR analysis reveals that the FI, II and III show characteristic vibration between 1637 - 1639 cm^{-1}(N-H for amines) and also between 1435 - 1440 cm^{-1} for (C-H for methyl group) (Fig. 12) (Table 17).

3.4.1.2. SDS-PAGE - Entomotoxin

The SDS-PAGE analyses of FI, FII and FIII revealed that the fractions contain different polypeptides having low molecular polypeptides, isolated through Sephadex G-25 column. FI (M.W. -16215 and -19104) and FIII (M.W. -3026 and -7004) contain two polypeptide bands and FII contains a single band (M.W. -10656) (Plate 3).

3.4.1.3. High Performance Liquid Chromatography (HPLC) analysis

Since FII has higher insecticidal activity, we are interested to analyze the same. FII was analyzed by HPLC for fractionation and separation of the active polypeptide. The samples were preliminarily subjected to Analytical HPLC. Two peaks were recorded with retention time of 4.262 min^{-1} with the area of 95.69% (FIIa) and 5.915 min^{-1} with the area of 4.1% (FIIb) (Fig. 4a). Then the fractioned HPLC sample FIIa was subjected to

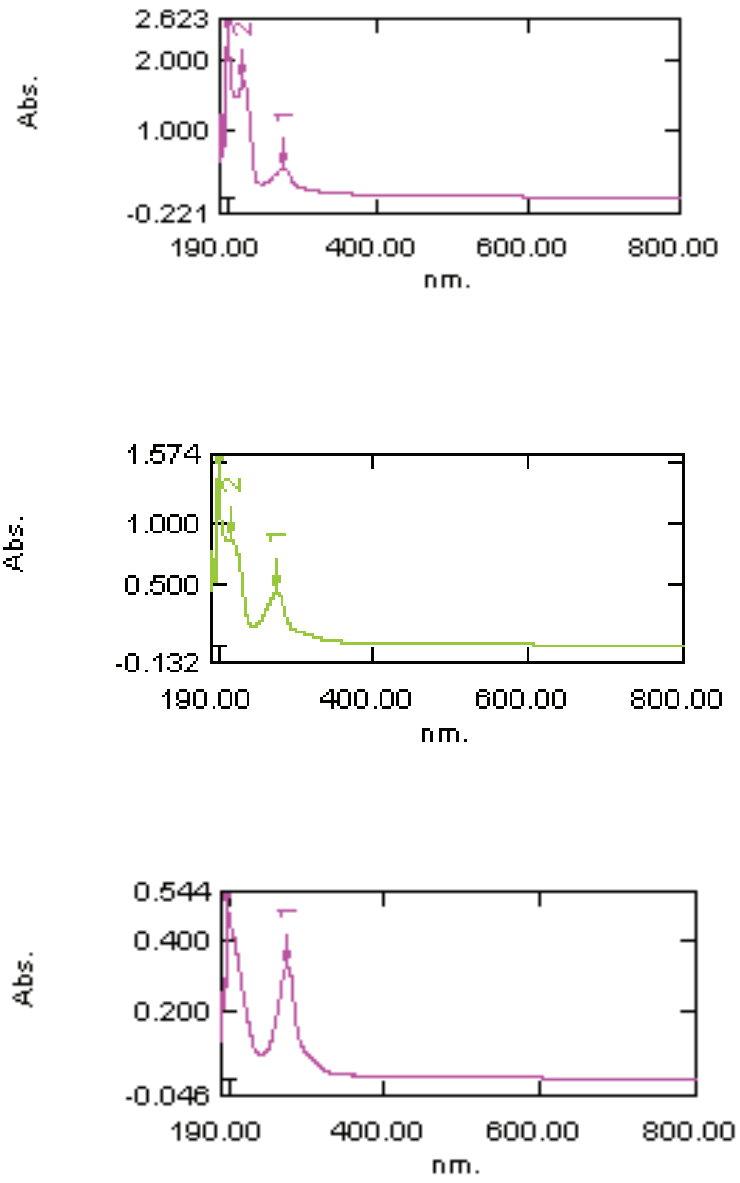


Figure 10. UV – visible spectroscopy analysis of *B. bassiana* protein fractions: fraction I (a), fraction II (b) and fraction III (c) eluted from Sephadex G-25 column

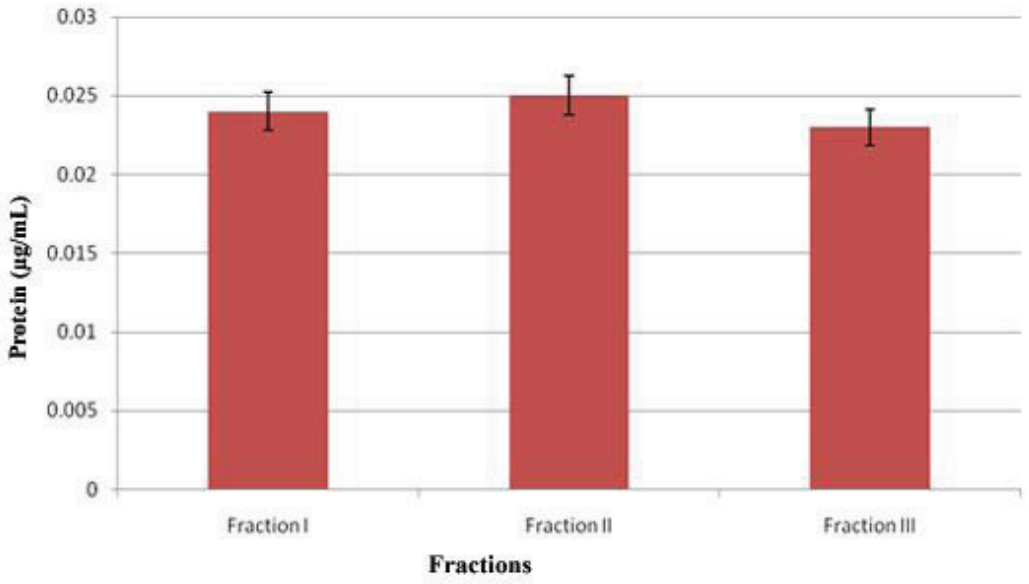
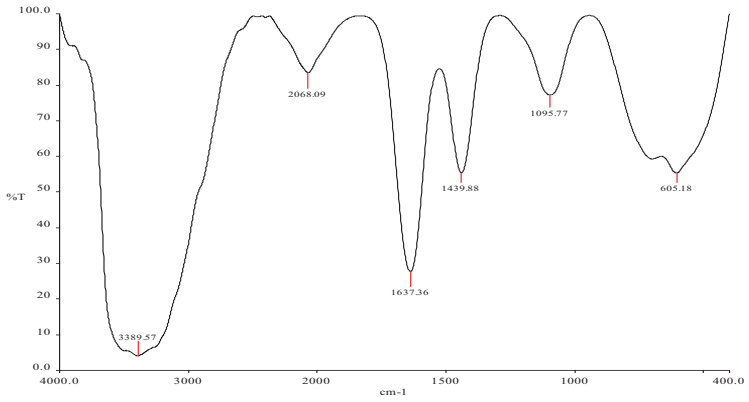
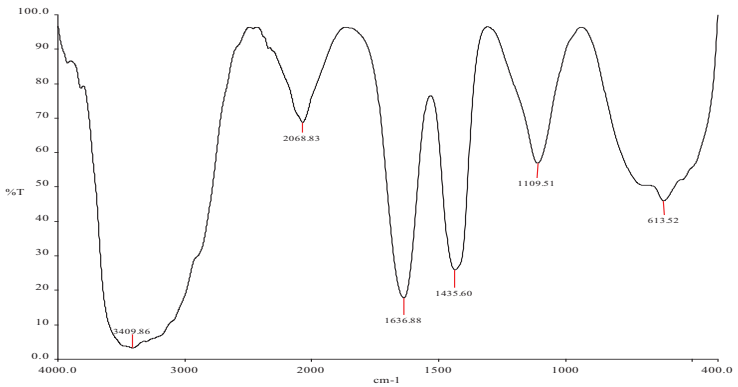


Figure 12. Protein quantity (µg/ml) of *B. bassiana* protein fractions: fraction I, fraction II and fraction III eluted by Sephadex G-25 column





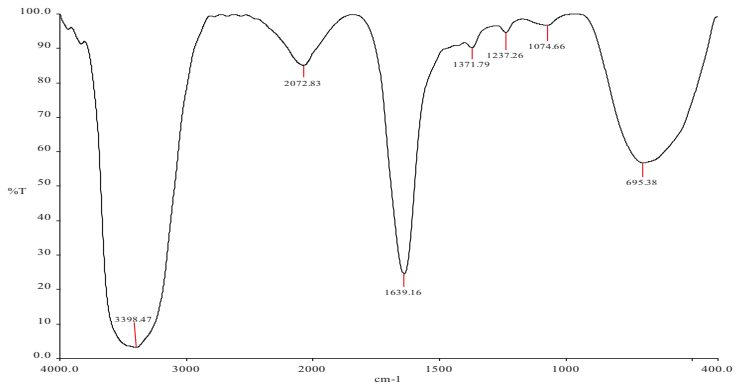


Figure 12. FT-IR analysis of *B. bassiana* protein fractions: fraction I (a), fraction II (b) and fraction III (c)

Table 17. FT-IR analysis of fungal fractions (I,II and III) eluted using Sephadex G-25 column

Fraction I		Fraction II		Fraction III	
Frequency (cm^{-1})	**Functional group**	**Frequency (cm^{-1})**	**Functional group**	**Frequency (cm^{-1})**	**Functional group**
3389	O-H stretching (H-bonded alcohols and phenols)	3409	O-H stretching (H-bonded alcohols and phenols)	3398	O-H stretching (H-bonded alcohols and phenols)
2068	Unknown	2068	Unknown	2072	Unknown
1637	N-H bend (Secondary amine)	1636	N-H bend (Secondary amine)	1639	N-H bend (Secondary amine)
1439	N-H bend (Secondary amine)	1435	N-H bend Secondary amine	1371	C-H bending (alcohol)
1095	C-O stretch (ketone)	1109	C-O stretch (ketone)	1237	C-O stretch (ketone)
605	C-Br bending (aldehyde)	613	C-Br bending (aldehyde)	1074	C-O stretch (ketone)
	-		-	695	C-Br bending (aldehyde)

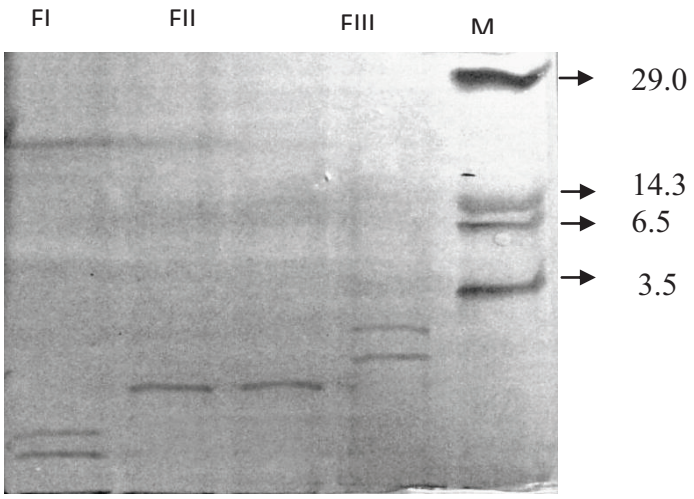


Plate 3. Protein banding pattern of *B. bassiana* entomotoxins eluted using Sephadex G-25 column. M - low molecular weight marker (kDa); FI – Fraction I; FII - Fraction II; FIII - Fraction III.

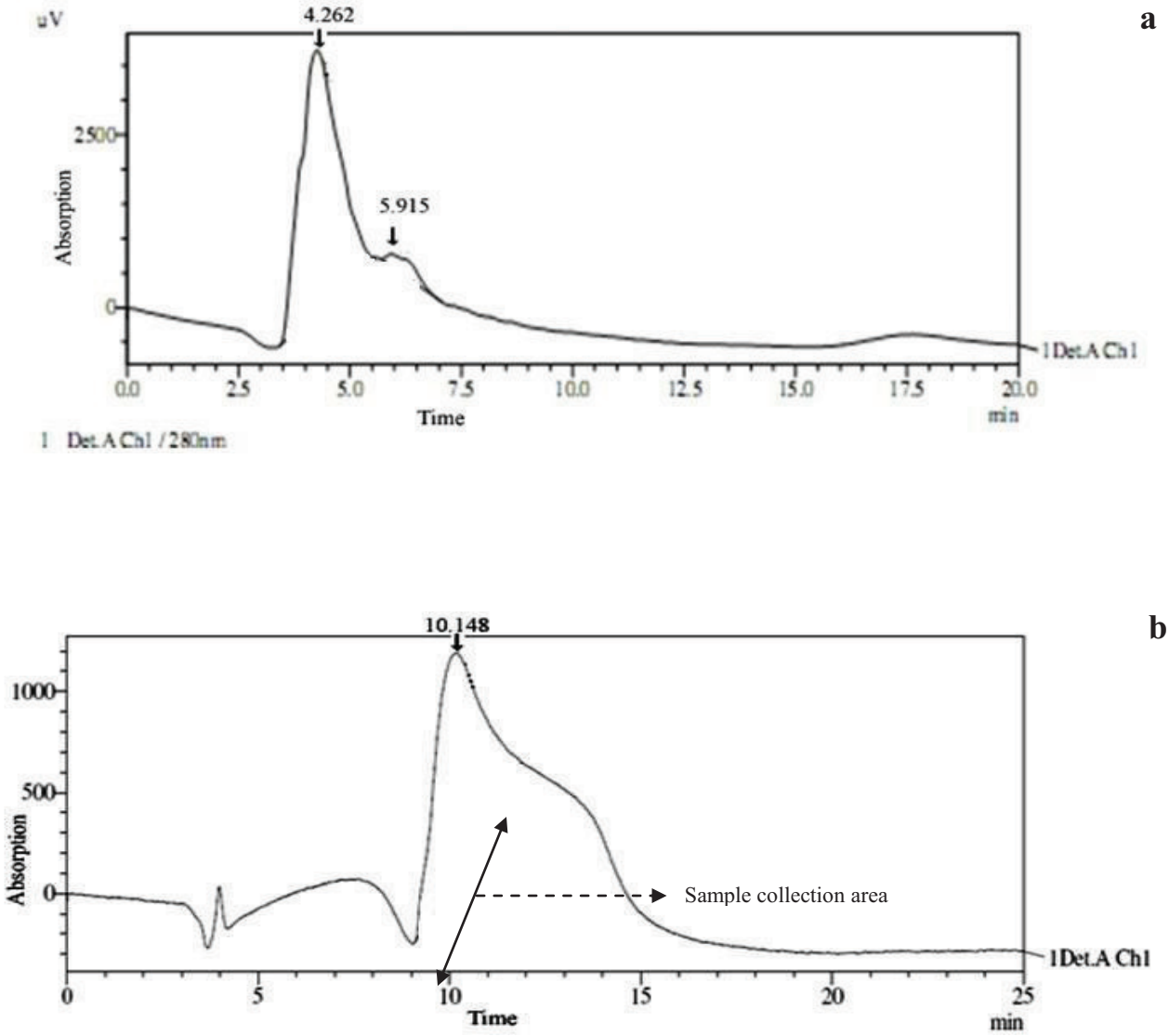


Figure 13. Chromatograms obtained by analytical (a) and preparative (b) HPLC (280 nm) for *B. bassiana* fraction II with peaks at approximately 4.262 and 5.915 min (analytical) and 10.148 min (preparative).

preparative HPLC to record major polypeptide/peak. It has a retention time of 10.184 min^{-1} (area 100%) with methanol as the solvent (Fig. 4b).

3.4.1.4. Matrix-assisted laser desorption/ionization-Time of Flight (MALDI–TOF)

The purified single peptide obtained by the preparative HPLC (FIIa) was analyzed by MALDI-TOF. A peak was obtained at the molecular weight of 174 Da and it was designated as BBF2 (Fig. 14). Unfortunately no specific molecules were identified by searching PMF databases using the MASCOT search engine.

3.4.2. Bioassay

Dose dependent mortality was recorded for FI, FII and FIII against the insects.FII was highly toxic to both insect rather than FI and FIII (Table 18, 19). When compared to the FIII, the LC_{50}values of FII were reduced by 27% and 38% at 96 hrs for *D. cingulatus* and *P. solenopsis* respectively. Significant difference in LC_{50} ($P<0.05$) was identified between FI and FII and FII and FIII in *D. cingulatus* and *P. solenopsis.*

3.4.3. Biochemical analyses

3.4.3.1. Digestive enzymes

The quantity of digestive enzymes such as amylase (F=54.50, df=5, 12, P=0.05), protease (F=60.20, df=5, 12, P=0.05) and lipase (F=9.827, df=5, 12, P=0.01) in *D. cingulatus* decreased while the concentration of FII was increased. Similar kind of observation was also recorded for *P. solenopsis* (F=43.60, df=5, 12, P=0.05; F=182.578, df=5, 12, P=0.05; F=9.926, df=5, 12, P=0.01 for amylase, protease and lipase respectively). Highly significant reduction was observed in 1600 ppm for both *D. cingulatus* and *P. solenopsis* (Fig. 15, 16, 17).

3.4.3.2. Detoxification enzymes

The quantity/activity of detoxification enzymes such as esterase (F=149.367, df=5, 12, P=0.05), glutathione s-tranferase (F=204.73, df=5, 12, P=0.05) and lactate

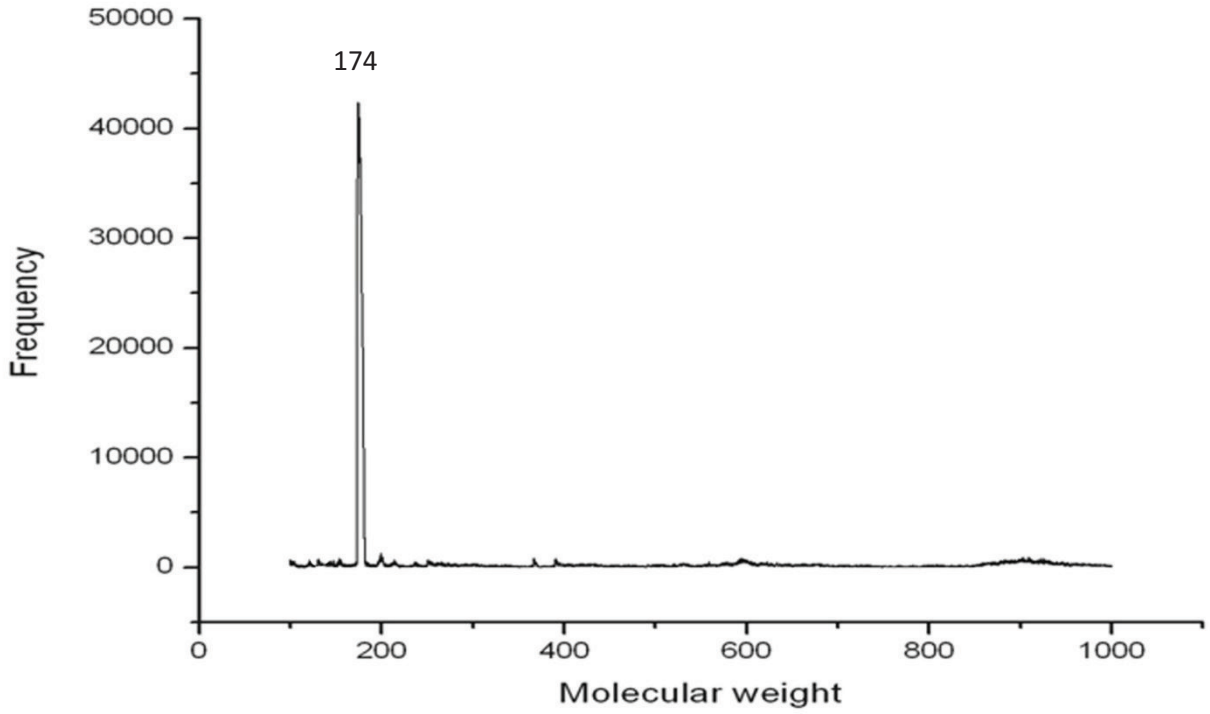


Figure 14. MALDI-TOF analysis of *B. bassiana* entomotoxic protein purified from fraction II (FII) using preparative HPLC.

Table 18. Corrected mortality of entomotoxin of *B. bassiana* (FII) fractionated with Sephadex G-25 column against *D. cingulatus* (third instar) at 96 hours using oral toxicity bioassay ($\overline{X}$±S.E; n=30)

Concentration (ppm)	Fraction I	Fraction II	Fraction III
100	10.3±0.33	10.3±0.33	3.3±0.33
200	21.9±0.45	33.3±0.14	10.3±0.33
400	33.3±0.33	45.6±0.14	18.7±0.24
800	41.2±0.12	51.3±0.21	20.5±0.02
1600	45.6±0.21	55.57±0.32	23.3±0.05
LC_{30}	637.3	240.1	1816.1
LC_{50}	1554.6	800.2	2991.3
LC_{90}	3796.5	3204.6	5865.5
Slope	5.3	5.7	3.6
Chi-square	13.38	26.3	10.3
Regression equation	Y=-486.1+36.3X	Y=-396.7+25.9X	Y=-297.3+60.3X

Table 19. Corrected mortality of entomotoxin *B. bassiana* (FII) fractionated with Sephadex G-25 column against *P. solenopsis* (adult) at 96 hours using leaf dip bioassay ($\overline{X}$±S.E; n=30)

Concentration (ppm)	Fraction I	Fraction II	Fraction III
100	21.43±0.03	20.69±0.02	6.90±0.02
200	25.00±0.03	27.59±0.03	10.34±0.12
400	39.29±0.05	44.83±0.14	17.24±0.03
800	50.00±0.04	51.72±0.12	20.69±0.03
1600	58.63±0.04	61.67±0.14	28.34±0.03
LC_{30}	227.0	119.9	1823.5
LC_{50}	871.14	713.3	9249.7
LC_{90}	23302.7	15970.4	489293
Slope	6.0	6.7	4.47
Chi-square	6.58	1.11	0.29
Regression equation	Y=-770.5+35.7X	Y=-729.5+32.7	Y=-525.9+68.6

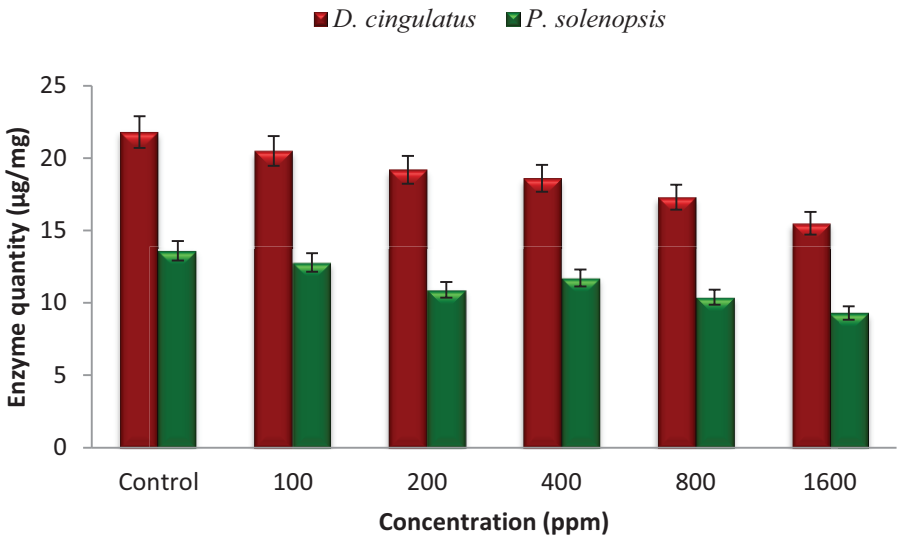


Figure 15. Quantitative analysis of amylase (µg/mg) in *D. cingulatus* and *P. solenopsis* treated with *B. bassiana* entomotoxic protein (FII).

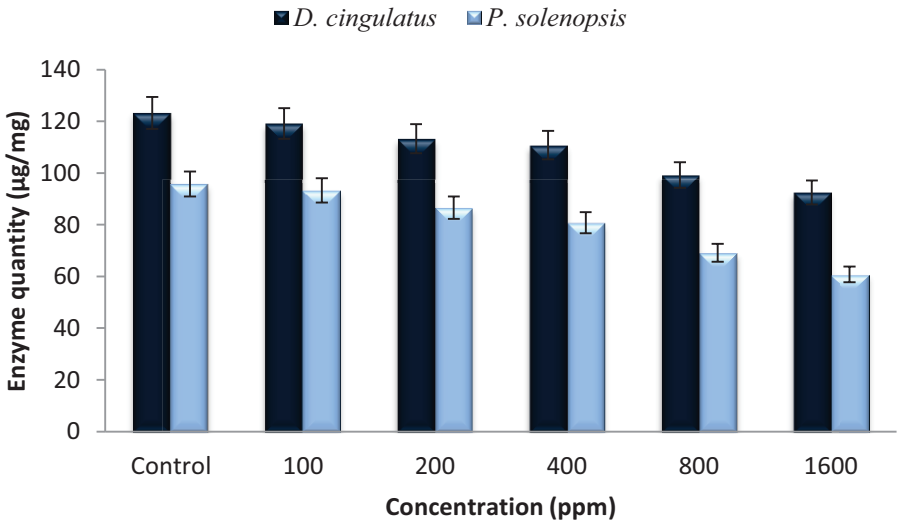


Figure 16. Quantitative analysis of protease (µg/mg) in *D. cingulatus* and *P. solenopsis* treated with *B. bassiana* entomotoxic protein (FII).

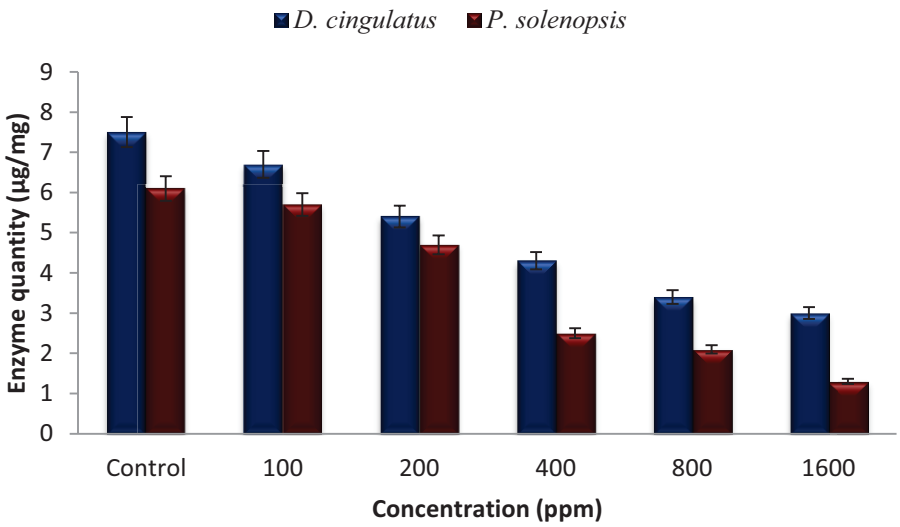


Figure 17. Quantitative analysis of lipase (µg/mg) in *D. cingulatus* and *P. solenopsis* treated with *B. bassiana* entomotoxic protein (FII).

dehydrogenase (F=17.185, df=5, 12, P=0.05) were increased when the concentration of *B. bassiana* fraction II (FII) increased in *D. cingulatus.* Also in *P. solenopsis,*the quantity/ activity of Esterase (F=36.706, df=5, 12, P=0.05), glutathione s-tranferase (F=132.73, df=5, 12, P=0.05) and lactate dehydrogenase (F=19.566, df=5, 12, P=0.05) were also increased at 1600 ppm (Fig. 18, 19, 20).

3.4.4. SDS-PAGE – Insect total body protein profile

The total body protein profile of *D. cingulatus* and *P. solenopsis* treated with different concentration of entomotoxin is shown in plate 4 and 5 respectively. The intensity of protein bands diminished when the concentration of the entomotoxin increased both in *D. cingulatus* and *P. solenopsis*. Totally nine peptide bands became visible for *D. cingulatus* in control category and it was sustained in lower concentrations (100 and 200 ppm) of FII. The concentration of FII increased the peptide band that disappeared and was the least in higher concentrations, such as 800 ppm (6) and 1600 ppm (7) (Plate 4). The same kind of result was observed for *P. solenopsis.* Eight bands appeared in control category and also in lower concentrations (100 ppm and 200 ppm) and 50% of bands were disappeared in higher concentration (1600 ppm) (Plate 5) (Table 20, 21).

3.5. DISCUSSION

Naturally occurring entomopathogenic fungi are important regulatory factors in insect populations. Many species are employed as biological control agents of insect pests under field and glasshouse crops, orchards, ornamentals, range, and lawn, stored products, and forestry and also used in vector insects of veterinary and medical importance (André *et al.,* 2007). Metabolites produced by entomopathogenic fungi would serve one or more of the following functions: (1) toxic to the host and help to cause death; (2) to aid the fungus overcome host defence; (3) to suppress competition from other pathogens and

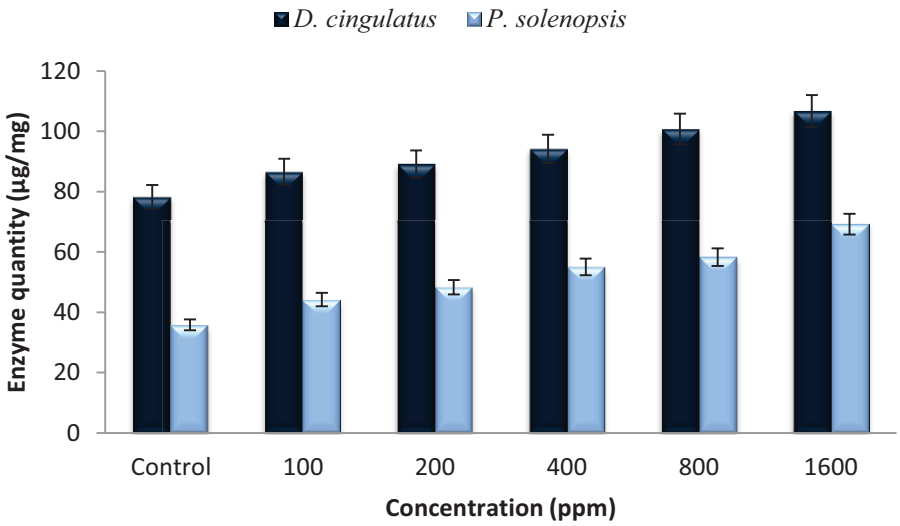


Figure 18. Quantitative analysis of esterase (µg/mg) in *D. cingulatus* and *P. solenopsis* treated with *B. bassiana* entomotoxic protein (FII).

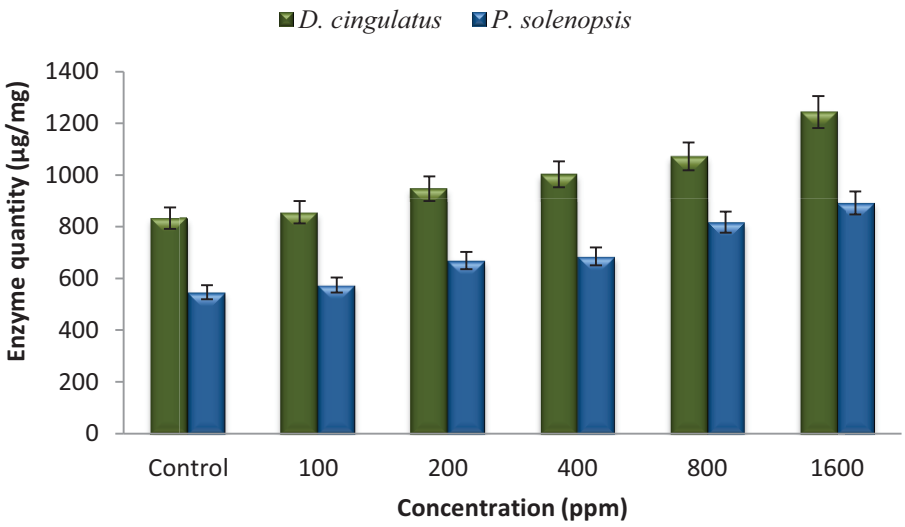


Figure 19. Quantitative analysis of glutathione S- transferase (µg/mg) in *D. cingulatus* and *P. solenopsis* treated with *B. bassiana* entomotoxic protein FII.

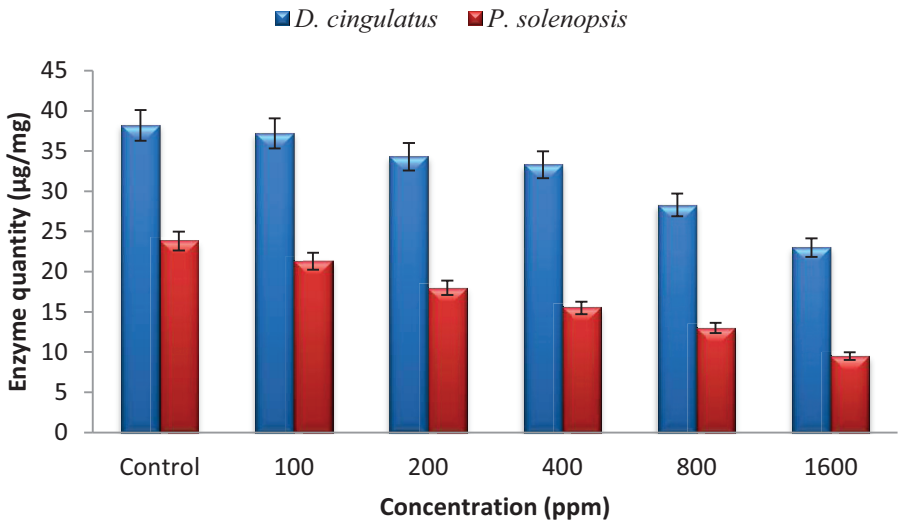


Figure 20. Quantitative analysis of lactate dehydrogenase (µg/mg) in *D. cingulatus* and *P. solenopsis* treated with *B. bassiana* entomotoxic protein FII.

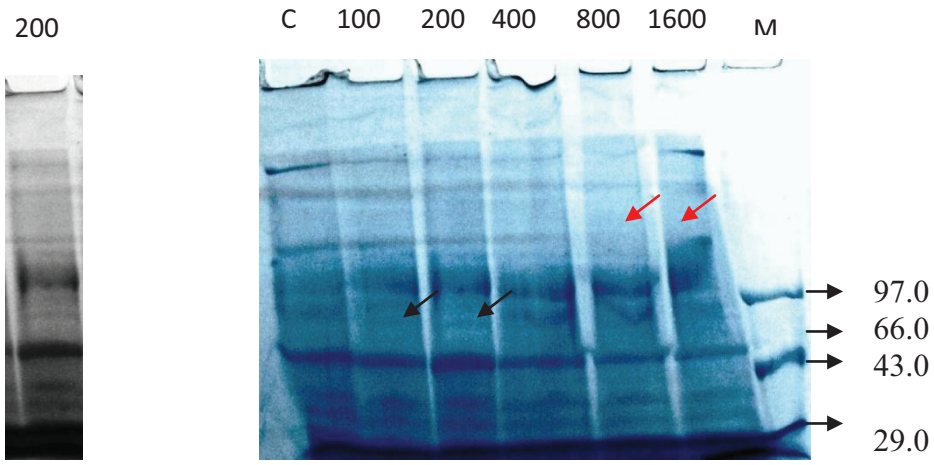


Plate 4. Protein banding pattern of *D. cingulatus* treated with *B. bassiana* entomotoxic Fracton II (FII) of Sephadex G-25 column at different concentrations (ppm). Dense appearance of protein pattern was showed separately. M - low molecular weight marker (kDa); C – control; ↙ - indicates induction of new polypeptides; ↙ - indicates disappearance of the polypeptides.

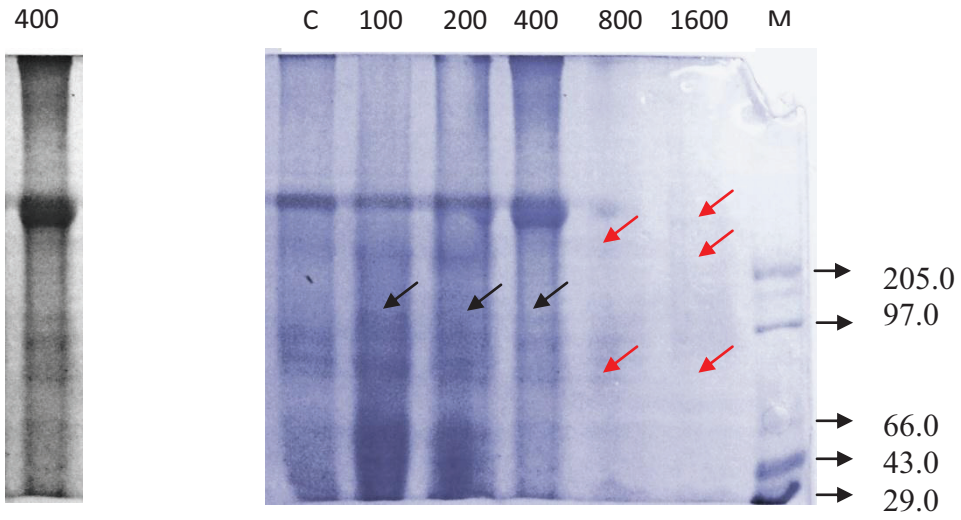


Plate 5. Protein banding pattern of *P. solenopsis* treated with *B. bassiana* entomotoxic Fracton II (FII) of Sephadex G-25 column at different concentrations (ppm). Dense appearance of protein pattern was showed separately. M - low molecular weight marker (kDa); C – control; ↙ - indicates induction of new polypeptides; ↙ - indicates disappearance of the polypeptides.

Table 20. Protein banding pattern (molecular weight in kDa) of *D. cingulatus* treated with *B. bassiana* entomotoxic Fracton II.

Band No	Marker	Control	100 ppm	200 ppm	400 ppm	800 ppm	1600 ppm
1	205.0	202.8	211.4	207.3	208.1	212.6	212.6
2	97.4	180.8	185.2	181.6	190.6	186.1	186.1
3	66.0	142.8	148.1	150.1	150.9	114.6	118.2
4	43.0	114.6	116.5	119.9	112.9	101.5	105.9
5	29.0	104.3	103.2	105.1	97.1	67.1	67.9
6		67.1	94.5	89.2	61.8	20.5	39.7
7		42.6	68.8	61.8	37.3		23.8
8		28.3	36.4	35.3	18.8		
9		16.9	23.0	21.3			

Table 21. Protein banding pattern (molecular weight in kDa) of *P. solenopsis* treated with entomotoxin FII.

Band No	Marker	Control	100 ppm	200 ppm	400 ppm	800 ppm	1600 ppm
1	205.0	227.4	224.6	225.5	212.2	221.8	143.4
2	97.4	200.6	189.2	190.1	145.3	139.7	129.1
3	66.0	131.9	142.5	149.1	124.5	129.2	117.6
4	43.0	116.7	138.8	136.9	102.4	113.9	69.1
5	29.0	104.3	117.6	115.7	69.2	66.3	-
6		68.2	110.2	99.6	45.2	-	-
7		39.6	75.6	67.2	-	-	-
8		33.7	64.2	45.2	-	-	-

saprophytes on the insect cadaver; (4) to provide a defence outside the host against mycophagous organisms (says Charnley, 2003).

Beauveria genera contain several pathogens which show enhanced pathogenicity against their host insects. The enhanced pathogenicity in these species is linked to toxin production, because nontoxin producing strains can be just as pathogenic as toxin producing strains if they are inoculated on toxin-sensitive host varieties in the presence of the host-selective toxin (Feng *et al*., 1994; Douglas *et al.,* 2000; Kaaya *et al*., 2002; Fang *et al*., 2005; André *et al.,* 2007; Fan *et al.,* 2007; Jiang *et al.,* 2008; Hamshou *et al.,* 2010; Bhagya Lakshmi *et al.,* 2010; Alex *et al*., 2012). All fungi probably produce mycotoxins, with low molecular weight and are generally considered nonvolatile (Vey *et al*., 2001; Kuhn and Ghannoum, 2003; Priyanka Dhar and Gurvinder Kaur. 2010). Quesada-Moraga and Vey (2004) isolated the intracellular proteins with specific medium under stress conditions and their insecticidal activity was tested against lepidopteran larvae. In the present study the intracellular proteins were precipitated using 90% saturated Ammonium sulphate solution and the efficacy evaluated against *D. cingulatus* and *P. solenopsis*.

Protein absorbs strongly at 280 nm due to a number of its constituent amino acids. The peptide bonds found in the amino acids also absorb at 205 nm (Urtz and Rice, 2000). In the present study the Sephadex column fraction was analyzed by UV-visible spectroscopy (Fig. 10). The result confirms that the fractions showed the absorption of proteins. In another analysis, the samples were subjected to FT-IR spectra. When the purification/specificity of the polypeptide increased, the quantity of protein content decreased (Urtz and Rice, 2000). The same findings were proved in the present study. The three entomotoxin fractions of *B. bassiana* showed low quantity level of proteins; on the other hand F II has the higher protein content (Fig. 11). The spectrum results showed the presence of proteins as secondary amines (Table 17, Fig. 12). Previous reports showed that

the filamentous fungi like *B. bassiana* had been shown to contain multiple types of enzymes (Vey *et al*., 2001; Quesada-Moraga and Vey, 2004; Fang *et al*., 2005; Fan *et al.,* 2007; Wang and St. Leger, 2007; Pava-Ripoll *et al.,* 2008; Molnar *et al*., 2010; Rohlfs and Churchill, 2011; Safavi, 2013). Vipul *et al.* (2005) reported the presence of protein in the metabolic mixture of *Fusarium oxysporm* which was identified using FT-IR spectra at the absorption range of 1500–1600 cm^{-1}. Present results also confirm the presence of protein in the range of 1400-1600 cm^{-1} in *B. bassiana* toxic fractions in the form of secondary amines/amides. FT-IR spectroscopy is a sensitive method to analyses the presence of protein in the fungal metabolites (Vipul *et al.,* 2005). In the present fractions, the peaks represent not only secondary amines, but also the presence of alcohols (3300-3400 cm^{-1}), ketons (1100-1200 cm^{-1}) and aldehydes (600-700 cm^{-1}) with respective ranges.

Analytical HPLC spectrum of *B. bassiana* entomotoxin revealed two polypeptides present in F II at the retention time of 4.2 min (95.6%) and 5.9 min (4.4%). Previously, Carlos *et al.* (2010) reported tyrosine betaine, a novel entomotoxic secondary metabolite from the fungus *Metarhizium* sp. having the retention time of 4.1 min. In another study Muraleedharan and Sheela Devi (1999) identified a neuropeptide from midgut of Colorado potato beetle by HPLC at the retention time of 4.4 min. These findings clearly showed that a toxic peptide was identified at the retention time of 4.1–4.4 min in HPLC spectrum. Hence, the result obtained from the present study confirms the toxic nature of *B. bassiana* fraction II.Themajor peak of FII was collected by preparative HPLC at the retention time of 10.1 minand later identified with MALDI-TOF showed 172 Da molecular weight. No previous report is available about this fraction.

Most fungal toxins are secondary metabolites and low molecular mass compounds (Charnley, 2003). Well-characterized classes of toxins include polyketides (e.g. aflatoxins

– 28 kDa), cyclic peptides, alkaloids and sesquiterpenoids (e.g. trichothecenes) (Chutao *et al.,* 2007).The same kind of result was reported by Andre *et al.* (2007).

In most cases, application of purified toxin is sufficient to cause death in susceptible insects (Si *et al.,* 2008; Cuthbertson *et al.,* 2009). This implies that by killing the insects with a secreted, soluble toxin, the fungi are able to circumvent the innate defenses of insects. Most of the studies reported the insecticidal activity of different strains of *B. bassiana* spores at different concentration (Alex *et al.,* 2012). Few reports are available about the insecticidal activity of *B. bassiana* toxins such as beauvericin, destruxins, bassianin etc. against insect pests (Russell, 2006; Li *et al.,* 2006; Lu *et al.,* 2008; Molnar *et al.,* 2010; Qian *et al.,* 2010; Safavi, 2013). In the present study, the *B. bassiana* entomotoxin FII showed high mortality against *P. solenopsis* (61.7%) and *D. cingulatus* (55.5%).Consumption oftoxic substances makes complicated physiological changes in insects (Griffitt *et al.,* 2008). There are several reports available about the insecticidal activity and after effects of *B. bassiana* toxic metabolites against insect pests (Hajek, and St. Leger, 1994; Feng *et al.,* 1994; Vey *et al*., 2001; Quesada-Moraga and Vey, 2004; Fang *et al*., 2005; Fan *et al.,* 2007; Wang and St. Leger, 2007; Pava-Ripoll *et al.,* 2008; Molnar *et al*., 2010; Rohlfs and Churchill, 2011; Safavi, 2013). Beauvericin (M.W. 28 kDa) (Safavi, 2013), destruxins(M.W. 22 kDa) (Strasser *et al*., 2000), bassianin (M.W. 18 kDa), bassianolide (M.W. 390 kDa), beauverolides (M.W. 72 kDa) (Vey *et al*., 2001), tenellin (M.W. 60 kDa) (Zimmermann, 2007), oosporein (M.W. 83 kDa) (Gupta *et al*., 1995) bassiacridin (M.W. 89 kDa) (Quesada-Moraga and Vey, 2004) are some of the important metabolites of *B. bassiana*.

Among them, Beauvericin is the most important virulent compound which was reported from *B. bassiana* (Quesada-Moraga and Vey, 2004).The preoteolytic enzymes from *B. bassiana* were intensively studied as the mortality factor (Bhagya Lakshmi *et al.,*

2010; Priyanka Dhar and Gurvinder Kaur, 2010; Uzma Mustafa and Gurvinder Kaur, 2010) Protease is one of the most important and earliest enzymes involved in the invasion followed by chitinase after the eventual exposure of chitin in the host cuticle after the proteolytic degradation of cuticular proteins (Shimizuy *et al*., 1993; Lacey *et al*., 2001). Dose dependent mortality was recorded in the present study. Higher mortality in *D. cingulatus* and *P. solenopsis*was observed after 96 hours of the exposure at higher concentration (1600 ppm) of FII. It must be said that very few pieces of literature are available about the effect of toxins from *B. bassiana* against live insects; most studies were proven enzymatically under spectrometric conditions (Savafi, 2013).

Amylase, protease (Uzma Mustafa and Gurvinder Kaur, 2010) and lipase (López-Rodríguez *et al.,* 2012) play a key role in the digestion of plant starch, proteins and lipids respectively in insects. Result reveals the digestive enzyme activities decreased with increasing concentration of *B. bassiana* FII fraction, leading to improper digestion of proteins to amino acid conversion.Further, the level of amylase decreased directly proportional to the *B. bassiana* entomotoxin FII concentration. Mycotoxins interrupted the secretion of digestive enzymes and further led to the disruption of gut physiology (Sahayaraj *et al.,* 2010). Similarly in this study, the protease levels decreased due to effect of *B. bassiana* entomotoxin FR II. Another suggestion proposed by researchers is decreased protease level can be due to the impact of mycotoxins on neurosecretory cells of the insects (Sun *et al.,* 2000; Kucharski and Maleszka, 2003; Isaka *et al*., 2005; James *et al*., 2010; López-Rodríguez *et al.,* 2012). The decreased lipase levels in the treated insects is due to the improper digestion of lipids and thus alter the physiological processes like reproduction, growth and defence against pathogens as recorded by Senthil Nathan *et al.* (2006a, b) and Zibaee and Bandani (2009).

Esterase and glutathione S-transferase play a significant role in insecticide metabolism which is supported by the results of Yang (1976) and Alizadeh *et al.* (2010), Marsha *et al.* (2010), Santo *et al.* (2011). Higher GST level in insects indicates, resistance developed by insects is not only due to esterase activity but also due to the activity of GST. In general, the increased levels of esterase and glutathione S-transferase due to more catalytically efficient enzyme able to hydrolyse the insecticides in insects (Conyers *et al.,* 1998; Isabela *et al.,* 2012) as recorded in this study.

Lactate dehydrogenase involved in the production of energy is particularly important when a considerable amount of additional energy is rapidly required (Senthil Nathan *et al.,* 2006b). Results shows in control category, significantly maximum LDH activity was observed in the midgut whereas in the treatment it was reduced. Therefore this enzyme may be a sensitive criterion for entomotoxin as reported for pesticide exposure by Diamantino *et al.* (2001).

The SDS-PAGE analyses of *D. cingulatus* and *P. solenopsis* revealed that *B. bassiana* entomotoxic fraction II has high impact on the total body protein content of the insects. When compared with the control category, in higher concentration treatments the protein bands disappeared and new protein bands appeared as reported by Vey *et al.* (2001), in *Galleria mellonella.* They observed that the toxin beauveriacin altered the total body protein profile of the animal.

3.6. CONCLUSION

From the study, it is concluded that the entomopathogen of *B. bassiana* can be utilized for cotton pest management. The application of toxin under field condition requires further processing. In the present study we identified the *B. bassiana* entomotoxic protein (FII) supported by UV-visible spectroscopy, FT-IR spectroscopy, HPLC and MALDI-TOF analyses and its insecticidal activity against two major cotton pests. The

molecular weight of the FII was identified as 172 Da. FII showed high impacts on *D. cingulatus* and *P. solenopsis* under laboratory condition. The quantities of digestive enzymes were decreased and detoxification increased in cotton pests which indicate that FII also affects the physiology of insects. Hence it is concluded that the entomotoxic protein and its SNP's can be utilized for the management of *D. cingulatus* and *P. solenopsis,* also it can be used as the component of BIPM.

CHAPTER 4

4.0. Biogenesis, characterization and application of biosilvernanoparticles

4.1. ABSTRACT

In the present study, biogenesis of silver nanoparticles and their activity on two cotton pests and one natural enemy were investigated. AgNPs were rapidly synthesized using protein fraction of *Beauveria bassiana* Bals. (Ascomycota: Hypocreales) and the formation of NPs was observed within 30 minutes. The result recorded from UV-Visible spectrophotometer, FT-IR, XRD and TEM support the biosynthesis and characterization of AgNPs. From the TEM analysis, the size of the AGNPs was measured 4-11 nm (mean 10.7±0.04 nm). It was evident from FT-IR result that primary amines (protein) (1435 cm^{-1}) act as capping as well as stabilizing agent. Further, the insecticidal activity of synthesized AgNPs causes 27.8 and 33.7 per cent mortality against *Dysdercus cingulatus* Fab. (Hemiptera: Pyrrhocoridae) and *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae) respectively within 96 hrs after exposure at 1600 ppm. However, *B. bassiana* toxic protein mediated AgNPs did not affect the predatory behavior and biocontrol potential of a natural enemy *Rhynocoris fuscipes* Fab. (Hemiptera: Reduviidae).

Key words: Biosafety, Biosilver nanoparticles, characterization, insecticidal activity.

4.2. INTRODUCTION

Development of biologically inspired experimental processes for the synthesis of nanoparticles is an important branch of nanotechnology. The biosynthesis of nanoparticles has received increasing attention due to the growing need to develop safe, cost-effective and environmentally friendly technologies for nano-materials synthesis. Among all nanomaterials such as silver (Ag), Gold (Au), Platinum (Pt), Palladium (Pd), Titanium (Ti), Zinc (Zn), Copper (Cu) etc. silver nanoparticles play a significant role in the field of biology, agriculture and medicine due to its active physiochemical properties.

Biological methods of nanoparticles synthesis using microorganism (Klaus *et al.*, 1999; Nair and Pradeep, 2002; Konishi *et al.*, 2007; Sangappa and Thiagarajan, 2012), and their enzyme (Wilner *et al.*, 2006; Jeevan *et al*., 2012) were well documented in the literature. However, exploration of pathogenic fungi as the potential nanofactories, has more interest in the biological synthesis of nanoparticles. Furthermore, fungi have been known to secrete heightened quantities of bioactive substances, which made fungi more suitable for large-scale production (Narayanan and Sakthivel, 2010). In addition, the extracellular biosynthesis of nanoparticles using fungi could make downstream processing much easier than bacteria (Mohanpuria, *et al.,* 2008). Though *B. bassiana* has been utilized for pest management worldwide (Russell, 2006; Nicolás *et al.,* 2007; Bonnie *et al.,* 2008; Fernando *et al.,* 2009; Wraight *et al.,* 2010; Alex *et al.,* 2012; López-Rodríguez *et al.,* 2012), it has not been utilized/tested for biosynthesis of silver nanoparticles or any other nanoparticles.

Biosynthesized or green nanoparticles are gaining momentum in the field of agriculture for pest (Graves, and Haystead, 2002; Chakraborty *et al.,* 2010; Nitai *et al.,* 2012) and disease management (Shankar *et al*., 2004; Mohanpuria *et al.,* 2008). Since the nanoparticles were prepared by biological methods, proteins were used as capping agents making a stand. The most commonly used proteins in the synthesis of nanoparticles are ferritin and apoferritin. Hence these methods have been changed for the bioreduction using fungi, including *Fusarium acuminatum* (Ingle *et al.,* 2008) and *Penicillium fellutanum* (Kathiresan, *et al.,* 2009). The extra cellular proteins produced by the fungi cause reduction and later they act as capping agents (Ahmad *et al.,* 2003). Despite these impressive results, there are no reports available for synthesizing silver nanoparticles using *Beauveria bassiana* entomotoxic proteins and the detailed mechanism have not been elucidated.

Keeping this lacuna in our mind, the present study was aimed at rapidly synthesizing AgNPs using protein fraction of *B. bassiana* and evaluate their antipest activity against cotton pests *viz. Dysdercus cingulatus* Fab. (Hemiptera: Pyrrhocoridae) and *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae). In addition, impact of this biological nanomaterial against a biological control agent *Rhynocoris fuscipes* Fab. (Hemiptera: Reduviidae) (Sahayaraj and Sujatha, 2012) was studied to validate the biosafety of synthesized silver nanoparticles.

4.3. MATERIALS AND METHODS

4.3.1. Protein precipitation and purification

The well sporulated fungal culture was filtered through a Whatman No. 1 filter paper. The matt was separated; freeze dried at $^{-}4^{0}$C and ground with ice cold 90% saturated Ammonium sulphate solution for protein precipitation (Quasada-Moraga and Vey, 2004). The precipitated protein was collected by centrifugation at 8000 rpm for 30 minutes. The crude protein was subjected to a gel filtration through a Sephadex G-25 (Sigma) column (1 x 10 cm) in 50 mM tris/HCl buffer at P^{H} 8.0.

4.3.2. Biogenesis of silver nanoparticles using fungal toxin

Silver nitrate was obtained from HiMedia, Mumbai, India (99.9 %). All glasswares were washed with deionised water and dried in an oven at 50-60^{0}C before use. The broth used for reduction of $AgNO_3$ molecules to Ag^0 was prepared by taking 10 mL of protein solution in a 250 mL Erlenmeyer flask. This protein solution was added to 90 mL of 10^{-3} M $AgNO_3$ aqueous solution.

4.3.3. Characterization of silver nanoparticles

4.3.3.1. UV-vis spectral analysis

The bioreduction of the $AgNO_3^-$ ions in a protein solution was monitored by periodic sampling of aliquots (2 mL) of the aqueous content and measuring the UV-visible

spectrum of the solution. UV-visible spectra of these aliquots were monitored as a function of time of reaction on the sample.

4.3.3.2. FT-IR Spectroscopy

For Fourier transform infrared (FT-IR) spectroscopy measurements, bioreduced silver nitrate solution drop coated on a Si (III) substrates was carried out on a Perkin Elmer, Spectrum (Japan) model RX-I, in the diffuse reflection mode at a resolution of 4 cm^{-1}.

4.3.3.3. Powder X-Ray diffraction

Powdered X-Ray diffraction (XRD) measurements of the bioreduced silver nitrate dried powder on to glass substrates were done on a Schumadzu, XRD 6000 (Japan) instrument operating at a voltage of 45 kv and a current of 30 mA with Cu Kα radiation. The particle size was calculated using Scherrer formula:

$$d = \quad 0.9\lambda / \beta\cos\theta$$

where d is the mean diameter of the nanoparticles, λ, the wavelength of X-ray radiation source and β, the angular FWHM of the XRD peak at the diffraction angle θ (Culity, 1978). Powder samples for analysis were prepared by centrifugation at 13,000 rpm for 15 minutes, redispersed in sterile distilled water to get rid of any uncoordinated biological molecules. Centrifugation and re-dispersion were repeated thrice in order to ensure better separation.

4.3.3.4. TEM analysis of silver nanoparticles

For Transmission Electron Microscopy (TEM) studies dried nanoparticle were placed onto carbon coated copper grids and measurements were taken on a JEOL model 3010 and Philips CM-200, Japan instrument operated at an accelerating voltage at 120 kV. Selected Area Electron Diffraction Pattern (SAED) was obtained using TEM. The size and

shape of bioreduced nanoparticles were manually interpreted separately by observing 100 particles randomly according to the shapes and sizes.

4.3.4. Insecticidal bioassay of biosilver nanoparticles

A colony of the test insects, *D. cingulatus* and *P. solenopsis* were established in the laboratory from cotton field collected and healthy insects reared under laboratory condition (28±2° C temperature, 70-75 RH, 11L: 13D photoperiod). Red cotton bugs were reared in plastic box (13 cm height x 7 cm diameter) on artificial diet (Sahayaraj *et al.,* 2011), whereas *P. solenopsis* was reared in plastic trough (13 cm height x 30 cm diameter) on fresh pumpkin (Venkatesha and Dinesh*,* 2012).

Bioassay for the effects of bioreduced silver nanoparticles on 0-day old third instar *D. cingulatus* nymphs (43.7±0.3mg) was determined by incorporating bioreduced silver nanoparticles in different concentrations (100, 200, 400, 800 and 1600ppm). A 5 mg of cotton swap dipped in artificial diet was provided to the red cotton bug continuously for 4 days. Instead of bioreduced nanoparticles distilled water was used in the control category. For *P. solenopsis* (0-day old) adults (17.4±0.3mg), young cotton leaf (SVPR IV variety) was dipped in above mentioned concentration of bioreduced nanoparticles (50 µL, 0.05% tween 80 was added for 10 mL sample as adjuvant) and provided to the insect continuously for four days. The experiments were carried out with 10 replicates each of them consisting of 3 insects. Insect mortality was recorded after 24 hrs till 96 hrs. Experiments were conducted in Petri dish (9 x 1 cm).

4.3.5. Biosafety

To assess the biosafety, bioreduced silvernanoparticles were sprayed on the *R. fuscipes* third instar (43.7±0.3mg) using hand sprayed (0.5 mL/nymph) and maintained on fourth and fifth instar larvae of *Corcyra cephalonica*. The mortality of insects was recorded for every 24 hrs up to 96 hrs continuously. Each treatment was replicated 6 times

with five animals each. After 96 hours of the exposure, the live predator was maintained with fourth and fifth instar of *C. cephalonica* larvae till their death. During the experiments, the nymphal developmental period, adult longevity, sex ratio, pre-oviposition period, oviposition period, post-oviposition period and fecundity of the reduviid predator were recorded. Along with silver nanoparticles, *B. bassiana* entomotoxic protein fraction II also was tested. Tris HCl buffer and silver nitrate solution were used as the controls. In each experimental sample 30 individuals were maintained.

4.3.6. Bioefficacy

In another experiment, 10 predators from each category were used to evaluate the bioefficacy under plastic cup enclosed with glass chimney as shown in Fig. 21a. A 20 days old cotton plant (SVPR IV) was maintained in a disposable glass pot (10 cm height X 8 cm upper diameter and 5 cm lower diameter) (Fig. 21b). Then the plant was covered with a glass chimney (15 cm height x 7 cm diameter) and was placed inside the plastic trough (15 cm diameter x 14 cm height). To evaluate the bioefficacy of *R. fuscipes* third instar nymphs (28.7±0.08 mg), 24 hours pre-starved predators were used. Five *P. ricini* third instar (38.5±0.04) larvae were introduced onto the cotton plant and allowed to settle for 15 min. Then a pre-weighed predator was introduced and maintained under room conditions as mentioned above. After 24 hrs, the number of preys consumed or killed was recorded. The weight gain of the predator was also calculated.

4.3.7. Statistical Analysis

Individual data of developmental time, adult longevity, fecundity, oviposition periods and bioefficacy of the reduviid treated with fungal toxin and bioreduced silver nanoparticles were compared using one-way analysis of variance (ANOVA) and post ANOVA Tukey's test using SPSS 20.0 version. All statistical tests were carried out at 5% level of significance ($\alpha = 0.05$).

Figure 21. Experimental arena for bioefficacy evaluation of *R. fuscipes* against *P. ricini* in glass chimney condition (a), cotton plant in dispossible glass pot (b).

4.4. RESULT

The biosilver nanoparticles were synthesized using entomotoxic protein fraction of *B. bassiana* (Fig. 22) biomass within 30 minutes after the addition of silver nitrate solution (Fig. 23a). The silver nanoparticles' surface plasmon resonance (SPR) bands occur in the range of 429 – 440 nm in an aqueous medium. The bioreduced silver nanoparticle was stable and highly intensified SPR bands initially occurred at 440 nm after 2 hrs (Fig. 23b) at room temperature. Initially turbid coloured solution turned into deep brown. After completion of reaction of the silver ions with the *B. bassiana* entomotoxic protein (*ie*, after 30 minutes of reaction), the silver nanoparticles solution was tested for stability. It was observed that nanoparticles solution was stable for more than 60 days (Fig. 23c) with little aggregation.

4.4.1. FT-IR spectroscopy

The silver nanoparticles synthesized using the *B. bassiana* protein fraction were subjected to FT-IR analysis to identify (if possible) the biomolecules stabilizing the nanoparticles in solution and also to provide clue as to what the reducing agent might be. The silver nanoparticles synthesized using *B. bassiana* protein fraction showed strong bonds at 1638 (Fig. 24a, 24b) (Table 22). This band corresponds to the amide II bands of polypeptide/protein.

4.4.2. XRD analysis

XRD analysis showed seven distinct diffraction peaks at 17.8, 33.6, 38.2, 48.3, 55.1, 64.6 and 77.6 which indexed the planes 111, 190, 300, and 378 of the cubic face centered silver. The lattice constant calculated from this pattern was $\alpha = 4.086\ ^{0}A$ and the data obtained were plotted (Fig. 25).

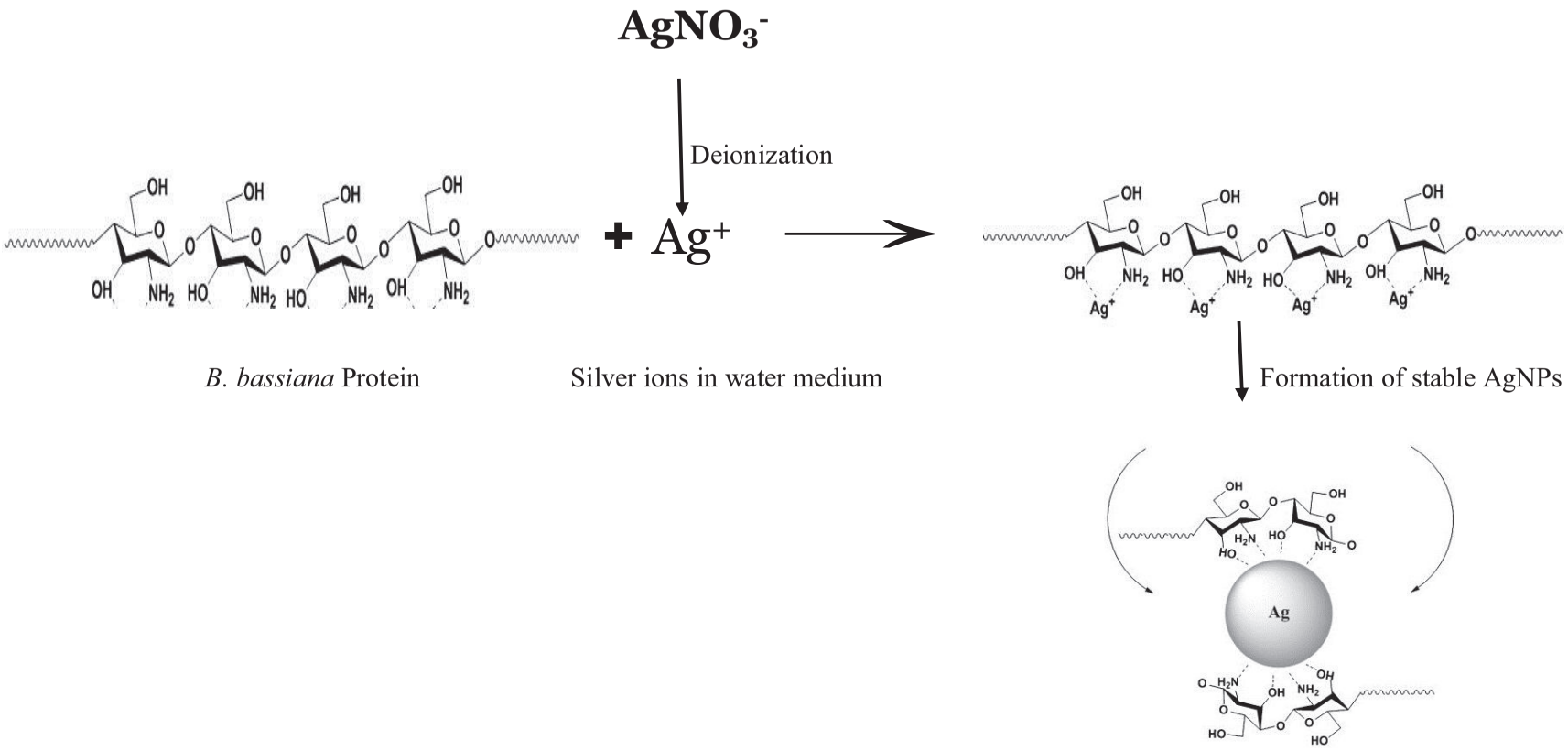


Figure 22. Schematic representation of formation of silvernanoparticles by *B. bassiana* protein (FII) separated with Sephadex G-25 column.

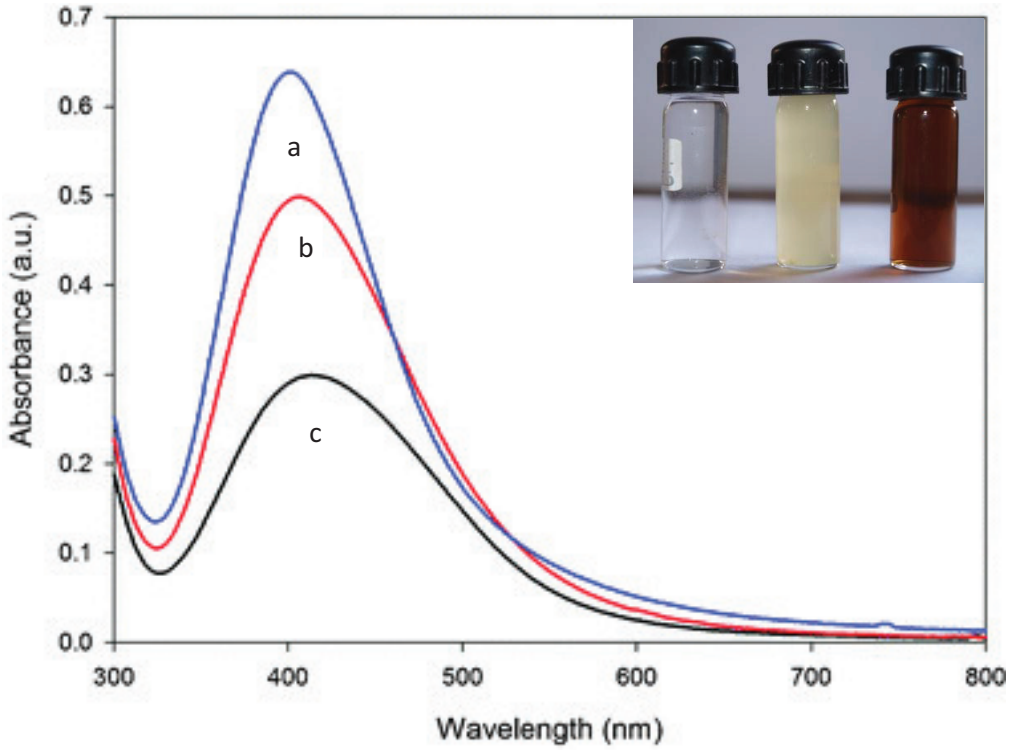


Figure 23. UV-visible spectrum of biosynthesized silver nanoparticles after 30 minutes (a), after 2 hours (b) and after 60 days (c).

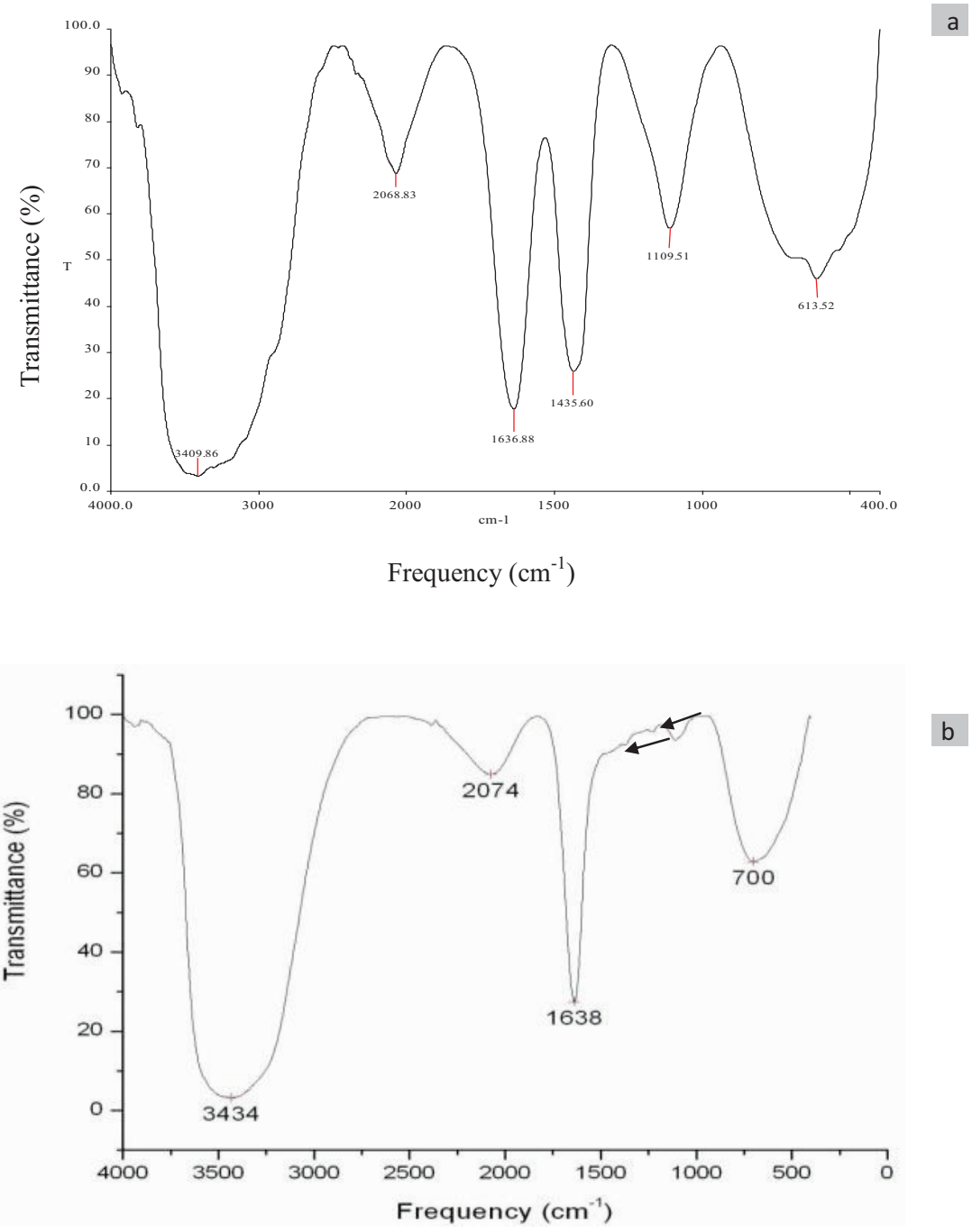


Figure 24. FT-IR analysis of *B. bassiana* protein fraction (a) and bioreduced silver nanoparticles (b). The arrow mark represents the replacements.

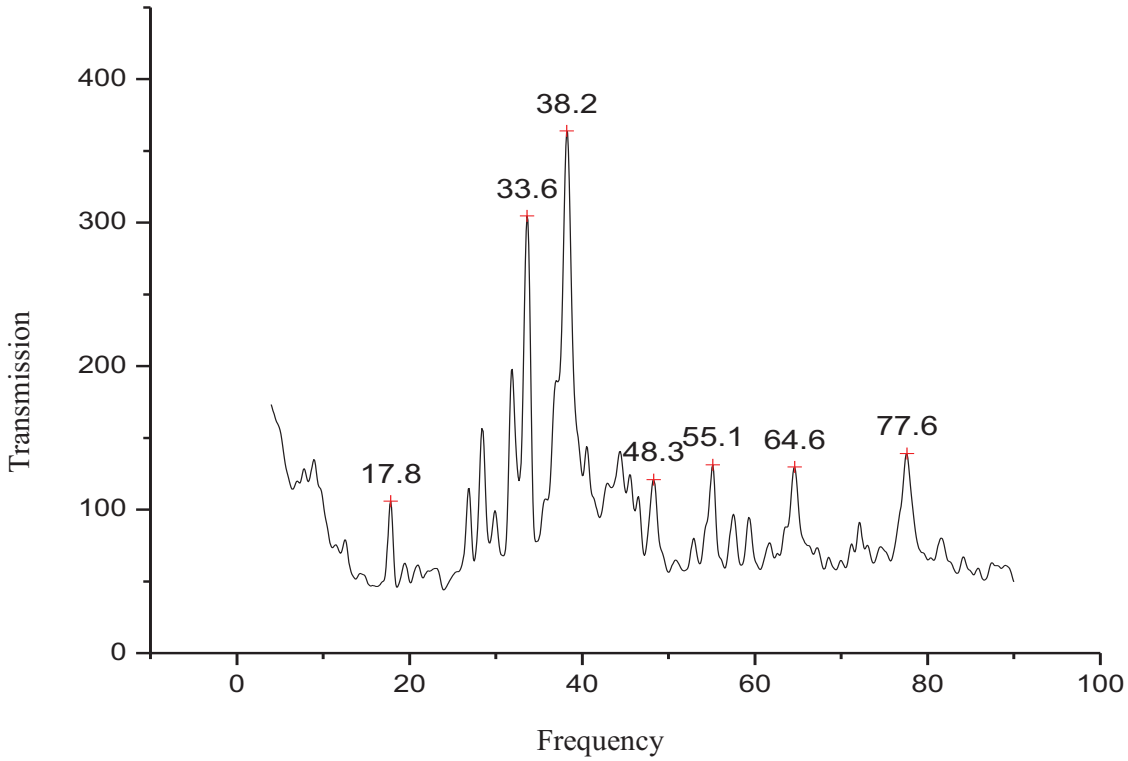


Figure 25. XRD pattern of bioreduced silver nanoparticles produced by *B. bassiana* protein fraction

Table 22 . FT-IR analysis of silver nanoparticles synthesized with *B. bassiana* entomotoxic fraction II eluted by Sephadex G-25 column separation

***B. bassiana* protein**		**AgNP's**	
Frequency (cm^{-1})	**Functional group**	**Frequency (cm^{-1})**	**Functional group**
3409	O-H stretching (H-bonded alcohols and phenols)	3434	O-H stretching (H-bonded alcohols and phenols)
2068	Unknown	2074	Unknown
1636	N-H bend (Secondary amine)	1638	N-H bend (Secondary amine)
1435	N-H bend (Secondary amine)	700	C-Br bending (aldehydes)
1109	C-O stretch (ketons)		
613	C-Br bending (aldehydes)		

4.4.3. TEM analysis

TEM analyses of the synthesized nanoparticles were clearly distinguishable owing to their size difference. From the TEM image the size of the synthesized silver nanoparticles was measured 4 – 12 nm (Fig. 26a, 27) with more number of spherical (88%) rather than hexagonal (10%) and triangular (2%) shapes (Fig. 26b, 26c, 26d, 28). The nanoparticles obtained are highly crystalline in nature.

4.4.4. Insecticidal activity of bionanoparticles

Bionanoparticles showed moderate or low toxicity against *D. cingulatus* (27.8%) (Table 23) and *P. solenopsis* (33.7%) at 96 hrs in 1600 (Table 24). The mortality data were analyzed with SPSS 20.0 software followed by One-way ANOVA and Tukey's test to compare effects among treatments. The results were expressed as mean (±S.E.) and considered significantly different at $P<0.05$. Probit analysis was used for estimation of the lethal concentrations (LC_{30}, LC_{50} and LC_{90}) by the SPSS software (version 20.0).

4.4.5. Biosafety

Fungal entomotoxin and silver nanoparticles showed very low mortality (6.66%) to *R. fuscipes* under laboratory condition at 1600ppm. The tested materials had no influence on the feeding behavior (approaching time and handling time) and bioefficacy (number of prey consumed) of the reduviid predator (Table 25) (Fig. 29).

4.4.6. Biology

The nymphal developmental time of the predator from third instar to adult was moderately affected by the treatments. The bionanomaterial slightly reduced the third instar ($F=5.030$, df=3,26, $P=0.007$), fourth instar ($F=0.115$, df=3,26, $P=0.976$) and fifth instar ($F=2.959$, df=3,26, $P=0.029$). BBTF2 also resulted in reduction of nymphal developmental periods of *R. fuscipes.* It reduced the third ($F=2.851$, df=3,26, $P=0.057$), fourth ($F=2.336$, df=4, 20, $P=0.091$) and fifth ($F=1.909$, df=4,20, $P=0.330$) nymphal

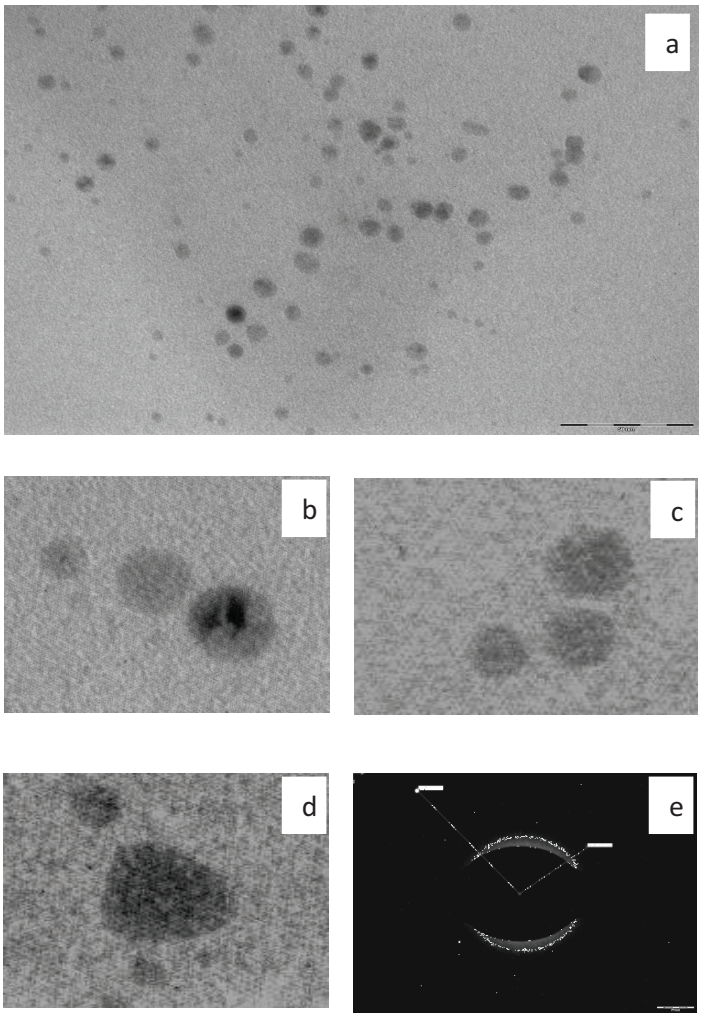


Figure 26. TEM image of synthesized silver nanoparticles at lower magnification (a), different shapes of nanoparticles, spherical (b), hexagonal (c) and triangular (d) and electron valance (e).

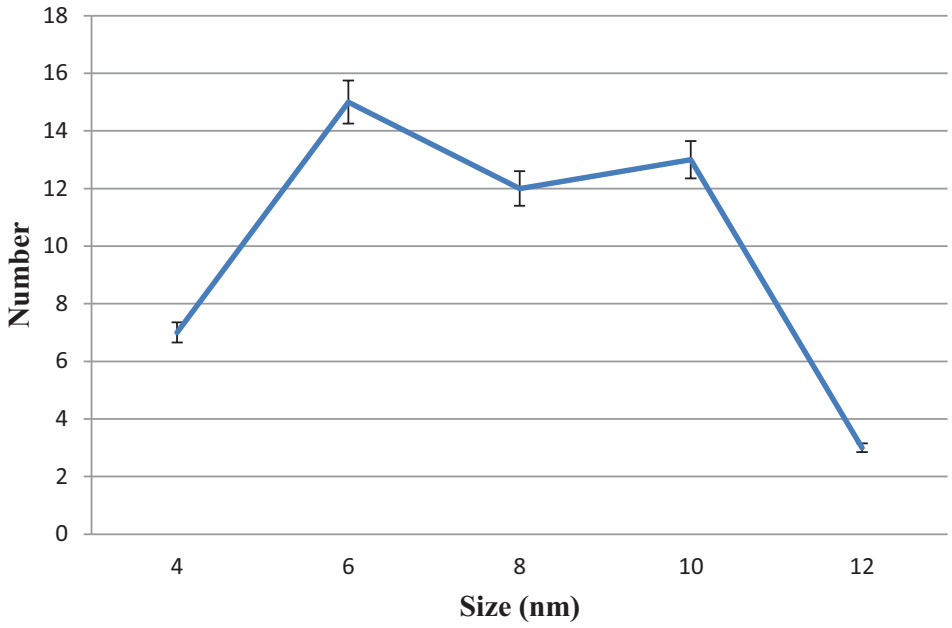


Figure 27. Different sized silver nanoparticles produced by *B. bassiana* entomotoxic protein.

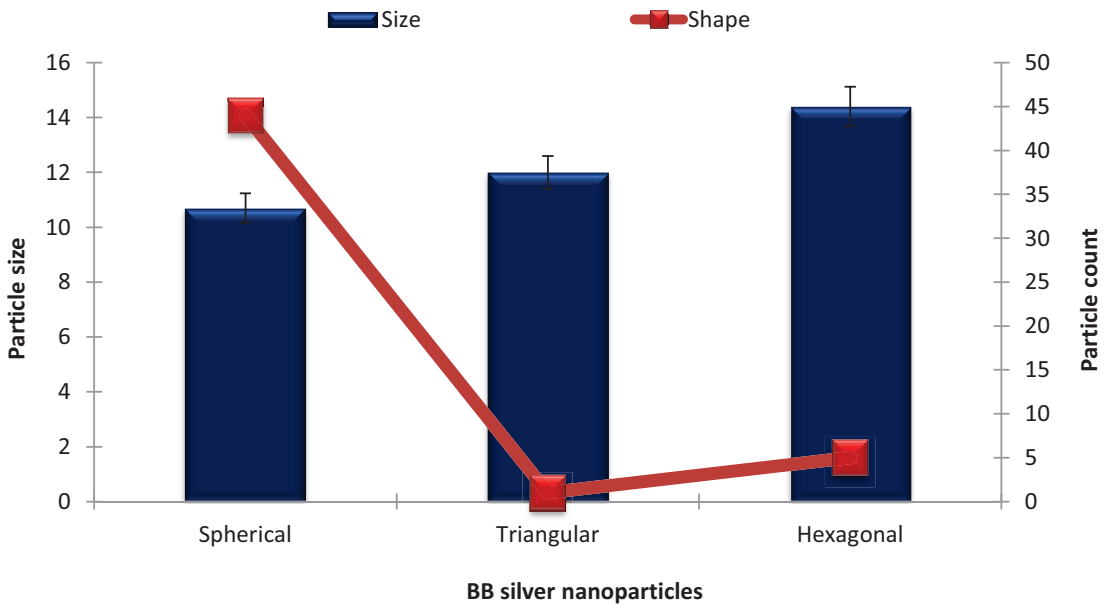


Figure 28. Spherical, triangular and hexagonal shape and their size of silvernanoparticles reduced by *B. bassiana* entomotoxic protein.

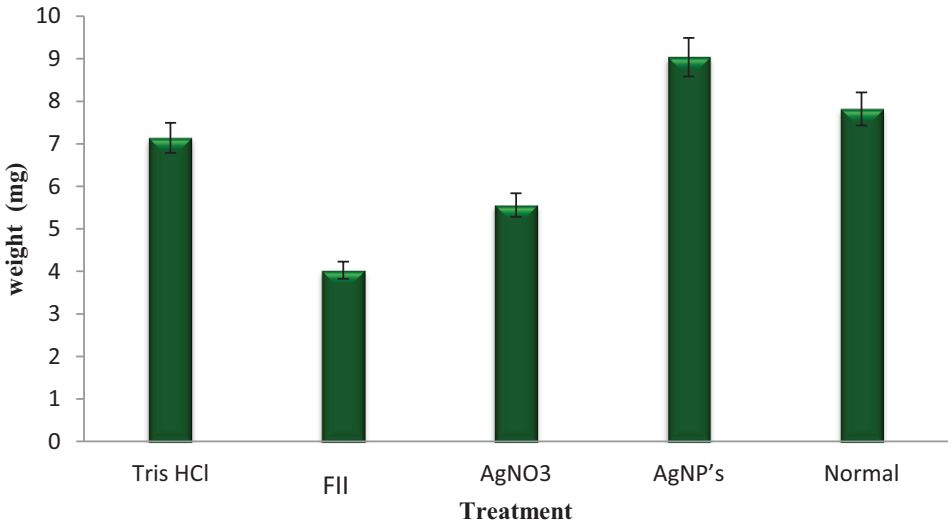


Figure 29. Weight gain of third instar of *R. fuscipes* treated with Tris HCl, *B. bassiana* toxic prtein fraction II (BBTF2), $AgNO_3$ and AgNP's

Table 23. Impact of bioreduced nanoparticles against *D. cingulatus* third, fourth and fifth instar nymphs corrected mortality at 96 hrs, lethal concentrations (LC_{30}, LC_{50} and LC_{90}), Chi-square value, slope and regression equation (n=30; $\bar{X}$ ± S.E.).

Concentration (ppm)	Third instar	Fourth instar	Fifth instar
100	3.3±0.3	0.0±0.0	0.0±0.0
200	3.4±0.3	3.5±0. 5	3.12±0.2
400	6.7±0.2	7.1±0.1	3.33±0.1
800	13.2±0.2	13.2±0.2	9.85±0.3
1600	27.8±0.3	17.9±0.5	11.53±0.3
LC_{50}	5971	8162	18611
LC_{30}	2143	2946	5863
LC_{90}	73017	98484	313132
Chi-square	2.42	2.11	2.64
Slope	5.67	4.97	3.93
Regression equation	Y=-23.7+59.2X	Y=-52.1+80.6X	Y=-20.2+115.1X

Table 24. Impact of bioreduced nanoparticles against *P. solenopsis* third instar and adult corrected mortality at 96 hrs, lethal concentrations (LC_{30}, LC_{50} and LC_{90}), Chi-square value, slope and regression equation (n=30; X ± S.E.).

Concentration (ppm)	Third instar	Adult
100	0.00±0.00	0.00±0.00
200	6.66±3.33	3.33±0.33
400	10.3±0.25	9.93±0.25
800	21.6±0.14	21.6±0.14
1600	39.9±0.33	36.6±0.33
LC_{50}	2310	2361
LC_{30}	1102	1186
LC_{90}	14093	12691
Chi-square	2.99	1.21
Slope	7.67	7.55
Regression equation	Y=11.4+38.8X	Y=42.1+40.4X

Table 25. Approaching time, handling time (mins) and predatory potential (number of prey consumed/predator/day) of Tris HCl, *B. bassiana* entomotoxic prtein fraction II (FII), $AgNO_3$ and AgNP's treated third instar of *R. fuscipes* against *P. ricini* under plastic cup condition.

Treatment	Approching time (min)	Handling time (min)	No. of prey consumed
Tris HCl	1.57±0.46^{abde}	14.28±5.80^{ade}	3.48±0.05^{abcde}
FII	1.67±0.72^{abd}	22.40±9.57^{abde}	3.54±0.02^{abcde}
$AgNO_3$	2.66±0.88^{bce}	24.20±3.55^{abcd}	3.61±0.02^{abcde}
AgNP's	1.20±0.63^{abde}	20.25±6.14^{abcd}	3.49±0.04^{abcde}
Control	1.96±2.77^{abcde}	10.66±2.76^{abde}	3.65±0.12^{abcde}

Mean followed by the same letters are not significant at 5% level by TMRT.

instars when compared with Tris HCl treated control category. The total nymphal development was significantly reduced more by bionanomaterials (F=18.462, df=5, 80, P=0.001) than by BBTF (F=6.325, df=6, 79, P=0.005) (Table 26).

Male longevity was reduced in bionano treated group (F=3.027, df=6, 5, P=0.022) as well as BBTF group (F=12.029, df=6, 5, P=0.008). The female longevity was almost similar in all the treatments; however, a slight difference was observed in bionano treated groups (F=3.597, df=4, 5, P=0.096) when compared with BBTF2 (F=1.151, df=4, 7, P=0.405), silver nitrate (F=0.957, df=4, 6, P=0.494) and tris-HCl buffer (F=1.140, df=4, 6, P=0.421) categories. The oviposition days in Bionanomaterial treated predators (F=2.635, df=5, 4, P=0.185), BBTF2 treated predators (F=1.484, df=5, 5, P=0.338), control categories [Tris HCl (F=1.259, df=6, 4, P=0.430) and silver nitrate solution (F=2.901, df=5, 5, P=0.134)] were almost similar. The same kind of result was recorded in the fecundity of the predator. The fecundity of the bionanomaterial treated predators (F=14.711, df=8, 1, P=0.199) and BBTF treated predators (F=1.210, df=8, 3, P=0.485) did not differ from control categories, Tris HCl (F=7.791, df=8, 2, P=0.119) and silver nitrate solution (F=12.235, df=9, 1, P=0.219) (Table 26).

4.5. DISCUSSION

Beauveria bassiana is a well known biocontrol agent worldwide (Adane *et al*., 1996; Rice and Cogburn, 1999; Kaaya *et al*., 2002; Russell, 2006; Nicolás *et al.,* 2007; Bonnie *et al.,* 2008; Fernando *et al.,* 2009; Wraight *et al.,* 2010; Molnar *et al*., 2010; Rohlfs and Churchill, 2011; Alex *et al.,* 2012; Safavi, 2013). The specific entomotoxic protein bassianolide (Mol.Wt. 390 kDa) (Vey *et al*., 2001), beauvericin (Mol.Wt. 28 kDa) (Quesada-Moraga and Vey*,* 2004) are responsible for the virulence of the fungi. The result reveals that *B. bassiana* protein fraction II consists of 172 Da polypeptide or protein. Literature survey reveals polypeptide or protein is responsible for the biogenesis of

Table 26. Biology of *R. fuscipes* treated with *B. bassiana* toxic prtein fraction II (BBTF2)and biosynthesizd silvernanoparticles (n=30; $\overline{X}$±S.E.).

Stage of the predator	BBTF2	AgNPs	Tris HCl	$AgNO_3$
Third stadium	8.2 ± 0.2^{acd}	7.8 ± 0.2^{abd}	8.7 ± 0.2^{cd}	8.8 ± 0.2^{acd}
Fourth stadium	7.3 ± 0.2^{abcd}	6.2 ± 0.2^{abcd}	7.7 ± 0.2^{bcd}	6.1 ± 0.2^{abcd}
Fifth stadium	8.3 ± 0.2^{abcd}	7.5 ± 0.2^{bcd}	10.1 ± 0.3^{bcd}	9.3 ± 0.2^{abcd}
Total nymphal development (days)	23.4 ± 0.2^{a}	21.3 ± 0.2^{b}	25.6 ± 0.4^{ac}	23.1 ± 0.3^{d}
Male longevity (days)	30.9 ± 0.8^{abc}	30.2 ± 0.6^{abcd}	32.1 ± 0.9^{abc}	32.1 ± 0.5^{abcd}
Female longevity (days)	39.3 ± 0.5^{abcd}	38.9 ± 0.6^{abcd}	39.5 ± 0.6^{abcd}	39.4 ± 0.6^{abcd}
Sex ratio (male: Female)	1: 0.5	1:0.4	1:0.4	1:54
Pre-oviposition (days)	16.1 ± 0.4^{abcd}	16.2 ± 0.4^{abcd}	17.4 ± 0.5^{acd}	17.8 ± 0.6^{abcd}
Oviposition (days)	11.5 ± 0.6^{abcd}	11.7 ± 0.7^{abcd}	10.3 ± 0.6^{acd}	11.1 ± 0.6^{abcd}
Post-oviposition (days)	9.40 ± 0.4^{abcd}	10.5 ± 0.6^{abcd}	10.4 ± 0.6^{abcd}	9.4 ± 0.4^{abcd}
Fecundity (No./female)	32.7 ± 2.4^{abcd}	31.8 ± 2.0^{abcd}	32.4 ± 1.7^{abcd}	32.1 ± 1.6^{abcd}

Mean followed by the same letters are not significant at 5% level by TMRT.

nanoparticles (Caruso *et al.,* 1998) particularly glutathiones of yeast (Mehra and Winge, 1991); Cystein residues of any protein (Gole *et al.,* 2001) of endophyte *Colletotrichium* sp. (Shankar *et al.,* 2004) acts as capping as well as stabilizing agents. It is a well known fact that proteins can bind to metal nanoparticles by surface bound proteins (Fig. 1) and this is in agreement with the information reported in the already available literature (Mehra and Winge, 1991; Caruso *et al.,* 1998; Gole *et al.,* 2001; Shankar *et al.,* 2004). The FT-IR results thus showed that the surface capping of silver nanoparticles synthesized using the pathogenic fungi, *B. bassiana* is predominantly by protein in capping as well as stabilization of nanoparticles.

Both transverse (370 nm) and longitudinal (440 nm) plasma vibrations were reported for silver nanoparticles (Shankar *et al.,* 2004). *Beauveria bassiana* entomotoxic protein-based AgNPs expressed longitudinal surface plasma resonance as observed for other fungi *Pencillium* (Sadowski *et al.,* 2008), *Aspergillus terrus* (Liu *et al.,* 2008). As indicated by the FT-IR data, the functional group responsible for the reduction of Ag^{+} was secondary amines (1636 and 1435cm^{-1}). Therefore, it is evident that these amines act as a reducing agent and also as a stabilizing or capping agent. Proteins play a major role in the reduction of silver ions by oxidation. These findings are in accordance with the results of Vipul *et al*. (2005) and Sadowski *et al.* (2008) in fungus mediated synthesis of AgNPs.

In addition to the Bragg peaks representative of silver nanocrystals, unassigned prominent peaks (33.6^{0} and 38.2^{0}) are also observed suggesting that the crystallization of bio-organic phase occurs on the surface of the silver nanoparticles. Similar results were reported in AgNP's synthesized using fungus (Vipul *et al*., 2005) and also using other microorganisms (Kaushik *et al*., 2010).

The low magnification TEM image (Fig. 4a) clearly shows a number of silver nanoparticles of a range of size and shapes (Fig. 6, 7). The structural features of the

individual silver nanoparticles are more clearly seen in the higher magnification TEM images (Fig. 4b, c, d). The particles are predominantly spherical (88%), rather than hexagonal (10%) and triangular (2%) in shape, ranging in size from 4 nm – 12 nm. Similarly, the regenerative capability of biological systems coupled with the discovery that fungi such as *B. bassiana* are capable of hydrolyzing metal complexes that they never encounter during their growth cycle shows enormous promise for development, particularly the large-scale synthesis of metal oxide materials.

The possible mechanism of biosynthesis of nanoparticles by biological system was reductases and any other equivalent reductants as reported earlier by Krishnaraj *et al.* (2010). The nitrate reductase from *F. oxysporum* has been documented to catalyze the reduction of $AgNO_3$ to silver nanoparticles utilizing NADPH as reducing agent (Darroudi *et al.,* 2011). Several naphthoquinones and anthraquinones having very high redox potentials have been reported from *F. oxysporum* that could act as an electron shuttle in metal reduction (Bansal *et al.,* 2011). To identify the fungal protein(s) responsible for hydrolysis of the aqueous anionic metal complexes FTIR was used. In case of the gold nanoparticles synthesized using *Colletotrichum* sp., strong bands at 1658, 1543 and 1240 cm correspond to the amide I, II and III bands of polypeptides/proteins respectively and agree with those reported in the literature (Ahmad *et al.,* 2003). Same kind of result was observed in the present study, which clearly indicates that the proteins are responsible for the reduction of AgNPs. It is well known that proteins can bind to metal nanoparticles either through free amine groups or cysteine residues in the proteins and therefore stabilization of the gold nanoparticles by surface (Gole *et al.,* 2001) bound proteins is a possibility in the case of metal nanoparticles synthesized using the fungal proteins.

The mortality of pest is the main factor in agriculture. In the present study the nanoparticles showed moderate toxicity against cotton pests and it is an advantage of using

bioreduced silver nanomaterial at low risk of developing resistance in long term usage as proposed by Mouchet *et al.* (2008). Previously, there are some reports available about the impact of various nanoparticles against higher organisms (Baun *et al*., 2008; Griffitt *et al*., 2008; Sakulku *et al.,* 2009; Mouchet *et al.,* 2009). Similarly, pesticidal activity of nanomaterials was tested by against *Sitophilus oryzae* (Griffitt *et al*., 2008), Chakraborty *et al.* (2010) against *Spodoptera litura* and Vinutha *et al.* (2013) against *Helicoverpa armigera*.

There is no report available about the impact of silver nanoparticles on reduviid predator as an important group of natural enemy distributed world-wide in many crop ecosystems. In the present study silver nanoparticles did not affect the bioefficacy of reduviid predator but silver nanomaterial has some impact on the development of reduviid. The current study result revealed that the insecticidal toxin BBTF and AgNPs have no severe impact on the predator. Hence the application of these bioinsecticides could be promoted for integrated pest management strategic programme.

4.6. CONCLUSION

A critical need in the field of nanotechnology is the development of a reliable and ecofriendly process for synthesis of metallic nanoparticles. The results clearly show that fungal entomotoxic proteins/polypeptide can lead to formation of biogenic nanoparticles in external environment of a cell, the fungi *B. bassiana* being a good candidate for such process. The results also showed protein secreted by *B. bassiana* is capable of binding with Ag ions and has a significant potential for development of nanoparticles. Irrespective of the biological system used, in order to exploit the system to its maximum potential, it is essential to understand the biochemical and molecular mechanism of nanoparticle synthesis.

5.0. SUMMARY

1. *Corcyra cephalonica* is the suitable factitious host for rearing *Rhynocoris fuscipes* under laboratory condition. The shorter nymphal period (42.5 days), higher survival rate (69.7%), longer adult longevity (male-40.5, Female-46.7) and higher oviposition index (0.086) were observed in *C. cephalonica* reared predator.
2. The life table of *R. fuscipes* was constructed by using biological parameters. Higher GRR (94.32) and higher innate capacity (0.080) were observed in *C. cephalonica* reared predator. The mean length of generation (Tc) was longer in *Phenacoccus solenopsis* (57 days).
3. The reduviid predator, *R. fuscipes* showed high biocontrol potentiality against cotton pests *Dysdercus cingulatus* and *P. solenopsis* under laboratory and pot condition. The pot study revealed that the predator is a diurnal animal.
4. The adult predators were group reared in a microenvironment cage method (MEC). The higher fecundity (46.9) and adult longevity (male-49.8 days; female-53.7 days) was observed in the predator while rearing in MEC.
5. The stage preference study revealed that the third instars of *D. cingulatus* were preferred by third (36.5%) and fourth (43.4%) instars of the predator. The fifth instar predator highly preferred fourth instars *D. cingulatus* (33.3%) and adult predators preferred fifth instars of *D. cingulatus* (34.5%). However, all the stages of the predator predate up on *P. solenopsis* adults (78.6%).
6. Third instar of *R. fuscipes* consumed a maximum of 3.3 prey at the prey density of 10, similarly fourth, fifth instars and adult were consumed/killed 4.0, 4.2 and 4.4 *D. cingulatus,* respectively.
7. *Rhynocoris fuscipes* highly reduced *D. cingulatus* (53.2%) population rather than *P. solenopsis* (51.2%), *Helicoverpa armigera* (50.0%) and *Aphis gossypii* (4.3%)

under field condition. Cost Benefit Ratio (CBR) was higher in chemical pesticides sprayed fields (1.72) than *R. fuscipes* released field (1.32) and control field (1.17).

8. The toxic protein of *B. bassiana* was precipitated with 90% saturated ammonium sulphate and the precipitate was eluted from Sephadex G-25 column. The eluents were categorized in to three fractions such as fraction I, fraction II and fraction III. The high protein content was recorded in fraction II. Significantly higher percent morality was observed in *D. cingulatus* third instar and *P. solenopsis* adult at 1600 ppm of fraction II. Hence, fraction II was subjected to SDS-PAGE, HPLC and MALDI analyses.
9. The biosilver nanoparticles synthesized using Sephadex column fraction II. The size and shape of the bionanoparticles were observed with the use of TEM. The spherical shaped bionanoparticles size was varied from 4nm to 10nm (6.87±0.04nm). Bionanoparticles showed moderate or low toxicity against *D. cingulatus* (27.8%) and *P. solenopsis* (36.6). BBTF II caused very low mortality (6.66%) to *R. fuscipes* under laboratory condition at 1600ppm.

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Impact of two pathogenic fungal crude metabolites on mortality, biology and enzymes of *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae)

K. Sahayaraj* and Majesh Tomson

ABSTRACT

Saprophytic fungi plays a crucial role in the pest management programme. Culture and mass production of the fungi is a tedious, laborious, time consuming and cost effective process. To minimize the use of chemicals, the metabolic products of the fungi have been utilized in the pest management progamme for the past one decade. A study was designed to evaluate the insecticidal activity of *Beauvaria bassiana* (Balasmo) Vuillimin.(BB) and *Metarhizium anisopliae* (Metchnikoff) Sorokin. (MA) crude metabolic extracts and fungal spores against *Dysdercus* cingulatus (Fab.) under *in-vitro* conditions. The toxicity bioassay revealed that MAF1 treated cotton seeds fed *D. cingulatus* showed high mortality (44.44%). Irrespective of the metabolic fractions and fungal spores, body weight of *D.cingulatus* gradually diminished when the nymph grew older. Maximum body weight reduction was recorded in BBF2 (44.3%) category followed by BBF1 (45.4%) and the metabolites showed higher activity than fungal spores. Treatments also reduced total body protein content. Maximum reduction was recorded in BBF2 (0.09mg/g) followed by MAF1 (0.102mg/g). Amylase level was highly reduced by MAF2 (0.036µg/mg) followed by BBF2 (0.085 µg/mg). Higher protease activity resulted in BBF2 ($9.1x10^{-5}$ µg/mg) followed by BBF1 ($6.5x10^{-5}$ µg/mg). The detoxification enzyme, glutamate oxalate transaminase (GOT) activity was highly reduced in BBF2 ($1.8x10^{-5}$ µg/mg) followed by MAF2 ($2.5x10^{-5}$ µg/mg). These observations indicated the potential of *B. bassiana* and *M. anisopliae as* the simple, inexpensive and accessible source of bioinsecticide to manage sucking pests like *D.cingulatus.*

Key words: *Beauvaria bassiana, Metarhizium anisopliae, Dysdercus cingulatus,* fungal metabolites

INTRODUCTION

Cotton, *Gossypium hirsutum* (Linn.) is the most economically important natural fiber material in the world and it is widely known as "The King of Fibers". Man has been utilizing cotton for his benefits since ancient times (Fryxell, 1992). Cotton is a multipurpose crop that supplies five basic products such as lint, oil, meal, seed hulls and linters. Lint is the most important product of the cotton plant and provides much of the high quality fiber for the textile industry. The economy of many countries like India, Pakistan, Egypt, Sudan etc. depends up on cotton and its products. In India, cotton is an important industrial crop, 24% of the total cotton production is cultured in India.

In recent years, yield of cotton has become static rather it is declining due to the infestation of insect pests and diseases. One of the major factors of low yield was the infestation of insect pests. Sucking pests are deleterious during early season of the cotton plant growth and development. Cotton stainer, jassids, aphids, white flies and thrips are constituted as important pests of cotton (Uthamasamy, 2004).

Dysdercus cingulatus (Fab.) (Hemiptera : Pyrrhocoridae) is a serious pest of cotton distributed in all the cotton growing regions of India (David and Ananthakrishnan, 2004; Sahayaraj and Ilayaraja, 2008). It is commonly known as red cotton bug and is an important pest of lady's finger, sambhal, hollyhock *etc*. Nymphs and adults of *D. cingulatus* feed mainly on developing or mature cotton seeds. It has been controlled by many synthetic insecticides. However they failed to controll this insect. because, both the nymphs and adults move from place to place very rapidly. Hence it is essential to find out an alternative method for the management of this economically important pest. For the past two decades, extension workers and pest management workers have been using fungal pathogens in pest management programme where *Beauvaria bassiana* and *Metarhizium anisopliae* have been play an important role (Arnold, 2005).

Beauvaria bassiana (Balasmo) Vuillumin (Ascomycota : Hypocreales) is one of the most ubiquitous and extensively studied entomopathogenic fungi (Dias *et al.,* 2008). Entomopathogens as biological agents are attracting increased attention because they provide environmentally safe insect control (E1-Mandarowy, 2005). *B. bassiana* infects larvae, pupae and adults of many insects successfully and at the time of insect death nearly all the internal organs of the insect are utilized by the fungus (Sabbahi *et al.,* 2008). *Metarhizium anisopliae* (Metchnikoff) Sorokin (Deuteromycotina : Hyphomy cetates) is a rather common agent causing infection in natural insect populations (Borisov *et al.,* 2001; Serebrov *et al.,* 2007). It has a very broad host range and is used as a good pest control agent for several pests (Borgio and Sahayaraj, 2007). Spontaneous variability of *M. anisopliae* should be considered as a reserve for selection of this biocontrol agent on high virulence towards pest insects (Serebrov *et al.,* 2007). Very little information is available on the metabolic products of the fungi on insects and none has studied their impact on *D. cingulatus*. The present study is aimed to investigate the impact of crude metabolic fractions of *B. bassiana* and *M.anisopliae* on *D.cingulatus*

MATERIALS AND METHODS

Pest Collection and Rearing

Adults and nymphs of *Dysdercus cingulatus* were collected from cotton field in Peikulam Tirunelveli District, Tamil Nadu, India. Collected insects were maintained under laboratory condition (27±2° C temperature, 70-75 RH, 11L:13D) on its natural host cotton. The third nymphal instars of *D. cingulatus* were used for the present study.

Fungal source culture and preparations of fractions

Isolated *Metarhizium anisopliae* and *Beauvaria bassiana* were obtained from CPRC, St. Xavier's College, Palayamkottai and used for the present study. The isolated fungi were cultured using standard potato-dextrose broth. 10 ml of 7 day-old fungal culture were taken in a test tube and centrifuged at 5000 rpm for 30 minutes. Separate the supernatant from the pellets. This supernatant was considered as fraction I (BBF1 and MAF1). The weight of the pellet was recorded using monopan balance. Add required amount (1 mg pellet /3 ml phosphate buffer) of phosphate buffer to the pellet, mix well and transfer into another test tube. Heat the mixture at 70 - 72° C for 20 minutes in a water bath. Centrifuge the sample at 5000 rpm for 30 minutes, and separate the supernatant and consider it as fungal fraction II (BBF2 and MAF2).

Bioassay

One gm of cotton seeds were soaked in 10 ml of water for 10 minutes shade dried for 5 minutes. Take 10 ml of fraction I in a beaker (50 ml capacity) and introduce the cotton seeds into the beaker and allow them for 40 minutes. These seeds were as food for *D. cingulatus*. Every day the cotton seeds provided were replaced and provided fraction I treated seeds continuously for four days. Then they were fed with water soaked cotton seeds till their death. The seeds soaked in phosphate buffer are treated as control. Similar procedure was followed for fraction II. The 10^8 fungal spores of *B. bassiana* and *M. anisopliae* were inoculated, and the cotton seeds were dipped in the culture and supplied to the animal. Mortality was observed during the nymphal stages. Moreover, weight of the nymphs has been recorded every day using monopan balance. Nymphal total life time was recorded. Irrespective of the treatments, 3 day-old adults were used for biochemical analysis.

The whole body protein quantities of insects were estimated by Bradford (1976) method. The enzymes were estimated by using standard procedures, the enzyme source was prepared by the freshly dissected gut homogenized with 1ml of phosphate buffer (pH 7.2). Add 3ml of distilled water to it and keep it in an eppondorf. Centrifuge the samples at 5000 rpm for 20 minutes and take the supernatant for enzyme studies. Amylase, protease and glutamate oxaloacetate tranraminase (Bernfield, 1955) enzymes were quantified. The whole body protein profile of the insect was analyzed by SDS-PAGE method (Laemmli, 1970).

Statistical Analyses

Experimental data was compared with control category data by Tukey's Multiple Range Test and their significance was expressed at 5 % level

Table 1. Impact of *M. anisopliae* (MA) and *B. bassiana* (BB) crude metabolic fractions on the corrected mortality (in %) of *D. cingulatus*

Treatments	Days after Exposure			
	Fifth day	Sixth day	Seventh day	Eighth day
MAF1	5.0	20.0	42.1	44.44
MAF2	10.0	25.0	31.58	33.33
BBF1	20.0	25.0	31.58	38.88
BBF2	15.0	25.0	26.32	33.33
B. bassiana	10.0	16.66	30.0	33.33
M. anisopliae	10.0	23.33	26.32	30.0

Table 2. Impact of different fractions of fungi on the body weight (in mg.) of *D. cingulatus* nymphs

Treatments	Observation After Treatments (in days)			
	Fifth day	Sixth day	Seventh day	Eighth day
Control	62.3 ± 0.7	63.2 ± 0.4	64.6 ± 0.5	66.5[a] ± 1.0
MAF1	61.8[NS] ± 1.3	60.2* ± 0.4	56.7* ± 1.6	48.6 ± 0.7
MAF2	58.6* ± 1.8	57.6* ± 1.8	52.4* ± 1.3	48.0 ± 0.9
BBF1	61.3[NS] ± 0.5	54.4* ± 0.7	52.8* ± 0.7	45.4 ± 0.6
BBF2	60.5 [NS] ± 1.0	56.1* ± 1.2	51.4* ± 0.8	44.3 ± 1.4
B.bassiana	62.1 [NS] ± 0.6	61.8 [NS] ± 0.5	60.4 [NS] ± 0.6	58.2 * ± 0.6
M.anisopliae	61.4 [NS] ± 0.6	59.5 [NS] ± 0.3	57.6* ± 0.8	56.2* ± 0.9

NS – Not significant, * Significant at 5% level by TMRT

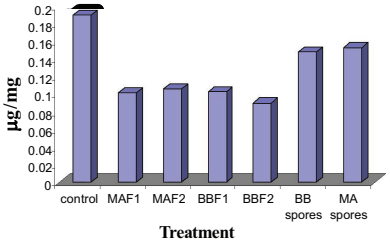

Figure 1. Protein quantity (µg/mg) of *D. cingulatus* fed with fungus and fungal fractions treated cotton seeds

RESULTS

Mortality

The percent mortality of *D. cingulatus* showed that the fungal crude fractions caused a moderate (44.44% for MAF1) or low mortality (33.33% for MAF2 and BBF2) to nymphal stages of insect. The fungal spores also showed moderate mortality (33% for *B.bassiana* and 30% for *M. anisopliae)*. The toxic effect increased with increase in the exposure time.

Body weight

Dietary utilization of *D. cingulatus* was severely affected, when it was fed with cotton seeds treated with fungal metabolites and spores (Table 2). In control the weight of the nymphs gradually increased and it attained a maximum weight of 66.5 mg on the eighth day of adult life. It was significantly ($P < 0.05$) reduced when the nymphs fed with BBF-2 (44.3mg) followed BBF-1, MAF-1 and MAF-2.

Nymphal development

Table 3 shows the nymphal period of *D. cingulatus* during the test period. It showed that insignificantly the fungal fractions and spores extend the nymphal period. However the fungal fractions and spores seriously affect the morphology of *D.cingulatus* during its molting.

Total body Protein

The total body protein of control *D. cingul*atus was 0.1896 µg/mg. Reduction of total bodyprotein was observed in fungal fractions treated *D. cingulatus* when compared with control category. Figure 1 shows the quantity of protein in animals decreased during the test period. The fraction of *B.bassiana* showed the higher activity than *M. anisopliae* in protein reduction. At the end of the eighth day, the maximum reduction was recorded in BBF-2, followed by MAF1, BBF-1, MAF2, *M. anisopliae* and *B. bassiana.*

SDS protein profile

SDS protein profile of *D. cingulatus* adults after feeding fungal metabolites showed in Plate 1b. In normal pest, four polypeptides were recorded (11832 Kd to 38128 Kd). It was reduced by *B.bassiana* fractions (Table 4). However, both MAF-1 and MAF-2 increased the molecular weight of the polypeptides. (Fig 2)

Enzyme Activity

Two digestive enzymes (amylase and protease) and a detoxification enzyme (GOT) were quantified. First fraction of both the tested fungi highly reduced amylase activity, whereas BBF2 increased the activity, in order to the fungal spores the enzyme activity is low. In contrast, invariably fractions and spores of these two fungi reduced protease level. In the case of GOT, higher activity was recorded in MAF1 followed by BBF1.

Table 3. Impact of fungal metabolites on nymphal life time (in days) of *D. cingulatus*

Treatments	Third instar	Forth instar	Fifth instar	Total nymphal period
Control	2.5±0.12	3.6±0.14	5.0±0.3	18.2±0.9
MAF1	2.6±0.13	3.6±0.14	5.6±0. 3	18.3±0.9
MAF2	2.5±0.12	3.6±0.14	5.6±0. 3	18.8±0.9
BBF1	2.6±0.13	3.6±0.14	5.6±0. 3	18.3±0.9
BBF2	2.5±0.12	3.6±0.13	5.6±0.3	18.8±0.9
B. bassiana	2.5±0.12	3.6±0.14	5.6±0.3	18.8±0.9
M. anisopliae	2.5±0.12	3.6±0.14	5.6±0.3	18.8±0.9

Table 4. Enzyme Levels of *D. cingulatus* in relation to various fungal fractions

Treatments	Amylase	Protease	GOT
Control	3.4×10^{-2}	4.3×10^{-5}	4.8×10^{-6}
MAF1	1.6×10^{-2}	5.5×10^{-5}	3.3×10^{-5}
MAF2	3.6×10^{-3}	6.0×10^{-5}	2.5×10^{-5}
BBF1	1.5×10^{-2}	6.5×10^{-5}	2.6×10^{-5}
BBF2	8.5×10^{-3}	9.1×10^{-5}	1.8×10^{-5}
B. bassiana	2.4×10^{-2}	1.2×10^{-4}	3.7×10^{-5}
M. anisopliae	3.9×10^{-2}	1.4×10^{-4}	3.9×10^{-5}

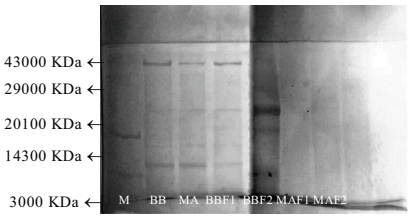

Figure 2. SDS - PAGE profile of *D. cingulatus* treated with fungal spores and the fractions

DISCUSSION

The development of pest control measures using microorganisms especially entomopathogens has attracted widespread attention in recent years. Fungi have considerable epizootic potential and can spread quickly through an insect population and cause its collapse. Because fungi penetrate the insect's body, they can infect sucking insects such as aphids and whiteflies that are not susceptible to bacterial and virus attacks. Our study reveals that two tested funguses are having the capacity to kill another sucking pest *D. cingulatus* (Borgio and Sahayaraj, 2007). However, recent scenario is to find out the insecticidal compounds form microorganism (Dowd *et al*., 1992; Essien, 2004). The mycopesticides based on deuteromycetous fungi *B. bassiana* is a common soil borne fungus that occurs worldwide and has been reported as a suppressive agent for several insect pests (Borisov and Serebrov, 2001) particularly pests having sucking type of mouthparts.

In the present study the metabolites of *B. bassiana* fraction 2 (BBF2) interfere with the digestive process, and the pest undergoes starvation, as a result the weight was reduced to 33.34 per cent when compared to the control. Simple mechanism proposed earlier that fungal metabolites can bind to a hydrophobic site remote from the catalytic site (Cheng and Ling, 1991). Weight reduction might be due the interference of these metabolic products affecting gut physiology events (*ie*., ion transport), reduce the palatability of the cotton seed to suck leading to the starvation of *D. cingulatus*, causing more body reduction which leads to death. It is essential to isolate and identify the chemical structure of these metabolic products and to integrate them in sucking pests management. Between the two digestive enzymes, crude fungal metabolites interfere more with the protease enzymes of *D. cingulatus* than with amylase. However, detoxication enzyme level was not much affected by the fungal metabolites, as observed by the action of botanicals in lepidopteran larvae of *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae) (Sahayaraj and Nirupa Antony, 2006).

The results revealed that the insect death mainly occurred at the time of moulting. We recorded different kinds of deformities like curling of wings, deformities in legs, retarded growth of the body, and haemolymph and/or body fluid oozing out from the abdomen. These changes affect further growth of the pests. Cadavers also showed the fungal hyphal growth of *B. basiana.* Although the exact mode of toxicity was not determined in the present study, earlier investigation have shown that fungi, particularly the aspergillus possess the ability to elaborate harmful metabolites which can induce acute and chronic mortality in insects (Dowd *et al*., 1992). This study also indicated that first fraction of *Beauveria bassiana* and *Metarhisium anisopliae* showed more impact than the second fractions. Hence these first fractions can be used for the sucking pest management either alone or in combination.

B. bassiana and *M. anisopliae* have the potential use as biological control agents against insect pests because they were relatively safe on non target insects, such as natural enemies and beneficial soil insects.

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Stage Preference and Functional Response of *Rhynocoris longifrons* (Stål) (Hemiptera: Reduviidae) on Three Hemipteran Cotton Pests

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ABSTRACT

In this work, the stage preference and functional response of the indigenous reduviid bug Rhynocoris longifrons *feeding on five different densities of the cotton aphid* Aphis gossypii*,* Phenacoccus solenopsis*, and* Dysdercus cingulatus *was examined in Petri dish arenas containing cotton leaves under laboratory conditions. The reduviid predator exhibited a Type II functional response at all hemipteran pests evaluated when data were fit to Holling's disc equation. Predatory rate gradually increased while the predator grew older and adults consumed maximum number of* D. cingulatus *and* P. solenopsis*. An opposite trend was observed, while the reduviid was provided with* Aphis gossypii*. The rate of attack on* P. solenopsis *was quite low but fairly consistent, with the different life stages of the predator generally more effective. Further investigation of the biological control potential of* R. longifrons *against cotton pests under pot and controlled filed should be done due to the predator's ability to kill adult stages of all prey species evaluated. These results indicated that* R. longifrons *could eat more aphids at high prey densities; however, predators also considerably reduced other cotton pests too so it could be considered a prospective candidate for use as a commercial biological control agent for cotton hemipteran pests in India.*

Key words: Cotton pests, functional response, reduviid predator, stage preference

INTRODUCTION

Dysdercus cingulatus(Fab.) (Pyrrhocoridae), *Phenacoccus solenopsis* (Tinsley) (Pseudococcidae) and *Aphis gossypii* (Glover) (Aphididae) are representative species of the three key economically important Hemipteran genera. Mealy bug, *P. solenopsis* is the most widely distributed species in tropical, subtropical and warm regions. *P. solenopsis* attacks the roots just below the level of the soil, especially where the root and the stem meet (Patel et al. 2010). Red cotton bug, native to Asia, is similarly widespread although it has not yet attained pest status in Central and South America, Europe, or North Africa. The red cotton bug or cotton stainer, *D. cingulatus* in particular causes serious damage by feeding on developing cotton bolls and ripe cotton seeds and transmitting fungi (Iwata 1975). It is difficult to control by insecticides because it is a highly mobile, polyphagous and polymorphic pest (Sahayaraj and Ilayaraja 2008) of many malvaceae crops. The cotton aphid *A. gossypii* is a polyphagous pest with worldwide distribution in tropical, subtropical, and warm temperate regions (Isikber 2005). This aphid is a vector of more than 30 plant viruses and has been observed feeding on more than 80 plant families (Blackman and Estop 1984; Ebert and Cartwright 1997; Ghabeish et al. 2010).

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Reduviid predators are considered as potential biocontrol agents against many insect pests (Sahayaraj 2007; Grundy and Maelzer 2000; Grundy 2007) and have been suggested to integrate in Bio-intensive Integrated Pest Management (BIPM). *Rhynocoris longifrons* (Stal) (Hemiptera: Reduviidae) is a voracious harpactorine reduviid predator (Ambrose et al. 2003) mainly distributed in India. This reduviid has been found in cotton ecosystems and predating on many insects pets (unpublished data). *Rhynocoris longifrons* is largely effective in predating upon the larval stages of cotton pests, such as *Helicoverpa armigera* that typically develop within the flowers and leaves (Ravichandran et al. 2003). Records of specific associations between *R. longifrons* and cotton sucking pests are limited.

Functional response characterizes the relationship between the number of prey consumed by the individual predators and the density of available prey (Solomon 1949; Holling 1959 a,b). The potential biocontrol efficacy of candidate agents can be extrapolated by quantifying the functional response, which serves as a predictor of attainable top-down, density-dependent regulation of a given pest species (Murdoch and Oaten 1975). Although some studies have investigated the functional response of *R. longifrons* against *Odontotermes obesus* Rambur (Kumar and Ambrose 1996), *Clavigralla gibbosa* Spinola (Claver et al. 2002), *Helicoverpa armigera* (Hübner) (Ravichandran et al. 2003), none of them has addressed the effect of hemipteran cotton pests on the functional response of the predator. Published accounts of reduviid functional response to cotton insect pests have thus far excluded the evaluations of the predator's potential to control the hemipteran prey (Grundy and Maelzer 2000; Grundy 2007). Functional response is an appropriate way to characterize the interaction of *R. longifrons* a number of three different hemipteran prey species and stages in a highly simplified environment. The current study investigated the stage preference and functional response of *R. longifrons* against three cotton pests with a view to optimize the biological control of these economically important sucking pests.

MATERIALS AND METHODS

Life stages of *R. longifrons* were collected from the scrub jungle bordering cotton agroecosystem of Kanyakumari district, Tamil Nadu. *R.longifrons* was maintained in the control temperature room at 32 ^{o}C and 75±5 % RH in plastic containers (one litre capacity) in a photo period of 11 h L and 13 h D. The pest *D. cingulatus*, *P. solenopsis* and *A. gossypii* were collected from the cotton agro-ecosystem of Tirunelveli district. They were maintained in the laboratory condition as mentioned above in plastic troughs (3 litre capacity). The predator was reared for one generation on an *ad libitum* supply of mixed life stages of these pests before starting the functional response study.

Stage preference

Stage preference studies were conducted in third, fourth, fifth nymphal instars and adult of *R. longifrons* against the life stages of *D. cingulatus* (second, third, and fourth nymphal instars), *P. solenopsis* (first, second, third nymphal instars and adult). To standardize the response, predators were starved for 24 h in plastic boxes before release into the test arena. The experimental arena consisted of a glass Petri dish (14 cm in diameter) lined with paper towel. Each dish contained a cotton leaf with its petioles inserted into an Eppendorf tube (2 ml) filled with sucrose water (1 %). The average leaf area (both sides) was estimated to be approximately 20-25 cm^2 (n = 5). Preys (2 in each stage) were gently transferred by a fine camel hair brush from plants of the stock culture to the leaves in the test arenas. The preys were allowed to settle and a third instar predator was introduced in each Petri dish. At each prey type, there were ten replicates for predator treatments and five controls (i.e., arenas without a predator). The preferred stage of the predator was recorded visually; similar procedure was followed for other life stages. Successfully preferred stage of the prey was used to record the functional response study.

Functional response studies

Experimental arena was prepared as mentioned for the stage preference studies. Second, third nymphal instars *D. cingulatus* (for nymphs and adult predator); adults of *P. solenopsis* and *A. gossypii* (all life stages of the predator) were used as prey in the experiment. The experiment was performed at five different densities of *Aphis gossypii* (5,10,20,30,40), *P. solenopsis* (2,4,6,8,10), and *D. cingulatus* (1,2,4,8,16). Appropriate numbers of aphids were gently transferred by a fine camel hair brush from the

plants of the stock culture to the cotton leaves in the test arenas. The aphids were allowed to settle and a third instar predator was introduced in each Petri dish. At each prey density, there were ten replicates for predator treatments and controls (i.e. arenas without a predator). The total number of prey killed during a 24-h period was recorded. Killed preys were replaced during the experiment. No mortality was recorded in the control category. Holling 'disc' equation (Holling 1965) was used to describe the functional response of *R. longifrons.*

Data analyses

Data were analyzed using SPSS (version 11.5) for the analysis of variance (ANOVA) and t-test, SAS for the analysis of functional response. Data were submitted to a two-way ANOVA 137 for the significance of the main effects of prey density and temperature on predation and their interaction. In the present study, the prey densities changed during the experimental period with each consumption event. To account for this prey depletion during the experiments, a generalized model of Rogers's random predator equation (Rogers, 1972) was used.

RESULTS

Stage preference

Results showed that third, fourth and fifth nymphal instars of *R.longifrons* significantly preferred second instar nymphs of *D. cingulatus* (df3,18; F= 8.70; P = 0.05), whereas the adult preferred third instar nymphs (df3,18; F= 8.68; P = 0.05) (Fig. 1A). However, nymphal instars and adult of *R. longifrons* selected adults of *P. solenopsis* (df3,18; F= 8.69; P = 0.05) (Fig. 1B) and *A. gossypii* (100%) (df3,18; F = 26.80; P = 0.01).

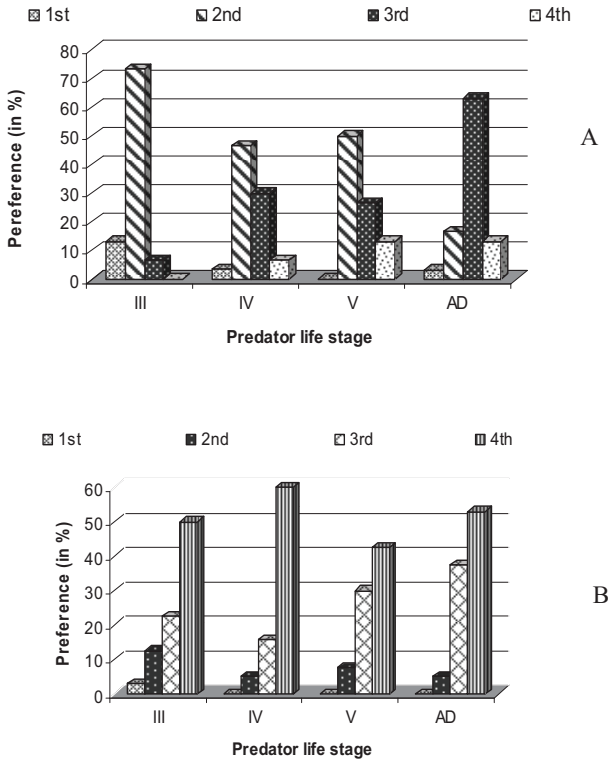

Figure 1 - Stage preference of *R .longifrons* on *D .cingulatus* (A), *P. solenopsis* (B).

Functional response

The proportion of prey consumed by the predator declined with increasing prey density. The coefficients of determination (R^2) indicated not much variation in predation rates against the tested preys (Table 1). Generally, searching efficiency

(E) gradually diminished while the prey density increased. When *D. cingulatus* (Table 2) and *P. solenopsis* (Table 3) were provided as preys, *R. longifrons* nymphs quickly searched rather than the adults. An opposite trend was observed when *A. gossypii* was offered as prey (Table 4). Attack rate decreased as prey density increased from 1 to 16 (*D. cingulatus*) or 5 to 40 (*Aphis gossypii*) or 2 to 10 (*P. solenopsis*) preys. At higher *D. cingulatus* (0.68 h^{-1}) and *P. solenopsis* (0.57 h^{-1}) densities, attack rate of adult predator was higher than that of third instar predator. An opposite response was observed while *A. gossypii* was provided as a prey. Maximum prey consumption (Na Maximum) gradually diminished as the predator grew older while offered with *Aphis gossypii.* However, the prey consumption increased when the predator was provided with *P. solenopsis* and *D. cingulatus.*

Table 1 - Correlation coefficient (R^2) between numbers of prey offered (N) and number of prey consumed (Na) by *R. longifrons* provided with three hemipteran pests of cotton

Life stage of the reduviid	Cotton Pests		
	D. cingulatus	***Aphis gossypii***	***P. solenopsis***
Third instar	0.92628	0.94034	0.94387
Fourth Instar	0.93105	0.976623	0.982511
Fifth instar	0.99294	0.97888	0.88211
Adult	0.97930	0.91317	0.98196

Table 2 - Functional response parameters recorded for the life stages of *R. longifrons* on *D. cingulatus***.**

Predator life stages	N	Na	E	T_h	Total Th	Ts	Na Maximum	a'
	1	1.0	1.0	0.29	0.29	0.71		1.40
	2	2.0	1.0	0.25	0.50	0.75		1.33
III	4	2.3	0.57	0.37	0.85	0.63		0.90
	8	3.1	0.38	0.33	1.02	0.67		0.56
	16	3.8	0.23	0.12	0.45	0.88	3.8	0.26
	1	1.0	1.0	0.40	0.40	0.60		1.66
	2	1.2	0.60	0.27	0.34	0.73		0.82
IV	4	1.8	0.45	0.43	0.77	0.57		0.78
	8	3.4	0.42	0.43	1.46	0.57		0.73
	16	3.8	0.23	0.41	1.56	0.59	3.8	0.38
	1	1.0	1.0	0.36	0.36	0.64		1.56
	2	1.6	0.80	0.07	0.11	0.93		0.86
V	4	2.4	0.60	0.52	1.25	0.48		1.25
	8	3.2	0.40	0.22	0.70	0.78		0.51
	16	5.4	0.33	0.30	1.62	0.70	5.4	0.47
	1	1.0	1.0	0.25	0.25	0.75		1.33
	2	1.6	0.80	0.25	0.40	0.75		1.06
Adult	4	2.3	0.57	0.12	0.28	0.88		0.64
	8	3.0	0.37	0.20	0.60	0.80		0.46
	16	8.0	0.50	0.27	2.16	0.73	8.0	0.68

Functional response parameters: N = Prey densities, Na = No. of prey consumed, E = Searching Efficiency, T_h = Handling time, Total Th = Na x Th, Ts = time of searching, Na Maximum, a = rate of discovery.

Table 3 - Functional response parameters recorded for the life stages of *R. longifrons* on *P. solenopsis.*

Predator life stages	N	Na	E	T_h	Total Th	Ts	Na Maximum	a'
III	2	1.0	0.50	0.15	0.15	0.85		0.58
	4	1.3	0.32	0.4	0.52	0.59		0.54
	6	2.3	0.38	0.06	0.13	0.94		0.40
	8	2.3	0.28	0.20	0.40	0.80		0.35
	10	2.6	0.26	0.17	0.44	0.83	2.6	0.31
IV	2	1.4	0.70	0.38	0.53	0.62		1.12
	4	1.8	0.45	0.53	0.95	0.47		0.95
	6	2.6	0.43	0.49	1.27	0.51		0.84
	8	2.8	0.35	0.21	0.58	0.79		0.44
	10	3.2	0.32	0.64	2.0	0.36	3.2	0.88
V	2	1.6	0.80	0.12	0.19	0.88		0.90
	4	1.6	0.40	0.05	0.08	0.95		0.42
	6	3.0	0.50	0.12	0.36	0.88		0.56
	8	2.6	0.32	0.19	0.49	0.81		0.39
	10	3.3	0.33	0.15	0.49	0.85	3.3	0.38
Adult	2	1.3	0.65	0.04	0.05	0.96		0.67
	4	1.6	0.40	0.16	0.26	0.84		0.47
	6	2.6	0.43	0.19	0.49	0.81		0.53
	8	4.0	0.50	0.09	0.36	0.91		0.54
	10	5.0	0.50	0.13	0.65	0.87	5.0	0.57

Functional response parameters: N = Prey densities, Na = No. of prey consumed, E = Searching Efficiency, T_h = Handling time, Total Th = Na x Th, Ts = time of searching, Na Maximum, a = rate of discovery

Table 4 - Functional response parameters recorded for the life stages of *R. longifrons* on *Aphis gossypii.*

Predator life stages	N	Na	E	T_h	Total Th	Ts	Na Maximum	a'
III	5	1.8	0.36	0.31	0.56	0.69		0.52
	10	3.0	0.30	0.27	0.81	0.73		0.41
	20	5.8	0.29	0.37	2.14	0.63		0.46
	30	5.4	0.18	0.26	1.40	0.74		0.24
	40	10.6	0.26	0.16	1.69	0.84	10.6	0.30
IV	5	2.4	0.48	0.15	0.36	0.85		0.56
	10	5.0	0.50	0.44	2.2	0.56		0.89
	20	6.0	0.30	0.15	0.90	0.85		0.35
	30	7.6	0.25	0.24	1.82	0.76		0.32
	40	10.4	0.26	0.37	3.85	0.63	10.4	0.41
V	5	1.0	0.20	0.16	0.16	0.84		0.23
	10	2.6	0.26	0.24	0.62	0.76		0.34
	20	5.0	0.25	0.09	0.45	0.91		0.27
	30	5.6	0.18	0.36	2.01	0.64		0.28
	40	7.6	0.19	0.30	2.28	0.70	7.6	0.27
Adult	5	1.0	0.20	0.40	0.40	0.60		0.33
	10	3.8	0.38	0.19	0.72	0.81		0.46
	20	3.6	0.18	0.37	1.33	0.63		0.28
	30	5.8	0.19	0.15	0.87	0.85		0.22
	40	6.2	0.15	0.20	1.24	0.80	6.2	0.18

Functional response parameters: N = Prey densities, Na = No. of prey consumed, E = Searching Efficiency, T_h = Handling time, Total Th = Na x Th, Ts = time of searching, Na Maximum, a = rate of discovery

DISCUSSION

Rhynocoris longifrons is a generalist predator; it occurs from India through the central Tamil Nadu. This species is commonly found in agro-ecosystems in India. Few studies have examined the effect of prey size on predator responses. Sahayaraj (1995a), Ambrose and Sahayaraj (1993), Sahayaraj and Ambrose (1994), Sahayaraj and Ambrose (1995), Cogni et al. (2002), Claver and Ambrose (2002) reported that small size reduviids preferred small size preys whereas large size predator preferred large size prey. As a rule, it could be supposed that larger preys were easier to be detected by a predator (Bell 1990). Similarly in the present study, all life stages of *R. longifrons* preferred only the adults of *P. solenopsis* and *A. gossypii.* However, nymphs and adults of *R. longifrons* preferred second and third instar nymphs of *D. cingulatus*. When compared to the predator body mass, all the tested prey's body was comparatively less, and hence predators invariable preferred stages were the largest size among the tested prey stages.

Although most predators attack the largest available individuals of their prey species, those species are generally smaller in body size than the predator. Predatory arthropods are known to be an exception to this limiting predator: prey relative body size ratios, because maximum prey size can be increased through the use of venoms, traps, or group hunting (Sabelis 1992). The results of the present study indicated that *R. longifrons* was capable of low level but fairly consistent success in killing its larger hemipteran prey. The results indicated that the percentage of hemipteran life stages of tested prey attacked by *R. longifrons* decreased as prey availability increased, typifying a Type II density independent functional response (Holling 1959, 1965; Gotelli 1995). A similar Type II functional response curves have been reported in a number of other reduviids (Sahayaraj 1995; Ambrose and Sahayaraj 1996; Claver et al. 2002; Ambrose et al. 2008, 2009, 2010; Sahayaraj and Asha 2010). However, Holling (1965) stated that predators showing a type III response were theoretically more capable of suppressing prey populations. It, therefore, could be expected that the equilibrium in predator: prey population dynamics, the theoretical hallmark of pest population regulation through the biological control, would not be attained following the release of *R. longifrons* in cotton field. But Schenk and Bacher (2002) reported that the evaluations performed under restrictive conditions (cages; single prey species) routinely indicated a Type II functional response in generalist insect predators. Van Alebeek et al. (1996) suggested that the constraints of experimental design might actually obfuscate the true nature of the functional response curve in the context of invertebrate predators, specifically citing how in a confined arena the increased chance of prey discovery might exaggerate the steepness of the response curve at the lowest prey densities. Finally, significant discrepancies in the outcome of laboratory vs. field evaluations of functional response have been reported (Schenk and Bacher 2002). Although the predator's response to life stages of *D. cingulatus* was particularly encouraging, the results presented here suggested that further evaluations of the predatory response of *R. longifrons* to hemipteran pest under more complexes experimental conditions should be done.

Functional responses may provide important information on the voracity of a biological control agent, and on the effects of abiotic (e.g. temperature) or biotic (e.g., host insect) factors on its foraging efficiency (Mohaghegh et al. 2001; Skirvin and Fenlon 2001; Mahdian et al. 2006; Li et al. 2007). However, functional response studies have been criticized because they are often performed in small artificial arenas using unrealistic prey densities and do not consider spatial habitat complexities or multispecies prey situations (Murdoch 1983; O'Neil 1989; Kareiva 1990; Wiedenmann and O'Neil 1991; Hardman et al. 1999). Furthermore, functional responses do not consider crucial life history parameters of a predator that may affect its value as a biological control agent.

Biological control programs should consider that although *R. longifrons* uses a large range of prey size, this predator prefers aphids and mealy bug adults with less than its own mass. This is important information to decide which part of the moth life-cycle could be more efficiently suppressed in the field by this predator. However, more field studies are needed to understand the foraging behavior *R. longifrons* in different cropping systems, in order to design the practical release strategies for this reduviid.

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