STUDY OF POPULATION DYNAMICS AND MOLECULAR CHARACTERIZATION OF FRUIT FLIES ASSOCIATED WITH CUCURBIT ECOSYSTEM

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THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science (Agriculture) in Entomology and Agricultural Zoology

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CERTIFICATE

To,
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Banaras Hindu University,
Varanasi-221005 (India).

Through: The Head, Department of Entomology and Agricultural Zoology, Institute of Agricultural Sciences, B.H.U., Varanasi.

Dear Sir,

I have great pleasure in forwarding the thesis entitled **"STUDY OF POPULATION DYNAMICS AND MOLECULAR CHARACTERIZATION OF FRUIT FLIES ASSOCIATED WITH CUCURBITS"** submitted by **Mr. Varun Arya, I.D. No. 20412EAZ017**, in partial fulfilment of the requirements for degree of **Master of Science (Agriculture) in Entomology and Agricultural Zoology**, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi and placing on record that he has completed the requisite residential requirements as contained in the statue of the university.

I certify that the entire scheme of investigation presented herein was planned and carried out solely by candidate under my guidance and supervision. The data presented in the thesis, to the best of my knowledge and belief, are genuine and original.

Thanking you

Yours faithfully
(**Dr. Srinivasa N**)
Supervisor

Forwarded by
Head of the Department

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Date: *June 2022*

Place: *B.H.U., Varanasi* ***(Varun Arya)***

CONTENTS

ABBREVIATION AND SYMBOLS

et al.	:	et alia (and associates)
%	:	per cent
ha	:	hectare
mha	:	million hectare
mmt	:	million metric tonne
bp	:	base pair
COI	:	Cytochrome oxidase 1
DNA	:	Deoxyribose nucleic acid
IPM	:	integrated pest management
ME	:	methyl eugenol
CL	:	cue-lure
ME/CL	:	combination of methyl eugenol and cue-lure
RK	:	raspberry ketone
ZN	:	zingerone
TL	:	trimedlure
FL	:	fruition lure
MAT	:	male annihilation technique
SIT	:	sterile insect technique
IIT	:	Incompatible insect technique
AW-IPM	:	area-wide integrated pest management
CI	:	cytoplasmic incompatibility
uni-CI	:	unidirectional cytoplasmic incompatibility
bi-CI	:	bidirectional cytoplasmic incompatibility
AA	:	ammonium acetate
MLST	:	multilocus sequence typing
ST	:	strain type

tsl	:	temperature sensitive lethal
PCI	:	pest control of India
SMW	:	standard meteorological week
@	:	at the rate of
km	:	kilo metre
m	:	metre
mm	:	mili metre
ml	:	mili litre
ft	:	foot
RCBD	:	randomized complete block design
RF	:	rainfall
Tmax	:	maximum temperature
Tmin	:	minimum temperature
RHm	:	relative humidity in morning
RHe	:	relative humidity in evening
SSH	:	sunshine hours
S.E.	:	standard error
SD (σ)	:	standard deviation
r	:	correlation coefficient
C.D.	:	critical difference
$\bar{x}$	:	mean
H	:	Shannon-Weiner function
E	:	Pielou evenness index
R	:	Margalef's richness index
^0C	:	degree Celsius
ATL	:	tissue lysis buffer
AL	:	lysis buffer
AW1	:	wash buffer 1
AW2	:	wash buffer 2
AE	:	elution buffer
µL	:	micro litre

nm	:	nano metre
rpm	:	revolutions per minute
CCD	:	coupled device ray
PCR	:	polymerase chain reaction
dH_2O	:	nuclease-free water
M	:	molar
TAE	:	Tris-acetate EDTA buffer
BLAST	:	Basic Local Alignment Search Tool
MEGA	:	Molecular Evolutionary Genetic Analysis
NCBI	:	National Centre for Biotechnology Information
R-M	:	radial-median vein
DM-Cu	:	dorsal median-cubitial vein
BCu	:	basal median-cubital vein
FTW	:	fruit fly per trap per week
Fig	:	figure
Avg.	:	average
MF	:	male to female sex ratio
BPH	:	brown plant hopper
DO	:	*B. dorsalis*
CO	:	*B. correcta*
ZO	:	*B. zonata*
DE	:	*B. digressa*
CU	:	*Z. cucurbitae*
TA	:	*Z. tau*
v/v	:	volume per volume
AT	:	Adenine and Thymine

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Chapter I

INTRODUCTION

Cucurbitaceae (also known as the "gourd family") is the largest family of fruits and vegetables, with approximately 125 genera and 960 species (Karaye *et al*., 2021), commonly called cucurbits (Christenhusz and Byng, 2016). Cucurbits are economically important vegetable crops, having higher adaptability from arid climates to humid tropics (Mondal *et al*., 2020), whose edible fruits have both medicinal and nutritional importance (Mukherjee *et al*., 2022), sharing about 5.6% of the total vegetable production in India (Chauhan and Chauhan, 2021). The edible plants derived from the family are grouped in 5 genera: *Cucurbita, Cucumis, Citrullus, Lagenaria* and *Sechium* (Rolnik and Olas, 2020).

The major cucurbit crops grown in India are: bitter gourd (*Momordica charantia* L.), pumpkin (*Cucurbita maxima* Duchesne.), bottle gourd (*Lagenaria siceraria* Molina Standl.), cucumber (*Cucumis sativus* L.), snake gourd (*Trichosanthes cucumerina* L.), watermelon (*Citrullus lanatus* Thunb.), pointed gourd (*Trichosanthes dioica* Roxb.), ash gourd (*Benincasa hispida* Thunb.), ridge gourd (*Luffa acutangula* Mill.), musk melon (*Cucumis melo* L.), ivy gourd (*Coccinia grandis* L. Voigt), smooth gourd (*Luffa aegyptiaca* Mill.), chayote (*Sechium edule* Jacq. Sw.) sponge gourd (*Luffa cylindrica* L.), kakrol (*Momordica dioica* Roxb.), squash melon (*Praecitrullus fistulosus* Stocks Pang), crookneck squash (*Cucurbita moschata* Duch.) and vegetable marrow (*Cucurbita pepo* L.) (Chakravarty *et al*., 2019). The largest area under cucurbits cultivation in India is of bottle gourd (0.189 million hectares), followed by watermelon (0.166 mha), cucumber (0.112 mha), bitter gourd (0.107 mha), pumpkin (0.104 mha), musk melon (0.061 mha) and pointed gourd (0.059 mha). In terms of production, watermelon (3.157 million metric tonnes), followed by bottle gourd (3.106 mmt), pumpkin (2.183 mmt), cucumber (1.656 mmt), muskmelon (1.368 mmt), bitter gourd (1.268 mmt) and pointed gourd (0.075 mmt) are the major cultivated cucurbits, with the area and production of other crops being negligible (DAC&FW, 2020).

Cucurbits are cultivated in tropical, sub-tropical and warm temperate regions (Chomicki *et al*., 2020). They have a higher economic and nutritional significance (Dhillon *et al*., 2020),

making them a highly preferred crop by vegetable growers throughout India. Cucurbit cultivation in India is done in the warm and rainy seasons mainly by seeds, but various off-season vegetable cultivation practices like plug-ray nursery raising (Singh *et al*., 2009) and Diara-land cultivation of cucurbits (Singh, 2012) which are limited to only a few areas in the country, are also gaining popularity. Some other off-season cucurbit production technologies including plastic low tunnel technology, found to be profitable for the cultivation of bottle gourd, bitter gourd, summer squash and muskmelon; and walk-in tunnels for muskmelon during peak winter periods (Singh *et al*., 2007).

The seeds of cucurbits are rich in proteins and minerals including iron, copper, phosphorus, zinc and magnesium (Rolnik and Olas, 2020). The seeds along with their defatted cakes have high protein content (28-40.49% and 61-73.59%, respectively), along with higher lipid levels comparable to other oilseeds (De and De, 2021), proving to be quite beneficial for those dealing with obesity and other inflammatory diseases (Carrera-Quintanar *et al*., 2018). Uttar Pradesh is the second largest vegetable producing state in the country, after West Bengal (Sarkar *et al*., 2021), with a record production of 10.86 million tonnes of cucurbits in the year 2017-18 (Kumari *et al*., 2021).

Abiotic and biotic restrictions limit agricultural output (Sahu and Samal, 2020), with one of the most important variables being the prevalence of major and minor pests infesting crops at various stages (Tripathi *et al*., 2020). The various pest complexes damaging cucurbits in accordance with the per cent yield loss are: Fruit fly complex *Bactrocera sp.* (more than 90%) (Paudel and Sah, 2020), melon thrips *Thrips palmi* (Karny) (80-90%) (Bhowmik and Saha, 2017), Hadda beetle *Henosepilachna vigitioctopunctata* (Fab.) and *Epilachna dodecastigma* (Wied.) (more than 80%) (Akinkunmi *et al*., 2021), red pumpkin beetle *Aulacophora foveicollis* (Lucas) (35-75%) (Ahmad *et al*., 2020), melon aphid *Aphis gossypii* (upto 60% due to Cucumber Mosaic Virus) (Thackray *et al*., 2004), pumpkin caterpillar *Diaphania indica* (Saunders) (25-50%) (Bhowmik and Saha, 2017), bottle gourd plume moth *Sphenarches caffer* (Zeller) (upto 13%) (Halder *et al*., 2020) and whitefly *Bemisia tabaci* (Gennadius). It is evident that the fruit fly infestation is a serious drawback limiting vegetable growth in the country, especially for cucurbits (Deguine *et al*., 2015).

Fruit flies (Diptera: Tephritidae) are also known as "peacock flies" due to a habitual strutting and vibrating wings (Kapoor, 1993) and to the knowledge, the most destructive pests of horticultural crops (Agarwal and Sueyoshi, 2005). The different fruit fly species infesting cucurbits in India are: *Zeugodacus cucurbitae* Coquillett (Mwatawala *et al*., 2015), *Z. tau* Walker (Nair and Pal, 2020), *Z. scutellaris* Bezzi (Prabhakar *et al*., 2007), *Z. diversa* Coquillett (Royer *et al*., 2018), *Bactrocera zonata* Saunders (Sohail *et al*., 2015), *B. dorsalis* Hendel (Darboe and Osekre, 2021), *B. correcta* Bezzi (Kunprom *et al*., 2015), *Z. caudata* Fabricius (Nair *et al*., 2021), *Z. cilifera* Hendel (Nair *et al*., 2017), *Dacus longicornis* Wiedemann (Nair *et al*., 2017), *D. ciliates* Lowe (Vayssières *et al*., 2008), *Pliomelaena udhampurensis* Agarwal and Kapoor (Prabhakar *et al*., 2012) and *Dioxyna sororcula* (Wiedemann) (Prabhakar *et al*., 2012). Among them, the most destructive pest is *Z. cucurbitae* Coquillett (the melon fruit fly) (Weems *et al*., 2012). The infestation especially due to melon fruit fly in cucurbitaceous hosts leads to a yield loss upto an extent of 30-100% depending on variety, growing season and other conditions (Dhillon *et al*., 2005). The infestation ranges from 41-89% in bitter gourd (Am *et al*., 2011) which is one of the most preferred hosts of the pest (Sen *et al*., 2019), followed by cucumber (56.88%) (Shivangi and Swami, 2017), bottle gourd (9-21%), ridge gourd (14-29%) and pumpkin (9-24%) (Sohrab and Prasad, 2018).

The fruit flies are developmentally holometabolous, with the fruit fly larvae (maggots) being the main damaging stage (Sarwar, 2018). These are internal feeders, being both frugivorous and florivorous (Doorenweerd *et al*., 2018). Generally, the adult female prefers to lay eggs in the soft and tender tissues of the plant (mainly fruits, but also flowers and stems) by piercing them with their sharp and pointed ovipositor. A watery fluid oozes out of the damaged/punctured spot, that becomes slightly concave and transforms into a brown resinous deposit. Sometimes, pseudo-punctures are also observed which are punctures without oviposition, with all these types of damage having a significant effect on the reduction of market value of the crops.

Conventional crop protection measures have some serious limitations. Cucurbits are picked up at short intervals of time for both marketing and self-consumption measures. It is difficult to manage the fruit fly only through foliar application of pesticides, which may kill the adults but is of no use to the maggots, which are internal feeders and the main damaging stage

(Nath *et al*., 2017). It also increases pesticide residue in final marketed fruits and vegetables due to a short waiting period (Anwar *et al*., 2011), destroys natural parasitoids and predators in the ecosystem (Khatun *et al*., 2016), leads to pesticide resistance in fruit fly species (Jin *et al*., 2011), and the resurgence of minor fruit fly and other pest species as major pests (Dutcher, 2007). These are some of the detrimental effects caused by the sole dependency on chemical control for fruit fly management.

Pest control strategies for fruit fly complexes, like any other insect, are totally based on accurate pest identification (Walter, 2003). Fruit fly identification utilizing published literature recommended by scientists and taxonomists is particularly useful in characterizing diverse species that are still prevalent and easily accessible to novice researchers. However, since they are members of cryptic/sibling complexes, numerous significant Tephritid pest species remain difficult to identify (Schutze *et al*., 2012). Despite the fact that sibling species can differ in crucial features including host usage and pest status, regional distribution and seasonal phenology; their research and control are complicated by their physical similarities (Barik *et al*., 2009). The mating behaviour will also differ across siblings, which has ramifications for the sterile insect management strategy, basically relying on the mating success of treated insects (Dyck *et al*., 2021). Furthermore, diagnostic features in taxonomic keys are frequently stated using subjective or relative vocabulary, such as 'narrow', 'slightly expanded', etc., making conclusive identifications more challenging (Lawson *et al*., 2003). For better identification of insect species, DNA sequence-based techniques are getting a lot of interest. The techniques, according to their advocates, have the potential to increase alpha-taxonomical research chances, namely: DNA barcoding (Hebert *et al*., 2003) and DNA taxonomy (Tautz *et al*., 2003). The DNA barcodes, also called the "Barcoding Life" consortium refers to the sequences consist of 650 base pairs (bp) of the mitochondrial cytochrome oxidase I gene (COI gene), which can be used for species identification; where the COI sequence of the unidentified species is matched with the publicly available database of identifiable DNA barcodes (Herbert *et al*., 2004). According to DNA taxonomy, DNA sequences will eventually offer the basic framework for a species-level taxonomy (Tautz *et al*., 2003). However, DNA barcoding is more popular than DNA taxonomy as per the interest of taxonomists and systematics throughout the world (Meier *et al*., 2006). Traditional identification approaches using available literature combined with DNA

barcoding will improve species recognition and provide a better grasp of the physical characteristics of the recognized insects.

Modern technologies such as integrated pest management (IPM) strategies are getting more attention as Indian farmers are now more responsive to changes and believe in sustainable development in agriculture. The use of parapheromones such as methyl-eugenol (ME) and cue-lure (CL) for male annihilation (Devi *et al*., 2021), population dynamics studies (Beer *et al*., 2021), fruit fly monitoring and mass trapping (Pramanik *et al*., 2021) has proven to be effective, economical and environmentally friendly pest management tactics. Of all the above-mentioned fruit fly species associated with cucurbits, the ones known to be attracted to CL are: *Z. cucurbitae* (Paripoorani *et al*., 2021), *Z. tau* (Zaelor *et al*., 2021), *Z. scutellaris* (Singh *et al*., 2017), *Z. cilifera*, *Dacus longicornis* and *Z. caudata* (Nair *et al*., 2017); while *B. dorsalis* (Liu *et al*., 2017), *B. correcta* (Orankanok *et al*., 2013), *B. zonata* (Ahmad and Begum, 2017) and *Z. diversa* (Royer *et al*., 2018) are attracted to ME. The attractant lures of *Pliomelaena udhampurensis* and *Dioxyna sororcula* are unknown (Prabhakar *et al*., 2012). As fruit flies associated with cucurbits are highly sensitive to both the parapheromones (ME and CL), combining the two lures in a single trap will save application costs and may synergize the individual effects of the lures used for monitoring and mass trapping programmes. However, testing the effect of parapheromones when applied in combination or mixed with each other has not yet been studied in the Indian context. It can be of great economic importance as many countries spend cash and capital for conducting wide ranged monitoring and surveillance programmes for fruit fly management. Combining different lures in a single trap may also have a relatively higher trap catches than the individual lures and might also be economical, as preparation and installation of individual traps is highly laborious and expensive.

The fruit flies group as mentioned associated with cucurbits are known to attract a wide range of hosts (Sarwar *et al*., 2013). Adult female fruit flies are well known to choose which fruit to oviposit, based on the fruit's suitability for their offspring's performance (Fontellas-Brandalha and Zucoloto, 2004), which can prove be a critical requirement for host shift in the ecosystem (Sarwar *et al*., 2013). Male annihilation technique (MAT) using parapheromone lures is also used to determine the host preference of fruit flies associated with different fruit crops (Iamba *et al*., 2021). However, placing parapheromone traps in cucurbit fields will also attract non-target

wild fruit fly species that are responsive to the same lures but are the active hosts of other fruits and vegetable crops found in the same area. This will interfere with the trap catches since the number and density of fruit flies in the traps might easily be confused with the ones that have actually emerged from the infested fruits, leading to uncertainty in results. Therefore, for the firm determination of fruit fly species infesting cucurbits as well as their host preference, it is necessary to examine adult emergence from the infested fruits.

Apart from parapheromone based pest management, another less popular but highly efficient tool is the autocidal techniques, which rely on the insect to manage itself, such as releasing sterile insects or inducing sterility in wild insects in the next or following generations (Handler, 2016). One of the most often utilized autocidal tactics against fruit flies is the sterile insect technique (SIT) (Hendrichs and Robinson, 2009). SIT works by mass-producing, sterilizing and releasing insects (preferentially males) of the targeted species on a regular basis (Mateos *et al*., 2020). Several Tephritids have had successful implementation of SIT programmes as a part of Area-wide Integrated Pest Management (AW-IPM) strategies; including *Ceratitis capitata* (Wiedemann) (Rempoulakis *et al*., 2016), *Anastrepha ludens* (Loew) (Rull *et al*., 2007), *Anastrepha obliqua* (Macquart) (Gallardo-Ortiz *et al*., 2018), *Zeugodacus cucurbitae* (Coquillett) (Miyatake, 2021), *Bactrocera dorsalis* Hendel (Aketarawong *et al*., 2011) and *Bactrocera tryoni* (Froggatt) (Collins *et al*., 2009). The incompatible insect technique (IIT) is another autocidal strategy that uses mating between insects which are mass-reared and wild, to control pest populations, mainly based on the principle of inducing female sterility by utilizing endosymbiotic bacteria (mainly belonging to genus *Wolbachia)* instead of radiation (Mateos *et al*., 2020).

Wolbachia is a maternally inherited, intracellular endosymbiotic bacteria, widely known to infect an estimated 65% of species of insects (Hilgenboecker *et al*., 2008). Feminization, parthenogenesis, male killing and egg-sperm incompatibility are some of the ways in which it affects the host biology, mainly for the enhancement of symbiotic transmission and persistence in the host (falling under the category of reproductive parasitism) (Werren *et al*., 2008). Among all the four methods of reproductive manipulations, the most prevalently induced by *Wolbachia* is cytoplasmic incompatibility (CI) (Mateos *et al*., 2020). All or part of the eggs from uninfected females that are fertilized by sperm from *Wolbachia*-infected male fail to develop in

unidirectional CI (uni-CI) (Telschow *et al*., 2007), whereas, crosses between two distinct (incompatible) *Wolbachia* strains result in bi-directional CI (bi-CI) where females and males infected with the same or suitable *Wolbachia* strains can successfully cross (Telschow *et al*., 2005). On the basis of uni-CI and bi-CI, the IIT can be implemented by two approaches: if the population of the target species lacks *Wolbachia* (uni-CI) by releasing males infected with one or more CI-inducing *Wolbachia* strains; or if the target species population harbour some or more CI-inducing *Wolbachia* strains (bi-CI) by releasing males with no *Wolbachia* infection (Mateos *et al*., 2020). However, there have been both positive (Asimakis *et al*., 2019) and negative (Manger *et al*., 2018) reports of their infection in fruit flies connected with cucurbits from India. It also interacts with the mitochondrial (COI) gene, a widely used marker for identifying and establishing evolutionary links between diverse invertebrate species (Hebert *et al*., 2004a). Thus, its presence in the infected species specimen may also leads to its misidentification on the basis of DNA barcoding.

Hence, the present study was undertaken to study population dynamics and molecular characterization of fruit flies associated with cucurbits, with the objectives given below:

- To study the population dynamics of fruit fly associated with bitter gourd.
- To study the trapping efficiency of combination of methyl-eugenol (ME) and cue-lure (CL) against responsive fruit flies.
- Molecular characterization and checking *Wolbachia* infection in fruit flies.

Chapter II

REVIEW OF LITERATURE

The present investigation entitles **"Study of population dynamics and molecular characterization of fruit flies associated with cucurbits,"** was carried out in the *kharif* of 2021 (from 35th to 44th SMW) at a farmer's filed in Ramna village, Varanasi. A concerted effort has been undertaken to evaluate the literature in this area under the following headings:

2.1. General biology of melon fruit fly, *Zeugodacus cucurbitae* Coquillett

The eggs are white, elliptical, flat ventrally and convex laterally and are laid either singly or in a cluster of 4-10. The incubation period of the egg ranges from 12-24 hours (Mir *et al*., 2014) or 24.4-38 hours (Waseem *et al*., 2012) with a mean of 16.8±6.9 hours in cucumber (Mir *et al*., 2014), 18.0±6.3 hours in bitter gourd (Gaddanakeri and Rolania, 2020) and 1.9±0.65 days in pumpkin (Das *et al*., 2017a).

The maggots are apodous and pass through three instars before going into pupation. They are elongated, transparent and have a pointed head with a mandibular hook. The total duration of the maggot period ranges from 5-7 days (mean of 5.80±0.78 days) in bitter gourd (Gaddanakeri and Rolania, 2020), 3.5-6.0 days (mean of 5.03±0.27 days) in sponge gourd (Desai *et al*., 2018), mean of 4.5±1.13 days and 6.45±1.38 days in cucumber (Mir *et al*., 2014). Matured 3rd instar maggots stop feeding, become sluggish and bend slightly, attaining a pre-pupal stage. The stage lasts for only 0.5-1 day (mean of 0.7±0.20 days) in bitter gourd (Gaddanakeri and Rolania, 2020), mean of 0.61±0.13 days in sponge gourd (Desai *et al*., 2018) and 0.8±0.25 days in cucumber (Mir *et al*., 2014).

The pupa is 11 segmented, barrel-shaped and yellowish-brown in colour. The pupal period ranges from 6-8 days (mean of 6.9±0.87 days) in bitter gourd (Gaddanakeri and Rolania, 2020), 8-9 days (mean of 8.4±0.51 days) in cucumber (Mir *et al*., 2014) and a mean of 7.13±0.71 days in sponge gourd (Desai *et al*., 2018).

Adults are reddish brown in colour, moderately sized, with lemon yellow markings on their thorax and spotted wings. The vitality of both sexes varies differently. The longevity of

adult females and males vary from 20-33 days (mean of 25.9±4.15 days) and 18-31 days (mean of 23.7±3.77 days) (Gaddanakeri and Rolania, 2020), 32-60 days (mean of 48.6±3.51 days) and 30-52 days (mean of 40.4±2.95 days) respectively, depending upon the availability of food and water sources in that particular bio-ecological system.

2.2. The population dynamics of fruit fly associated with cucurbits.

Banerji *et al*. (2005) reported that the population of *Z. cucurbitae* was present throughout the year with its incidence was found to be the highest during *kharif* season (peak population in the season was observed at the 39th SMW with 63.33% fruit infestation) followed by summer season (population peak observed at the 42nd SMW with 43.33% fruit infestation) and the lowest in *rabi* season (population peak observed at the 14th and 15th SMW with 33.33% fruit infestation) on the basis of per cent fruit infestation in Meghna variety of bitter gourd in West Bengal, India.

In a study conducted by Alim *et al*. (2012) in three places in Bangladesh: Goloar char, Pubail and Ganakbari during the years 2007 and 2008, it was found that the population of *Z. cucurbitae* was present throughout the years. In Goloar char, the highest population was observed in the month of May (381.37±22.40 in 2007 and 472.62 ±42.63 in 2008) and the lowest in November (19.12±3.01) in 2007 and October (18.37±4.37) in 2008. In Pubail, the highest population was observed in June (388.75±50.34 in 2007 and 319.37±47.67 in 2008) and the lowest in December (37.25±7.16) in 2007 and November (16.12±2.5) in 2008. In Ganakbari, the peak in population was in June (278.00±26.65) in 2007 and April (251.62±25.73) in 2008 and the lowest recorded population was in October (55.62±6.88 and 35.87±4.38, respectively) both in 2007 and 2008 as per fruit flies collected from cue-lure traps with the data of mean fruit flies per trap per 15 days ± SD.

As per the studies conducted by Lekshmi *et al*. (2014) regarding the population dynamics of major pest species in bitter gourd, *Z. cucurbitae*, the population was first observed during the 4th week of March. The fruit flies were the highest in the 4th week (4.6 adults/plant) and 3rd week (4.3 adults/plant) of April in 2012 and 2013, respectively, as per the count of adults resting on the bitter gourd fruits (variety: Pusa Vishesh) during the morning time in New Delhi, India.

Stanley *et al*. (2015) studied the population dynamics of *B. zonata, B. dorsalis* and *B. diversa* through ME traps in vegetable gardens (including cucurbits) and orchards in Uttarkashi, Uttarakhand, from the month of October 2007 to September 2009. The population of *B. zonata* in vegetable gardens were the highest during the study period with the population peaked during the month of July 2008 and 2009. The peaks in *B. dorsalis* population observed, was in the month of September 2008 and 2009, whereas, the population of *B. diversa* was very low with the highest population observed in the month June and August of 2008. The mean trap catches of fruit flies were higher in orchard than compared with vegetable garden, proving fruit crops being the better preferred hosts for *B. dorsalis*, *B. zonata* and *B. diversa*.

In research conducted by Abro *et al*. (2017), the incidence of melon fruit fly *Z. cucurbitae* was highest in ridge gourd (124.86), followed by bitter gourd (114.11) and bottle gourd (104.73) as per the data of the mean population of fruit flies/trap in the CL traps. The population was present throughout the year with the maximum and statistically significant population observed in the months of May (300-350) and October (150-200) as per the total adults captured from all traps and different crop fields at district Hyderabad and Sindh, Pakistan.

As per the study by Dubale *et al*. (2018), the infestation of fruit flies started after fruit setting in the 33rd SMW with a minimum population reached at the 36th SMW (16.67±18.25) and a maximum at the 40th SMW (50.00±18.25) on the basis of per cent fruit infestation ± standard deviation (SD) in a field of ridge gourd (cultivar: Konkan Harita) in Maharashtra, India.

Wazir *et al*. (2019) conducted a study on summer squash (variety: DON 17) from the 18th to 29th SMW in Jammu, India, and reported that the incidence of melon fruit fly *Z. cucurbitae* was present throughout the study period, with the lowest population recorded being in the 19th SMW (20 adults/trap/week) and the highest in the 26th SMW (317 adults/trap/week), trapped in Green Victory ME traps.

According to a study by Nair *et al*. (2020), the adults of *Z. tau* were found to be abundant during the study period from July 2015 to June 2017 under different cucurbitaceous crops in Tripura, India. The adults were collected in CL pheromone traps. The trap catches with the lowest population were recorded in the 52nd SMW of 2015 (5 flies/trap/week) and the 51st SMW

of 2016 (9 flies/trap/week). The highest populations were observed in the 14th SMW of 2016 (83 flies/trap/week) and 2017 (72 flies/trap/week).

Nahid *et al*. (2020) conducted studies in cucumber fields from the months of March to May and June to August, 2017 in Gazipur, Bangladesh. The flies were collected using ME traps at a four-day interval. The population of *Z. cucurbitae* was prevalent throughout the study period in the area with the highest population in the summer season was observed on the 11th of April (34 flies/trap/4 days) and the lowest on the 7th of April (21 flies/trap/4 days). In the autumn season, the highest population was observed on July 15th (19 flies/trap/4 days) and the lowest on July 11th (10 mean population/trap/4 days). The incidence was higher in the summer than in the autumn season.

According to Beer *et al*. (2021), the peak in the population of *Z. cucurbitae* was observed during the 41st SMW (70.00 flies/trap/week) and of *Z. tau* in the 32nd SMW (15.50 flies/trap/week), which were collected in the CL traps. The population of *B. zonata* reached its peak during the 40th SMW (65.00 flies/trap/week), *B. dorsalis* in the 42nd SMW (58 flies/trap/week), *B. correcta* in the 44th SMW (10.50 flies/trap/week) and the mean population of *Z. diversa* 1.24 fruit flies/trap/week during the 43rd to 46th SMW collected in the ME traps. The study was conducted in a bitter gourd crop field in Uttar Pradesh, India.

As per the research conducted by Patel and Das (2021), the peak in the population of *Z. cucurbitae* was observed at the 33rd SMW (57 flies/trap/day) in the year 2016, the 33rd and 36th SMW (17 flies/trap/day) in the year 2017 and the 8th SMW (19 flies/trap/day) in 2018 in CL traps. Almost a same trend in decline was observed in the 42nd to 12th SMW (4-8 flies/trap/day) in all the three years in a farm area in West Bengal, India where no regular cucurbits were grown within a radius of 1 km.

A study was conducted by Bose *et al*. (2021) in a vegetable garden cultivated with cucurbits, Rajshahi, Bangladesh during the months of July 2014 till January 2015 to study the population dynamics of *Z. cucurbitae* using CL traps. The population followed an irregular trend throughout the study period, with the highest fruit flies/trap/month recorded in the month of August (77.19±19) whereas, the least population was recorded in the month of January

(12.29±0.70). The results revealed that the population of *Z. cucurbitae* varied significantly between months ($p<0.001$).

2.3. Trapping efficiency of combination of methyl eugenol (ME) and cue-lure (CL) against responsive fruit flies.

Hooper (1978) conducted an experiment to study the effect of combining ME and CL in a single trap in eucalypt forest area in Queensland for 16 weeks. Four treatments were prepared and replicated three times at 0.5 km intervals: (i) and (ii) single traps of ME and CL, (iii) double traps of ME and CL hung side by side on the same tree, and (iv) a mixture trap of ME and CL in separate wicks. The total number of fruit flies of different species captured in single traps of ME was significantly higher than those in double and mixed traps. However, in the case of CL, the trap catch of fruit flies was the highest (but not significant) in double traps compared with single and mixture traps, showing that combining ME and CL on a single wick in one trap will reduce the catch of species responding to either attractant.

A study was conducted by Keng-Hong and Soo-Lam (1982) on the species diversity and abundance and of fruit flies in Penang Island, West Malaysia for 18 weeks in diverse ecosystems by using different attractants, namely: Capilure (mixture of Trimedlure and extender), CL, Dorsalure (mixture of ME and CL), ME and Trimedlure in traps separated by a distance of 30 m (with no chemical details or proportion of the mixtures). Dorsalure captured a significantly lower number of *B. dorsalis* and *Z. cucurbitae* males than individual ME and CL traps, respectively.

Vargas *et al*. (2000) studied the consequences of combining ME and CL in different proportions: (i) 100% ME and 100% CL in separate traps, (ii) a mixture of 25% ME and 75% CL in a single trap, (iii) 50% ME and 50% CL in a single trap, and (iv) 75% ME and 25% CL in a single trap in terms of trap catches of *B. dorsalis* to *ME* and *Z. cucurbitae* to CL in Kauai Island, Hawaii for four seasons. The traps were separated by a distance of 20 m and placed at 1 m height above the ground in the papaya orchard. The population of *B. dorsalis* and *Z. cucurbitae* captured in 100% ME and 100% CL traps were found to be the highest than in all the lure combinations, throughout the study period, respectively. The study recommended the use of individual lure traps, proving to be advantageous over combining different lures in a single trap for fruit fly management practices.

The following study was conducted by Shelly *et al*. (2004) in Oahu, Hawaii from January to November, to check the effectiveness of combining ME and CL in a single trap by testing its response in the trap catches of *B. dorsalis* in ME baited trap due to the presence of CL and *Z. cucurbitae* in CL baited trap due to the presence of ME. In a mixed fruit orchard, traps baited with ME and CL in the same trap (same trap with a mixture of both lures in the same wick and same trap with both lures in separate wicks) and single lure traps baited with either ME or CL (traps separated 1 m away and 3 m in the same tree) were used. Trap baited with ME and CL (separate or mixed) captured significantly fewer males of *B. dorsalis* or *Z. cucurbitae* than traps baited with ME or CL alone, respectively. As a result, combining CL and ME in a single trap will reduce the trap catches of subsequent fruit fly species populations attracted to it.

An experiment was conducted by Dominiak *et al*. (2011) by mixing ME and CL at different volumes in a single trap in order to check the potential advantages of fruit fly surveillance programmes. The study was conducted in mixed fruit orchards in Griffith and Sydney, Australia from the months of March to December, 2003 by preparing lure traps for four treatments: (Treatment: A) CL (4.4 ml), (Treatment: B) CL (4.4 ml) + ME (0.5 ml), (Treatment: C) CL (4.4 ml) + ME (2.2 ml), and (Treatment: D) ME (2 ml), separated by an average distance of 5.8 m in ten (Griffith) and nine (Sydney) experimental sites. The trap catches of CL responsive fruit fly species were the highest for treatment C followed by B and A; i.e., more fruit flies were captured in mixed lures (CL+ME) than in individual CL traps in various sites in Griffith, but the number of CL responsive fruit flies captured in individual lures was greater than in mixed lures in different sites in Sydney. However, the ME responsive fruit fly species were more attracted to individual lures (treatment D) than compared with combinations, providing a mixed response, which is more in favour of using parapheromone traps with mixed lures for fruit fly surveillance than in male annihilation.

According to Wee and Shelly (2013), to check the better fruit fly capturing ability among liquid versus solid lure formulations, a detailed study was conducted in an orchard with different fruit trees in Malaysia for 12 weeks, where ME and raspberry ketone (RK) (a synthetic analogue of CL) were mixed in liquid and wafer separately and compared with individual ME and RK lure traps, kept as standards. ME traps in liquid form captured more males of *Bactrocera* sp. (4 out of 5 ME responding species of fruit flies in the study area) than traps with a mixture of ME and RK

containing wafers. Out of the six cue-lure responsive fruit fly species in the study area, the populations of *Z. cucurbitae* in the trap catch were significantly higher in liquid than compared with a mixture of ME and RK wafers baited traps.

Shelly and Kurashima (2016) conducted a study to compare the efficacy of individual parapheromones raspberry ketone (natural analogue of CL) (RK) and ME for male annihilation of *Z. cucurbitae* and *B. dorsalis*, respectively, when applied in combination with one another. Three ME (liquid) and RK (powder) mixtures by weight in various proportions (85:15, 90:10 and 95:5, respectively) were prepared along with individual lure traps and were installed in a coffee field in Hawaii at 1.5-2 m above the ground, for 8 weeks at a distance of 50 m. The males of *B. dorsalis* captured in mixed lures were higher (but not significant) than compared with individuals captured in ME traps, whereas the population of *Z. cucurbitae* captured had no significant difference in combined and individual lures. But the given study suggests combining ME and RK for multi-species trapping of fruit fly species.

Inskeep *et al.* (2018) studied the effectiveness of combining zingerone (ZN) and CL in a single trap versus separate ZN lure in trap captures of *Z. cucurbitae* in a commercial farm (cultivating eggplant, cucumber and tomato) in Oahu, Hawaii, from the month of July to September, 2015. The field experiment was conducted using five treatments with both the lures (ZN and CL) mixed in different ratios, which were: (Treatment: 1) 100% ZN, (Treatment: 2) 75% ZN + 25% CL, (Treatment: 3) 50% ZN + 50% CL, (Treatment: 4) 25% ZN + 75% CL and (Treatment: 5) 100% CL. Five treatments were installed in each host randomly in blocks (trap distance of 100 m between the blocks and 35 m withing a block). The fruit fly trap catches per week was the highest for 100% CL (1724±242), which significantly reduced in each treatment the per cent share of CL was reduced, with the lowest in 100% ZN (39±9). However, the trap catches were the highest in tomato (1105±151 flies/trap), followed by cucumber (877±150 flies/trap) and eggplant (544±72 flies/trap). It proved mixing ZN with CL have an inhibitory role in subsequent trap catches of *Z. cucurbitae*, with tomato being the highly preferred host.

An experiment was conducted by Royer and Mayer (2018), to examine the effect of combining CL and ME in a single trap to study its influence on attractiveness of different fruit fly species in Papua New Guinea and Australia. CL and ME were placed on separate wicks hung

in a single trap (ME/CL) along with ME and CL hung in separate traps, were field tested. The traps were placed at rainforest, transition forest, coastal forest and urban areas in order to cover a range of habitats. 24 of the 27 fruit fly species caught were substantially more attracted to ME or CL alone than to the ME/CL, in Australia. Similar findings were observed in Papua New Guinea, where 13 of 16 fruit fly species caught were significantly more attracted to individual lures than to ME/CL, proving that combining ME and CL reduces trap catches for most of the Dacini fruit fly species.

Stringer *et al*. (2019) combined different lures and studied their impact on surveillance of fruit fly species in mixed fruit orchards in Hawaii (USA), New South Wales and West Australia (Australia) throughout the year. The various lures (in wafer formulation) combined in a mixture were raspberry ketone (RK) (2.0 g), CL (2.0 g), Trimedlure (TL) (3.5 g), ME (5.5 g) in traps, which were compared with single, double and triple combinations of TL, ME and CL (RK only kept in triple combination) isolated by a distance of 20 m. The population of fruit fly species responsive to individual lures was less (but not significant) in mixed lures than compared with individual lures in all the locations, proving it to be effective for surveillance programmes of fruit fly species present in a given area, whose sensitivity can be increased by increasing the trap density.

Divya *et al*. (2019) conducted research to assess the effectiveness of combining different lures and traps for trapping melon fruit fly *Z. cucurbitae* for both monitoring and mass trapping purposes in pumpkin and bottle gourd orchards in Tamil Nadu, India from the month of March to May, 2011. Traps were placed at a distance of 10 m and tied 6" below the pandal wire (bottle gourd orchard) and 60 cm above ground (pumpkin field). The following treatments were created using various traps and lures: (T1) Jar trap + CL (disc), (T2) Jar trap + CL + Ammonium acetate (AA) (disc), (T3) Jar trap + AA (disc), (T4) Jar trap + CL (vial and wick), (T5) Collapsible trap + CL (disc), (T6) Jar trap + CL + ME (disc), (T7) Jar trap (with gum inside) + CL (disc), (T8) Jar trap (with gum inside) + CL {Pest control of India (PCI) – disc}, and (T9) Dome trap (with water inside) + CL (PCI - disc). The greatest overall trap catches of *Z. cucurbitae* was of T6 (Jar trap + CL + ME) (20.85% of the total trap catch), then compared with T1 (Jar trap + CL) (17.11%) and T2 (Jar trap + CL + AA) (16.23%) proving the use of CL in combination with ME for both monitoring and mass trapping of melon fruit flies.

Younus *et al*. (2022) performed an 18-week experiment (June to September, 2021) to evaluate several traps and lures for monitoring fruit flies belonging to the genus *Bactrocera* at a peach orchard in Swat district, Pakistan. Three traps (cylindrical bottle trap, fruition NOVA trap, and yellow sticky trap) were made with four different lure treatments, including ME, CL, methyl eugenol + cue-lure (ME/CL) and fruition lure (FL), and were installed at a rate of 15 traps per acre in a 3-acre area with a height of 5 feet on trees. The best results were obtained with cylindrical bottle traps (155 mean fruit flies/trap/week), while ME traps (134 mean fruit flies/trap/week) outperformed ME/CL (95 mean fruit flies/trap/week), with CL having the fewest trap catches (14 mean fruit flies/trap/week), demonstrating the superiority of ME lure traps over ME/CL for monitoring purposes.

2.3. *Wolbachia* infection detection in fruit flies.

In a study by Jamnongluk *et al*. (2002) to identify *Wolbachia* infection in different fruit fly species found in Australia based on *wsp* gene sequence, fifteen *Wolbachia* strains were found to be infecting nine fruit fly species, namely: *Z. cucurbitae, Z. caudata, B. dorsalis sp.* A[a], *Z. diversa, Z. modicus, B. pyrifoliae, Z. ascites, B. dorsalis sp.* A1[a] and *Dacus destillatoria* collected from cucurbitaceous hosts. Among them, *Z. ascites* harbored five different *Wolbachia* strains and *B. dorsalis sp.* A1[a] and *Z. diversa* were infected with two different strains.

Karimi and Darsouei (2004) conducted a study in order to check the presence of *Wolbachia* in different insect species of the Tephritidae family. Fruit flies were collected as larvae in infested fruits and reared in insect chambers filled with a 5 cm soil layer from Razavi, North and South Khorasan provinces in Northeastern Iran during the spring and summer season of 2011. DNA extraction and molecular analysis was conducted by *Wolbachia* detection based on multilocus sequence typing (MLST), under which internal segments of five single copy having stable genes per strain were routinely sequenced. *Carpomya vesuviana* and *Rhagoletis cerasi* showed infection by two separate strains of *Wolbachia* (wVes1 and wCer6, respectively). The use of MLST and *wsp* gene sequence to genotype *Wolbachia* strains in 12 populations out of 5 fruit fly species revealed the presence of 2 novel strains and a new strain type (ST) belonging to the supergroup A (Zhou *et al*., 1998). The phylogenic analysis of the

identified strains strongly supported the hypothesis of horizontal inter-species and intra-specific transmission of *Wolbachia*

Coscrato *et al*. (2009) performed research to study the infection of *Wolbachia* in fruit flies (*Ceratitis capitata* and *Anastrepha* spp). The individuals were collected from infested fruits from different host crops including orange, tropical almond, loquat, guava, apricot, pumpkin, etc., from different location within Brazil. The molecular analysis was done on the basis of PCR assay using *wsp* gene primers (amplifying 586-610 bp fragments) followed by *136A-F* and *691A-R* (amplifying 556 bp fragments). The *wsp* gene was found to be the most variable and informative for phylogenic studies, with all the individuals of *Anastrepha* spp were found to be infected with *Wolbachia* strain belonging to supergroup A. According to phylogenic analysis, *Wolbachia* infected strains found in *Anastrepha* spp was found belonging to the same group as wMel, infecting *Drosophila melanogaster* studied at different locations providing the evidences for horizontal transfer of *Wolbachia.*

In an experiment by Sarakatsanou *et al*. (2011) to study the effect of *Wolbachia* strain (wCer2) on the fitness of laboratory reared lines of *Ceratitis capitata* and the effect of 2 different *Wolbachia* strains (wCer4 and wCer2) on single fruit fly line. The experiment was conducted in Greece from the month of March 2009 to January 2010 and the fruit fly lines used were Benakeio, WolMed 88.6, WolMed S10.3, Vienna 8 and Vienna 8-E88. The results stated that infection with *Wolbachia* affects the vital fitness component in two strains (Benakeio and Vienna 8 GS) and the two sexes respond separately to the infection, with different strains of *Wolbachia* eliciting different response in same strain of fruit fly genotypes.

Sun *et al*. (2014) in an experiment to identify the diversity of *Wolbachia* were studied by collecting wild populations of *B. dorsalis* from five different provinces of China, such as Guangdong, Guangxi, Fujian, Hainan and Yunnan by ME lure traps from the months of June to August 2004 and 2005. Results reveled infection of *Wolbachia* at lower rate (0.7-3%) in nineteen wild males of *B. dorsalis* and the Wolbachia belongs to three groups such as *Mel, Kue* and *Cuc.* This showed a possibility that bacteria may have been transferred horizontally across species giving evidence of natural gene flow.

Morrow *et al*. (2015) conducted and experiment to study the horizontal transmission of *Wolbachia* into uninfected host lineages of fruit flies by collecting fruit flies belonging to the genera *Ceratitis*, *Bactrocera, Dirioxa* and *Dacus*, via parapheromone traps in two sampling periods (1996 to 2001 and 2012 to 2013) from different regions in New South Wales. *Wolbachia* sequences were detected in nine of the 24 species (37% of the total population studied) with seven species (29%) displaying four distinct *Wolbachia* strains (*wsp*-11, *wsp*-662, *wsp*-661 and *wsp*-16) and rest two species contained pseudogenes and incomplete sets of marker genes, based on characterization of full multi locus sequencing (MLST) profiles. Overall, *Wolbachia* strains were limited to tropical areas (two strains mainly, ST-289 and ST-285) and a low frequency of the four *Wolbachia* strains were observed in infected species (2-17%). In the absence of comprehensive MLST profiles, the discovery of *Wolbachia* pseudogenes infection at high frequency in 2 species might indicate the imprints of lost past infections. The identical low prevalence strains discovered in limited number of individuals from 7 different species doubted their role in vertical inheritance and as a reproductive manipulator. Instead, the study might be evidence of transitory infections caused by unidentified spillover occurrences.

Bakovic *et al*. (2018) investigated the spatial spread of *Wolbachia* in the European cherry fruit fly, *Rhagoletis cerasi* collected from Austria, Czech Republic (2015) and Hungry (2016) through infested fruits of *Prunus avium*. A standard non-linear equation described the spatial distribution of *Wolbachia* through fruit fly adults representing a standard wave (using standard wave model). The results showed that wCer2 strain of *Wolbachia* is known to spread in *R. cersai* within Central European countries with an adult dispersion rate of 3.8 km per generation in Czech Republic (with a wave width of 11.4 km) and 2.0 km in Hungry (width of 5.8 km) representing a fast spatial spread of *Wolbachia* in Europe.

An experiment conducted by Gichuhi *et al*. (2019), to estimate diversity of *Wolbachia* in a population of *B. dorsalis* males collected from mango farms at different locations in Sudan and females collected from infested mango fruits in Kenya in 2017, which were later reared in an insect rearing cage filled with sand to check adult emergence. Ten samples detected *Wolbachia* sequences and four different variants were discovered in African populations of *B. dorsalis* based on their *coxA* and *wsp* sequences: WdorTzc13, WdorTg6, WdorNg3/WdorMu2 and WdorKi1. WdorSl11, a sample discovered from Sri Lanka was a fifth variation. All of these variations

differed from those previously identified in *B. dorsalis*, suggesting that this species harbors a great variety of low prevalence *Wolbachia*. However, the sexual variations in this host have no effect on the *Wolbachia* detection rate.

Yong *et al*. (2019) investigated *Wolbachia* endosymbiont prevalence in the microbiome of the *B. latifrons* chilli host crop across life stages in Malaysia. With five bacterial phyla discovered in larva stages, four in adult females and two in adult males, the infection rate was substantially greater in immature stages than in adults. Only *Wolbachia* and *Halomonas* were present throughout the insect's life stages, out of a total of 77 bacterial taxa. The abundance of the *Wolbachia* endosymbiont *Culex quinquefasciatus* Pel was found to be 98.64% in the larval stage, 98.53% in the pupal stage, 97.66% in adult males and 99.89% in adult females, indicating that the bacteria is transmitted from the larval stage to emerging adults. This was the first report on the microbiota associated with *B. latifrons*.

A study was conducted by Asimakis *et al*. (2019) to investigate the presence of endosymbiotic reproductive parasites including *Wolbachia, Spiroplasma, Arsenophonus* and *Cardinium* in 9 fruit fly species under to genus *Bactrocera, Zeugodacus* and *Dacus* collected from Southeast Asian countries of India, China and Bangladesh. Out of the total population, *Wolbachia* infection was the most prevalent (64 out of 801 infected individuals i.e., 8% of the population) but only 1.5% of the population (12 out of 801) was infected with *Cardinium*. *Spiroplasma* infection was detected only in two individuals (*B. dorsalis* and *Z. cucurbitae*) and no individuals were found to be infected with *Arsenophonus.* The fruit fly species found infected with *Wolbachia* include *B. scutellaris* (42.9% of the total individuals found infected), *B. correcta* (30%), *B. dorsalis* (13.2%) and *B. zonata* (12.2%). No symbiotic bacterial infection was found in individuals of *B. nigrofemoralis, B. minax,* and *D. longicornis*. Three *Wolbachia* pseudogenes including 16S *rRNA, wsp* and *ftsZ* were observed in all the species detected with *Wolbachia* primarily responsible for reduction in the size of their gene sequences. 16S *rRNA* was prevalent in all the species whereas, *ftsZ* and *wsp* was only found in *B. zonata*.

Schebeck *et al*. (2019) studied the dynamics of *Wolbachia* strains spread in the cherry fruit fly, *Rhagoletis cerasi* collected from infested fruits of *Lonicera xylosteum* and *Prunus avium* from 19 different locations from Germany in the month of July 2016. As a result, an

average of 30.2% of the population (89 out of 295) was found to be infected with wCer2 strain ranging from 0% (Rosbach, Wallenrod and Lich) to 100% (Dossenheim). The results showing the spread of *Wolbachia* was comparable with the historical data of the last two decades and spreading from wCer2-infected to wCer2-uninfected individuals rather showing a gradual spread showed a scattered geographical pattern. This led to assumption that due to sufficiently higher infection rate, wCer2 strain will get fixed in some following years just as wCer1 strain is already fixed in Europe (Arthofer *et al*., 2009).

Dionysopoulou *et al*. (2020) investigated the reaction of two *Ceratitis capitata* fruit fly lines to *Wolbachia* strains, as well as the differential impacts of two *Wolbachia* strains in the same lines of fruit fly, at a laboratory at the University of Thessaly, Greece, from September 2017 to April 2018. The following four populations of *C. capitata* were used: Banekeio, 88.6, S10.3 and Wildish, reared in two hosts (apple and bitter gourd). Wildish lines had a greater survival rate than laboratory-adapted lines (Banekeio), especially in bitter oranges. The *Wolbachia* infected lines, particularly the *w*Cer4 infected line, had lower survival rates and longer developmental durations. The *Wolbachia* infected *w*Cer4 line population was killed by high temperatures, which stifled immature growth. In both host fruits, lower temperatures predicted longer developmental durations for juvenile stages of all fruit fly populations studied. This research, for the first time showed the demographic changes caused by *Wolbachia* when *C. capitata* immature development occurs on natural host fruits.

Chapter III

MATERIALS AND METHODS

"Study of population dynamics and molecular characterization of fruit flies associated with cucurbits" was the goal of the thesis. This chapter discusses how to apply materials and methods that are consistent with research.

3.1. General details about the experiment

The experiment was carried out in a bitter gourd (*Momordica charantia* L.) field of 60 biswa area (0.75 hectare) at a farmer's field in Ramna village, Varanasi, Uttar Pradesh (India), during the *kharif* season, from the months of August to November of 2021, i.e., 35th to 44th Standard Meteorological Week (SMW). Ramna village is situated 3 km away from Varanasi city (25.3176^0 North, 82.9739^0 East) and at an elevation of 80.71 m (282 ft) above the main sea level. The crop was raised by farmer and the details he followed are here. 3.75 kg seeds of bitter gourd, variety Pragati (Brand: East West Seeds) was sown during 3rd week of July at a seed rate of 4 to 5 kg per hectare (ha). The seeds were sown at a distance of 150 cm (row-to-row) and 150 cm (plant-to-plant) (1.5 m × 1.5 m) spacing and at depth of 2-5 cm in pandal training system. A full pandal was erect at a height of 2 m (approximately 6 ft) from the ground with the help of bamboo poles buried into the soil at a depth of 45 cm, separated by a distance of 5 m along the pits. Nylon threads was tied on the poles, extended and passed on across the field at a distance of 1-1.5 m above the ground.

3.2. Preparation of low-cost trap bottles

Transparent plastic water bottles (1 litre capacity) were used to prepare low-cost traps. Empty bottles were washed properly and the plastic wrapper was removed. Two windows of size 2 cm × 5 cm (length × breadth) were cut parallel to each other with the help of scissors and a sharp knife, at a height of 2/3rd of the length of the bottle from the cap. Four holes were punched at the base of the bottle with the help of a needle and sharp knife, two of them being 5 cm and the other two being 8 cm apart (Plate 1.), along with a small hole at the centre of the cap for draining out of excess water collected in traps during rainy season (Plate 2). A piece of thread

was guided through the holes at the base of the bottle (8 cm apart) and tied to form a loop for hanging the bottles upside down. Another thread was passed through the holes 5 cm apart and was used to tie a lure wick made up of wooden blocks of size 5 cm × 5 cm × 2 cm (length × breadth × width) with a hole at the centre (Plate 2). Handling of sharp knife, pair of scisssors and needle was done cautiously. A rubber cork was kept at the base to punch holes in the bottle caps. The cut edges of the bottles were also handled carefully.

3.3. Preparation of parapheromone lure solutions

Parapheromone methyl eugenol (ME) (4-allyl-1,2-dimethoxybenzene) and cue-lure (CL) {4-(p-acetoxyphenyl)-2-butanone} were obtained from Sisco Research Laboratories, Mumbai, India. Two separate solutions of ME and CL were prepared in different glass beakers (1 litre capacity each) by mixing 100% ethyl alcohol (60 ml), ME or CL (40 ml) and Malathion 50 EC (20 ml) with the help of a measuring flask, in the ratio of 6:4:2 as per the design based on the experiment by Mariadoss (2020) (Plate 3). The beakers were labeled to prevent misidentification. The wooden blocks were soaked in the lure solutions for the duration of 24-48 hours for complete absorption of the solution. 15 wooden blocks were added in each solution containing individual lures. The mouth of both the beakers were covered with aluminum foil tied around with the help of rubber bands (Plate 4). While preparing parapheromone lure solutions, proper precautions were taken, such as wearing double masks and disposable rubber gloves (GM polynitrile rubber gloves). The table used was cleaned and covered with double layer of blotting paper to prevent chemical spillover without any harm. After adding each chemical, separate sterilized wooden sticks for each solution were used to slightly stir the mixtures which were disposed after use. The mixtures prepared were kept away from light in a separate room for mentioned duration of time to prevent any respiratory or allergic reaction. The following procedure was followed under the supervision of my advisor.

3.4. Final preparation of lure traps

The soaked wooden blocks were then hung inside the prepared trap bottles almost to the level of the cut window with the help of a thread passing through the centre of the blocks. The then prepared trap bottles containing individual lures were correctly labelled with the name of

lures installed in it (Plate 5). For traps with a combination of lures, two wooden blocks each of ME and CL solution were hung together at the same level, separated by a distance of 2-3 cm from each other, within the trap bottle. Proper mask and rubber gloves were used while handing soaked wooden blocks and tying them inside the bottle. To soak off excess dripping solution from the wooden blocks, they were kept over tissue papers for a few seconds. The implements used for the above process were then wiped with 70% alcohol and washed in detergent solution. ME, CL and combination lure (ME/CL) were the three treatments prepared, each with five replications.

3.5. Field placement and observations

A total of 15 parapheromone based lure traps were installed in the field (five traps each of ME, CL and ME/CL) separated by a distance of 10 m, from flowering till the harvesting of bitter gourd fruits (35^{th} to 44^{th} SMW). The traps were installed in the filed in a randomized complete block design (RCBD) manner using threads, which were tied upside-down at 1.5-2.0 m above the ground (Plate 6). The traps were installed under the crop vines so that rain and excessive sunshine would have the least effect. Observations of fruit flies collected from each trap were taken at weekly intervals by uncapping and emptying the bottle in an air-tight polybag. While handling the traps, proper mask and rubber gloves were worn. The fruit flies collected were taken to the laboratory where the weekly population count was done by using a Dewinter Zoomstar-III binocular stereoscope, Dewinter Optical Inc., with the magnification range of 8X to 50X. The fruit flies were observed in a petri plate, examined and separated with the use of forceps and needles (Plate 7). The observations regarding the identification of fruit flies on morphological basis were made as per the available literature by David and Ramani (2011), Leblanc *et al.* (2019) and Nair *et al.* (2018). Individually caught fruit flies of each species were preserved in vials containing 90% ethyl alcohol for genetic investigations (Plate 8). The traps were monitored in every 15 days for possible damage by fast flowing winds, heavy rainfall or attack by ants and birds. The lures needed to be changed in every 30-40 days as per the recommendations by Arya *et al*. (2022b), for which the traps along with the lure were replaced with new ones.

3.6. Population dynamics analysis of fruit flies

Adult males were collected from their responsive parapheromone lure traps (5 traps) and ME/CL traps (5 traps) to analyze the population dynamics of fruit fly species associated with the cucurbit ecosystem. Males were identified using available literature and calculated in the laboratory under a stereoscope to determine the actual population at weekly intervals. The weekly fruit fly population was estimated as the average of the population collected in ten traps.

3.7. Construction of insect chamber

To identify fruit fly species that are genuinely infesting the crops from the total fruit fly species collected in traps, 100 infested bitter gourd and cucumber fruits were collected from the fields and housed in insect chambers for pupation and monitoring of fruit fly emergence. The insect chamber was made from a cardboard box with dimensions of 1 m × 0.25 m × 0.25 m (length × width × height) that was opened from the top. A thick layer of polythene (1 m × 0.25 m) taped correctly across the sides was placed within the box's base. The box was filled with soil up to a height of 10-15 cm from Agriculture Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. The top section of the box was covered with a transparent mosquito net fabric with a 1.2 mm mesh size that was stapled to the borders of three sides of the box permanently and temporarily using clips that could be removed when adding fruits and collecting emerging flies (Plate 9). The infested fruits were identified on the basis of small light-colored punctured patches on the fruits through which watery fluid either oozed out or hardened, hollow fruits packed with pulps, or fruits broken down into pieces that fell to the ground (Plate 10). The infested fruits were weekly added to the chamber from the time of first fruit development till harvesting. The emerged fruit flies were collected in a plastic container (500 ml volume) containing 2-3 cotton pieces saturated with ethyl acetate as killing agent. The opening from a single side of the chamber was used to get the killing jar inside for fruit fly collection. Proper mask and rubber gloves were worn as excess exposure to ethyl acetate may also cause dizziness and headache to the user as well. No fruit flies were damaged during the collection, which might make identification difficult. The freshly collected individuals were kept inside the killing jar for 3-4 hours for proper killing. The individuals were then taken out and kept on a tissue paper for 15 minutes for removal of volatile chemical in a separate room.

3.8. Collection of data regarding weather parameters

The weekly data regarding the weather parameters including rainfall (RF) (mm), maximum temperature (Tmax) (^{0}C), minimum temperature (Tmin) (^{0}C), relative humidity in morning (RHm) (%), relative humidity in evening (RHe) (%) and sunshine hours (SSH) was collected from the Department of Agronomy, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, from 35th to 44th SMW.

Table 1. Weekly meteorological observation during experimental period from August (2021) to November (2021).

SMW	Month	Rainfall (RF) (mm)	Temperature (^{0}C)		Relative humidity (%)		Sunshine hours (SSH)
			Maximum (Tmax)	Minimum (Tmin)	Morning (RHm)	Evening (RHe)	
35	August	59.5	34.3	23.6	91	68	5.8
36	September	31.3	32.9	25.0	91	77	5.7
37		199.8	31.7	23.1	93	75	4.6
38		34.0	32.2	22.8	95	88	5.6
39		38.8	32.6	25.5	92	81	5.8
40	October	46.4	31.9	24.4	93	73	5.9
41		0.0	34.4	23.4	92	61	7.6
42		74.3	33.0	23.1	96	70	6.3
43		0.0	31.8	18.9	93	53	8.2
44	November	0.0	30.7	14.9	94	46	8.6

Calculations

3.9. Statistical analysis

By comparing the mean of fruit flies/trap/week data of individual species along with the standard errors (S.E.), the effect of combining lures (ME and CL) in the same trap on the attraction of responsive fruit flies in ME, CL and ME/CL combination was investigated by Paired t-test was calculated using R-4.2.0 software. The correlation coefficients (r) of mean fruit flies/trap/week data of different fruit fly species with mentioned weather parameters was calculated in R-4.2.0 software and multiple linear regression equations were constructed at a critical difference (C.D.) of 5% (0.05) level of significance with the help of Microsoft Excel 2016, for studying the population dynamics. The Shannon-Weiner function (H) was used to calculate species diversity (Shannon and Weiner, 1949), Pielou evenness index (E) for species evenness (Pielou, 1966) and Margalef's richness index (R) for species richness (Margalef, 1958). The formulae used for the above calculation are mentioned below:

(i) Correlation coefficient (r)

$$r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[n\sum x^2 - (\sum x)^2][n\sum y^2 - (\sum y)^2]}}$$

n = number of observations

x = first variable in the context

y = second variable in the context

(ii) Linear regression equation

$$y = a + bx$$

$$b = \frac{n\sum xy - (\sum x)(\sum y)}{n\sum x^2 - (\sum x)^2}$$

$$a = \frac{\sum y - b(\sum x)}{n}$$

b = slope of the line

a = Y-intercept

x = first variable in the context

y = second variable in the context

n = number of observations

(iii) Sample standard deviation (σ)

$$\sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

x = variable in the context

$\bar{x}$ = mean

n = total number of observations

(iv) Standard error of mean (S. E. mean)

$$S.E.(mean) = \frac{\sigma}{\sqrt{N}}$$

σ = standard deviation

N = number of observations

(v) Critical difference (C. D.)

$$C.D. = S.E.(mean) \times t\ (5\%)$$

t = 't' value at 5% probability level

(vi) Shannon-Weiner diversity index (H) (Shannon and Weiner, 1949)

$$H = -\sum_i pi \log_{10} pi$$

$$pi = \frac{\mathrm{Ni}}{\mathrm{N}}$$

Ni = total number of individuals in a species of taxon "i"

N = total number of individuals in all species of taxon "i"

(vii) Pielou's evenness index (E) (Pielou, 1966)

$$E = \frac{e^H}{S}$$

H = Shannon-Weiner diversity index

S = Number of taxa

(viii) Margalef's richness index (R) (Margalef, 1958)

$$R = \frac{S - 1}{In(N)}$$

S = Number of taxa

N = Number of individuals

Molecular characterization

3.10. DNA extraction

The DNA was extracted from the hind leg portion of fruit flies using the Qiagen DNeasy Blood and Tissue Kit, Qiagen India Pvt. Ltd.

Equipment used:

Qiagen DNeasy Blood and Tissue Kit {DNeasy mini spin columns (2 ml), collecting tubes (2 ml), tissue lysis buffer (ATL), lysis buffer (AL), wash buffer 1 (AW1), wash buffer 2 (AW2), elution buffer (AE) and proteinase K}, Dewinter Zoomstar-III binocular stereoscope (Dewinter Optical Inc.), microcentrifuge (BEZIF India Pvt. Ltd.), BI-VM-2500 vortex mixer (BR Biochem Life Sciences Pvt. Ltd.), mini-centrifuge (BR Biochem Life Sciences Pvt. Ltd.), hot water bath (Green Genome India Pvt. Ltd.), Kimble Pellet Pestle Cordless motor (DWK Life Sciences), ethyl alcohol (70%, 90% and 100%), pestles, Accupipet micro-pipettes (100 µL and 1000 µL) (Singhla Scientific Industries), micro tips (100 µL and 1000 µL), centrifuge tube (Eppendrof, India) (1.5 ml), needle, microscissors, forceps, brush, thermometer, tube stand, marker pen, surgical masks and GM polynitrile rubber laboratory gloves.

Procedure:

(i) Wear rubber gloves and sterile surgical mask before starting. Wipe the platform along with needles, microscissors, forceps, tube stand, tip boxes and micro-pipettes with 70% ethyl alcohol using tissue.

(ii) Wash the fruit fly samples in 90% ethyl alcohol with a clean brush to remove unwanted dust.

(iii) Remove the hind legs using microscissors and wash the specimen in 90% ethyl alcohol under stereoscope using needle and forceps.

(iv) Put the sample in 1.5 ml centrifuge tube with the help of brush. Label the tubes with the name of the species.

(v) Add 30 µL ATL buffer and crush the sample with cordless pestle till fine. Then add 150 µL of ATL buffer slowly (50 µL +50 µL +50 µL).

(vi) Add 20 µL proteinase K-mix. Vortex the mixtures separately and keep them in water bath at 56^0C for 2-3 hours.

(vii) Add 200 µL buffer AL and immediately mix by vortex, then add 200 µL (100%) ethyl alcohol and mix immediately by vortex.

(viii) Spin the centrifuge tubes in mini-centrifuge to settle down the unwanted particles (spin slightly).

(ix) Take supernatant (i.e., 600 µL) and add to DNeasy mini spin column placed in 2 ml collecting tube. Centrifuge it at 8000 revolutions per minute (rpm) for 1 minute and discard the collecting tube.

(x) Place the spin column in a 2 ml collection tube. Add 500 µL buffer AW1, centrifuge at 8000 rpm for 1 minute and discard the collecting tube.

(xi) Place the spin column in a 2 ml collecting tube. Add 500 µL AW2 and centrifuge for 3 minutes at 14000 rpm. Dispose the supernatant liquid only and dry spin without adding anything at 14000 rpm for 1 minute. Discard collection tubes.

(xii) Transfer the spin column to centrifuge tube and keep it open at room temperature for 3-5 minutes for evaporating the ethanol.

(xiii) Elude the DNA by AE buffer by adding 30 µL (15 µL +15 µL) to the centre of spin-column in a tube and leave it for 2 minutes (the amount of AE buffer added depends upon the size of sample).

(xiv) Centrifuge the samples at 14000 rpm for 1 minute at room temperature and discard the spin column.

3.11. Checking the quality of DNA fragments and Polymerase Chain Reaction (PCR)

The extracted genomic DNA was stored at -20^0C until for further use. To measure the concentration and purity of recovered DNA, a NanoDrop microvolume spectrophotometer (ThermoFisher Scientific Pvt. Ltd., India) was used. 1 µL DNA was pipette out which was placed on the receiving fiber (onto the end of an optic cable on the lower pedestal). The liquid sample was brought into contact with the source fibre (second fibre optic cable on the upper pedestal), bridging the gap between the two optic ends. Spectrophotometer with a charge-coupled device ray (CCD ray) was used to analyse the light after it passes through the sample using a pulsed xenon light source. The data is logged in an archive file on the PC, and the

instrument is managed by PC-based software. Both the optic ends were wiped out with the help of clean tissue after the readings were taken. The procedure to operate the PC-based software is as follows:

Procedure:

(i) Switch on the instrument including desktop.

(ii) Click on the 'ND 1000 V3.7' icon by clicking it on the desktop.

(iii) Set 'default user' and click 'nucleic acid' after arriving at the programme's main page.

(iv) **Set blank:** A blank must be measured and saved before producing a sample measurement; a straight line will display on the screen after making an initial blank measurement. It's ideal to start every measurement session with blanking (11 cycle for the most consistent results). This ensures that the instrument is in good operating order and that the pedestal is free of debris. To execute a blanking cycle, we followed the procedure:

(a) A blank sample (1 µL AE buffer) was loaded onto the lower pedestal and the arm of the instrument was lower down.

(b) Click on 'blank' button.

(c) The buffer was wiped with the help of clean tissue from both the pedestals right when the measurement was completed.

(v) Quantify the unknown sample:

(a) Move the sampling arms apart and 1 µL of DNA was added onto the lower pedestal.

(b) The sample arms were closed and the PC's operating software was used to start a spectrum measurement.

(c) The samples were wiped-off from both the pedestal with the help of clean tissue after the measurements were completed.

(d) A subsequent sample was measured 5-6 times each.

The absorbance of all samples was measured at a wavelength of 260 to 280 nm. The OD260/OD280 ratio was used to determine the sample's purity. A value close to 1.8 demonstrated the extracted DNA's purity and proved that it was protein and contaminant free and a ratio less than ≤1.6 indicated the presence of proteins or other UV absorbers in the sample, under which it is advisable to reprecipitate the DNA.

After the DNA was analysed for quality check, the extracted genomic DNA was again stored at -20^{0}C until it was used in the polymerase chain reaction (PCR). A 25 µl PCR reaction mixture was prepared which contained 12.5 µl of PCR master mix (Takara's EmeraldAMP GT PCRMaster Mix), 9.5 µl of nuclease-free water (dH_2O), 2 µl of template DNA and 0.5 µl of each primer (forward and reverse). To amplify the mitochondrial cytochrome oxidase I barcode region, LCO1490 (5′-GGTCAACAAATCATAAGAATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) primers were used (Folmer *et al*., 1994). All the reaction mixtures were prepared in PCR-Cooler 0.2 ml (Eppendorf, India) and a T100 thermal cycler (Bio-Rad, India) was used to run the samples. The thermal cycling conditions for the mixture were as follows:

Thermal cycling steps	Temperature (^{0}C)	Duration
Initial denaturation (hot start)	95	5 minutes
Denaturation	94	30 seconds
Annealing	47	40 seconds (35 cycles)
Extension	72	40 seconds
	72	8 minutes
Cooling	4	Infinite (∞)

3.12. Preparation of agarose gel, gel electrophoresis and visualization of PCR products

Reagents used:

1. 50X TAE (Tris-acetate EDTA) buffer

Reagents	Quantity
Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M)	100 ml

All of the components were mixed together in a beaker and stirred until a clear solution was created. The volume was reduced to one litre and autoclaved for 20 minutes at 15 psi for

sterilization. To make 1X TAE buffer, it was diluted 50 times with sterile double distilled water.

2. Ethidium bromide

Reagents	Quantity
Ethidium bromide	100 mg
Double distilled water	10 ml

The solution was stirred until the dye was completely dissolved and stored in a dark bottle at room temperature.

3. Gel loading dye

Reagents	Quantity
Bromophenol blue	0.025 g
Xylene cyanol	0.025 g
Glycerol	1.0 ml

All the reagents were mixed and the volume was made up to 10 ml by adding sterilized distilled water.

4. Agarose gel (1%)

1 g of Analytical Grade (Ultra Pure DNA Grade) agarose was dissolved in 100 ml of 1X TAE buffer.

3.12.1. Procedure for agarose gel electrophoresis

The quality of DNA samples was assessed using 1% agarose gels. In a conical flask, the needed amount of agarose was mixed with 1X TAE buffer and melted in a microwave oven for 1 minute, until the agarose was completely dissolved and a clear solution produced. About 2 µL of ethidium bromide (10 mg/ml) was added to the solution upon cooling and mixed well. The solution was poured on to the gel casting tray containing comb to a thickness of about 5 mm,

allowing it to solidify for 30 minutes at room temperature. The comb was removed carefully from the tray and the gel was placed in a tank filled with electrophoresis buffer (1X TAE). DNA sample was mixed with 1 µL of loading dye at 5:1 ratio. The samples were mixed well and loaded at a rate of 6 µL per well, with a DNA ladder (100 bp) as a molecular weight marker of placed in the first lane. The power unit was linked to the cathode and anode of electrophoresis unit and the gel was operated at a constant voltage of 70 volts until the dye (bromophenol blue) moved to 2/3rd of the gel's length. The gel containing electrophoresed DNA was observed and recorded using the UV transilluminator gel documentation Image Lab software Version 6.1. (Bio Rad). The ethidium bromide dye was responsible for the DNA fluorescence under UV light. The gel profile was checked for integrity, DNA clarity and RNA and protein contamination.

3.13. DNA sequencing and refining using BioEdit software 7.2.5.

The samples were sequenced in both directions at Eurofins Genomics India Pvt. Ltd. (Bengaluru, India). The obtained sequences were checked for stop codons using expasy translate (Gasteiger *et al*., 2003), trimmed and aligned using BioEdit 7.2.5 (Hall, 1999). Fruit fly consensus sequences were aligned using ClustalW software.

3.14.1. Construction of maximum-likelihood tree using Molecular Evolutionary Genetic Analysis (MEGA 6) software

(i) Open “MEGA 6” software icon from the desktop.

(ii) Click on “Align” then on “Edit/Build Alignment”. An alignment editor window will appear.

(iii) Click on “Create a new alignment” and then on “OK” button.

(iv) An option will appear asking for “Data type for alignment”, select “DNA”.

(v) A blank window named “M11: Alignment Explorer” will appear. Open the Microsoft Word document containing aligned DNA in a different window.

(vi) Select and copy all the DNA sequences from the Word document and paste it in the “M11: Alignment Explorer” window, along with the name of species it belongs to. Also add a DNA sequence of a different individual as an outgroup (*Nilaparvata lugens*, in our study).

(vii) Select all the sequences (Ctrl + A) and then click on "Muscle Alignment". A window will appear, click on "OK".

(viii) Click on "Data" and then on "save session", the file will be saved in a '.mas/.masx' format.

(ix) Click on "Phylogeny" option mentioned on the main window of the MEGA 6 and select "Construct/Test Maximum Likelihood Tree", upload the file saved in the above step in '.mas/.masx' format.

(x) "M11: Analysis Preferences" will appear. Select "Bootstrap method" and "2000" number of bootstrap replications from the "Phylogenetic test" column.

(xi) Select substitution type as "Nucleotide" and "Kimura 2-parameter model" from the "Substitution model" option (Kimura, 1980).

(xii) Keep all the options same and click on "OK".

(xiii) It will take few minutes to construct and a phylogenetic will be developed. Save it in '.png/.pdf' file format file.

3.14.2. Computing pair-wise genetic distance using Molecular Evolutionary Genetic Analysis (MEGA 6) software

(i) Open "MEGA 6" software icon from the desktop.

(ii) A blank window will appear, click on "Distance" option and select "Compute Pairwise Distance"

(iii) Upload the file previously saved in '.mas/.masx' format.

(iv) Select substitution type as "Nucleotide" and model/method as "Kimura 2-parameter model" (Kimura, 1980). Keep rest option as it is and click on "OK".

(v) Pairwise genetic distance will be displayed in a separate window.

(vi) Select "Export distance to an excel file" and the table of genetic distance will be saved as an MS Excel document (.xlsx format).

3.15. Checking for *Wolbachia* infection in fruit flies

Wolbachia infection was tested in three specimens from each species of fruit fly using the wsp81-F and wsp691-R primers (Braig *et al*., 1998). We used the PCR composition and thermocyclic conditions as per Braig *et al*. (1998):

Thermal cycling steps	Temperature (^{0}C)	Duration
Initial denaturation (hot start)	94	5 minutes
Denaturation	94	30 seconds
Annealing	55	1 minute
Extension	72	1 minute
	72	10 minutes
Cooling	4	Infinite (∞)

(35 cycles)

The amplified PCR products were resolved on a 1% agarose gel and then visualized using Image Lab software Version 6.1 under UV light (Bio Rad), with *Wolbachia* infected *Nilaparvata lugens* as positive control.

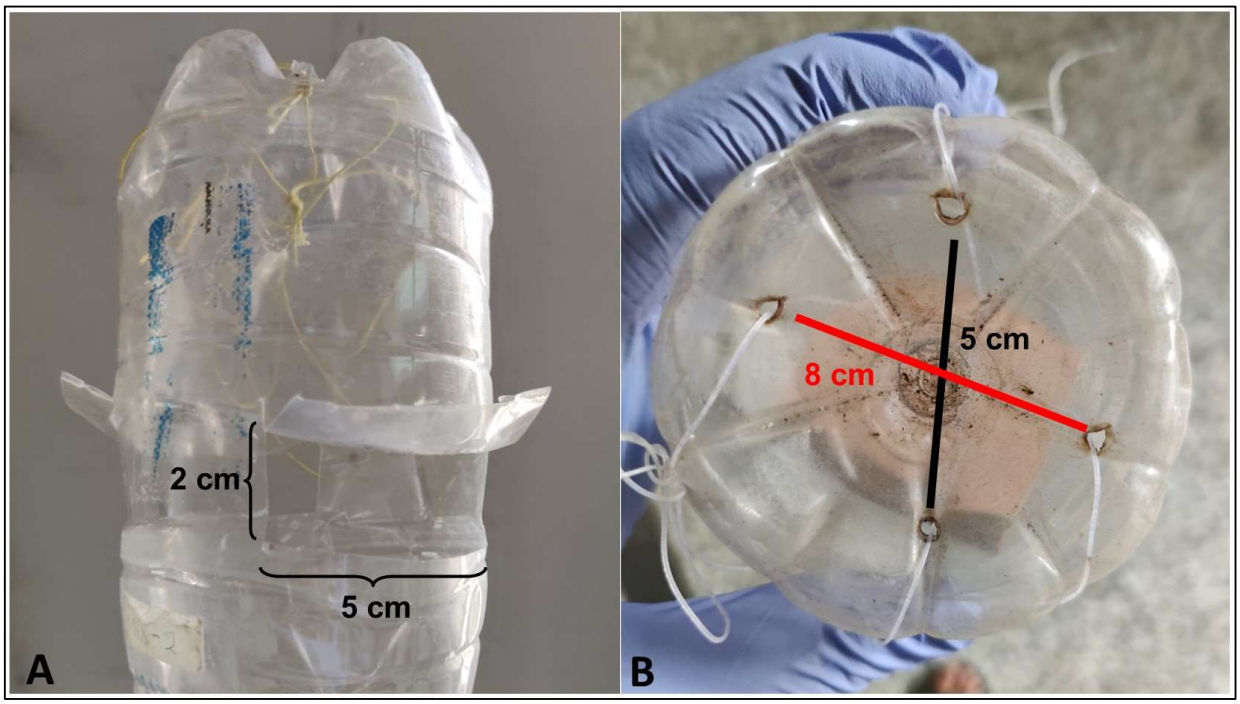

Plate: 1 (A) Vertical section of the trap bottle showing windows. (B) Horizontal section showing holes at the base of the bottle with thread passed through them.

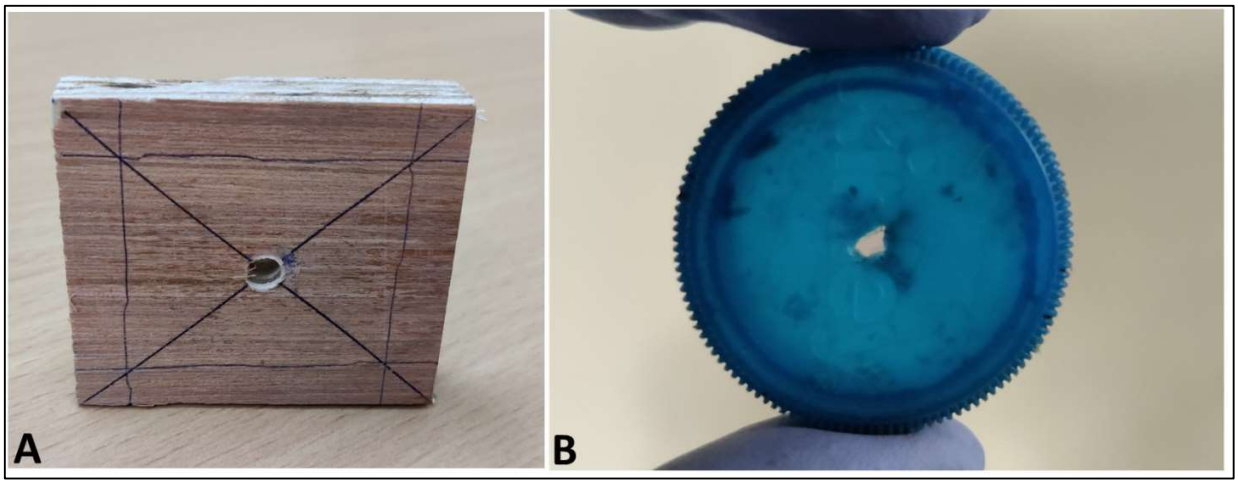

Plate: 2 (A) Wooden blocks of size 5 cm × 5 cm × 2 cm (length × breadth × width) and (B) bottle cap with hole at the centre.

Plate: 3 (A) Parapheromone cue-lure and methyl eugenol. (B) Chemicals and apparatus used in the preparation of parapheromone lure solutions.

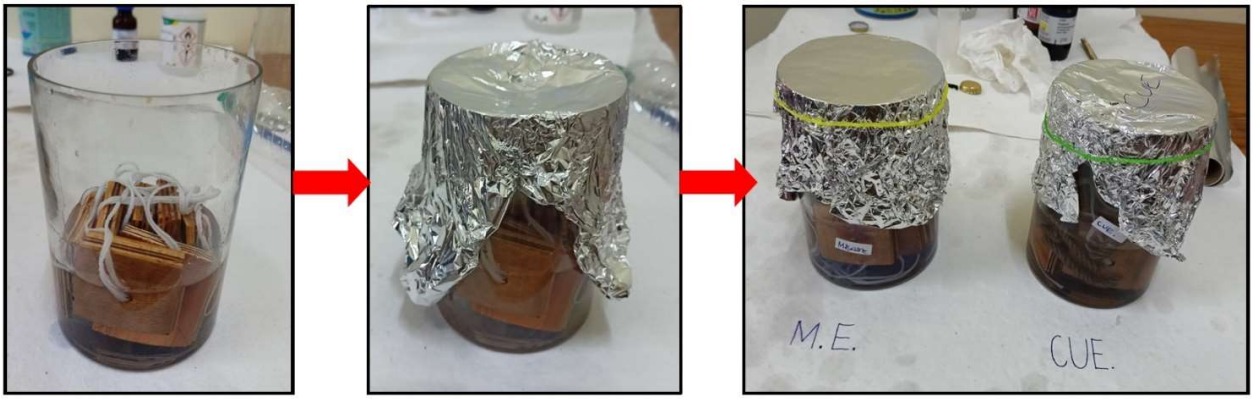

Plate: 4 Wooden blocks were added into the lure solution containing beaker and covered with aluminium foil. The foil fastened with rubber band and labeled.

Plate: 5 Prepared and labeled parapheromone trap lure bottles.

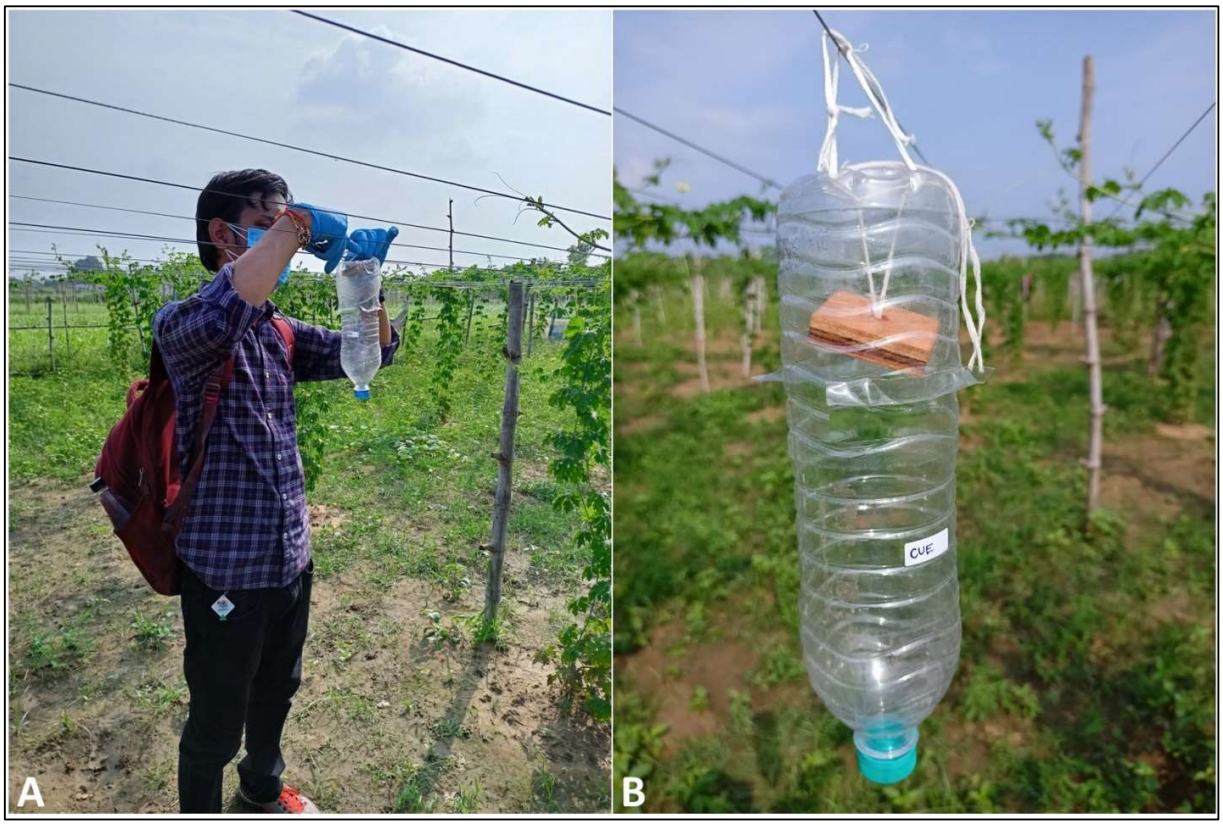

Plate: 6 (A) Hanging parapheromone trap lure bottles in cucurbit field. (B) An installed trap bottle.

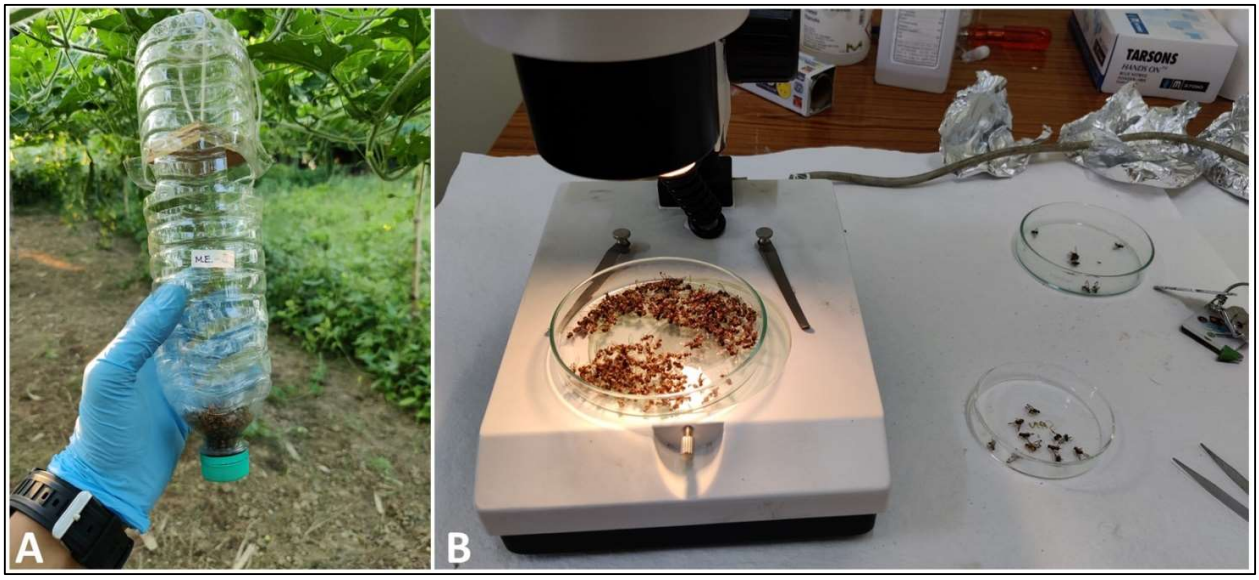

Plate: 7 (A) Fruit flies collected in parapheromone trap bottles in field. (B) Collected fruit flies observed under stereoscope.

Plate: 8 Fruit fly species stored in 90% ethyl alcohol for genetic investigations.

Plate: 9 Insect rearing cages used for keeping infested fruits of (A) bitter gourd and (B) cucumber for observing fruit fly emergence.

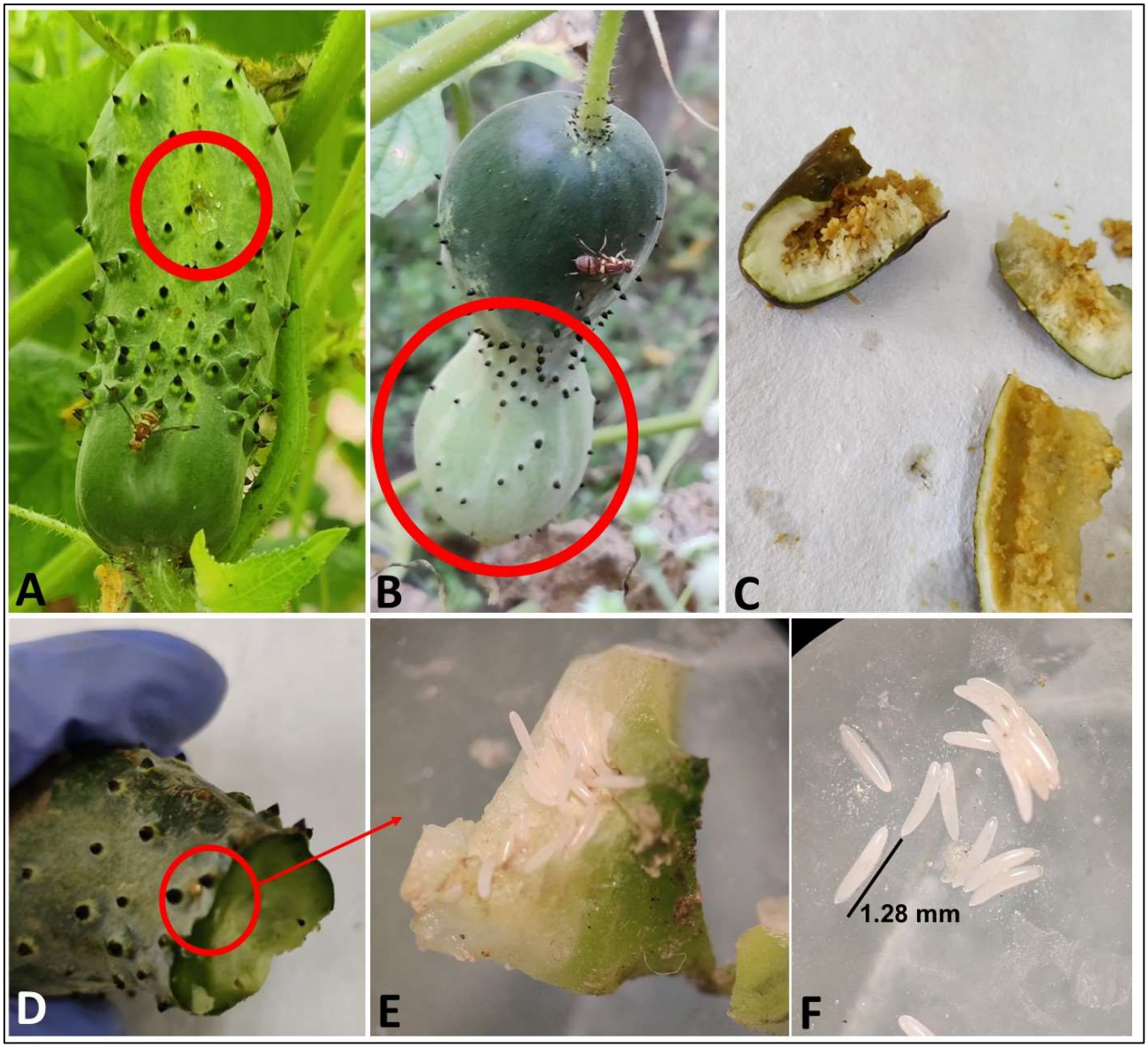

Plate: 10 Identification of damaged fruits infested by fruit flies. (A) Watery fluid oozing out of the punctured spot getting solidified like a gel. (B) The lower portion of the fruit got crinkled to excessive feeding by the maggots. (C) Internal rotting of fruits along with fruit fly maggots when split opened. Slicing an identified punctured spot (D) and observing fruit fly eggs under stereoscope (E) and (F).

Chapter IV

EXPERIMENTAL FINDINGS

Present investigation entitled **"Study of population dynamics and molecular characterization of fruit flies associated with cucurbits"** was carried out during *kharif* of 2021 from 35th SMW to 44th SMW on a farmer's field in Ramna village, Varanasi, Uttar Pradesh (India). The results obtained from the investigation have been summarized under the following heads:

4.1. Morphological and molecular identification of fruit flies

Fruit flies were collected weekly from the cucurbit crop fields using lure traps containing ME, CL and ME/CL for 10 weeks (from the 35th to 44th SMW). Fruit flies were cleaned using hair brush and were sorted based on similarity. The identity of fruit flies was established by observing external morphology by following the literature of David and Ramani (2011), Leblanc *et al*. (2019) and Nair *et al*. (2018). The morphological identification of fruit flies was based on unique traits of the thoracic, wings and abdominal section. The fruit flies identified were: *Bactrocera dorsalis, Zeugodacus cucurbitae, B. zonata, Z. tau, B. correcta* and *B. digressa*. Further, fruit fly identities were confirmed by molecular barcoding. The DNA sequences of fruit flies collected showed 99-100% identity with reported sequences, were submitted to the National Centre for Biotechnology Information (NCBI) GenBank and accession number obtained are mentioned in Table 2.

4.1.1. Identification characteristics of *B. dorsalis:*

(i) ***Wings:*** Wings are transparent and membranous with the costal band being dark coloured with tiny hairs. The costal band of the wing is confluent with the radial vein (R_{2+3}) and is not extended or very slightly expanded apically.

(ii) ***Thorax:*** Colour pattern of the scutum is quite diverse, ranging from different shades of black to blackish-brown with varying lanceolate orange-brown patterns to fully orange-brown. Postpronotal lobe, lateral postsutural vittae and scutellum are creamish-yellow in colour. Median postsutural vitta is absent.

(iii) ***Abdomen:*** Brown coloured abdomen with a prominent T-shaped marking from the third segment and a pattern on the abdomen with a thin medial band and large lateral dark marks. A narrow light horizontal band on the second terga (Plate 11).

4.1.2. Identification characteristics of *Z. cucurbitae:*

(i) ***Wings:*** Infuscations along the radial-median vein (R-M) and dorsal median-cubitial vein (DM-Cu) cross-veins, as well as the costal band and anal streak, on the wing. Costal band expanding into a large apical spot extending half-way to the median. Dark spots along DM-Cu cross-vein and basal cubital vein (BCu-Cu_2).

(ii) ***Thorax:*** Yellowish orange in colour. Postpronotal lobe, lateral postsutural vittae and scutellum are all bright yellow in colour with a prominent median postsutural vittae.

(iii) ***Abdomen:*** Yellowish-orange coloured with a short light horizontal band on the second terga. A narrow dark coloured horizontal band on third segment with two distinctly narrow lateral band along with a median band on fourth terga. The median band extends down (Plate 11).

4.1.3. Identifying characteristics of *B. zonata:*

(i) ***Wings:*** Transparent wing with light coloured costal band without tiny hairs. A small dark spot on the apical angle, between R_{2+3} and R_{4+5}.

(ii) ***Thorax:*** Scutum reddish-brown in colour. Postpronotal lobe, lateral postsutural vittae and scutellum are light-yellowish in colour. Median postsutural vitta is absent.

(iii) ***Abdomen:*** Orange brown coloured abdomen with two spots on the third abdominal terga and a light median band on the terminal segments (Plate 11).

4.1.4. Identifying characteristics of *B. correcta:*

(i) ***Wings:*** Transparent wing with light coloured costal band without tiny hairs. A small dark spot on the apical angle, between R_{2+3} and R_{4+5}.

(ii) ***Thorax:*** Scutum black or dark brown in colour. Postpronotal lobe, lateral postsutural vittae and scutellum are light-yellowish in colour. Median postsutural vitta is absent.

(iii) ***Abdomen:*** Reddish-Brown coloured abdomen with a prominent T-shaped marking on the third and fourth abdominal terga with lateral spots on fourth terga. A narrow light horizontal band on the second terga (Plate 11).

4.1.5. Identifying characteristics of *Z. tau:*

(i) ***Wings:*** Transparent wing with overlapping vein R_{2+3} and expanding into an apical area on the wing costal band. Another spot is present at the BCu till Cu_2 with no spot on the DM-Cu cross-vein.

(ii) ***Thorax:*** Mix coloured scutum with median yellowish-brown and lateral black bands. Yellowish orange in colour. Postpronotal lobe, lateral postsutural vittae and scutellum are all bright yellow in colour with a broad median postsutural vittae.

(iii) ***Abdomen:*** Yellowish-orange coloured with a short light horizontal band on the second terga. A narrow dark coloured horizontal band on third segment with two distinctly narrow lateral band along with a median band on fourth terga, expanding downwards (Plate 11).

4.1.6. Identifying characteristics of *B. digressa:*

(i) ***Wings:*** Wings are transparent and membranous with light coloured costal band being with fine hairs. The costal band of the wing is confluent with the R_{2+3} vein and is not extended or very slightly expanded apically.

(ii) ***Thorax:*** Scutum reddish-brown in colour. Postpronotal lobe, lateral postsutural vittae and scutellum are yellowish in colour. Median postsutural vitta is absent.

(iii) ***Abdomen:*** Orange brown coloured abdomen with a tiny dark spot at the centre of the third abdominal terga (Plate 11)

1.2. Fruit flies attracted to different lures

Of the total six fruit fly species collected during the study period, *Z. cucurbitae* and *Z. tau* were observed to be attracted to CL whereas, *B. dorsalis, B. correcta* and *B. zonata* to ME. *B. digressa* were found to be equally responsive to both the lures (Table 2).

Table 2: Fruit flies found in cucurbit ecosystem collected from different lures along with their accession number (ME: Methyl eugenol, CL: Cue-lure)

Fruit fly species	Parapheromone attraction	Accession No.
B. dorsalis	ME	OK559996
B. zonata	ME	OK559997
B. correcta	ME	OK559995
Z. cucurbitae	CL	OL701253
Z. tau	CL	OL701270
B. digressa	CL and ME	OK559998

4.3. Population dynamics of fruit flies associated with cucurbits

According to data acquired from fruit flies trap captures in cucurbit fields from the 35th to the 44th SMW, the population fluctuated dramatically during the crop season. The population of *B. dorsalis* was the most abundant (with 7667 individuals constituting 80.50% of the total population), followed by *Z. cucurbitae* (1661 individuals, 17.44%), *B. correcta* (93 individuals, 0.97%), *B. zonata* (52 individuals, 0.54%), *B. digressa* (38 individuals, 0.39%) and *Z. tau* (0.13%) respectively. The variation in population of individual fruit fly species are as follows:

4.3.1. *B. dorsalis*

The population of *B. dorsalis* declined steadily during the cropping season, with the population peaking in the 37th SMW (2005 individuals) and with 200.5±46.43 fruit fly per trap per week ± standard error (FTW). The population began to fall, and in the 40th SMW (783 individuals) with an FTW of 78.3±19.83, another modest peak was observed. The population eventually plummeted to 42 individuals with 4.2±1.33 FTW, the lowest value recorded during 35th to 44th SMWs (Fig 1a).

4.3.2. *Z. cucurbitae*

Throughout the crop season, the population of *Z. cucurbitae* increased at an increasing rate. The fruit fly population increased significantly until the 39th SMW (169 individuals, 16.9±5.77 FTW), after which it decreased slightly in the 40th SMW (134 individuals and

13.4±3.37 FTW). The population again increased in the 43rd SMW, reaching a peak of 378 individuals (37.8±8.28 FTW), before declining slightly to 367 individuals (36.7±10.65 FTW) on the 44th SMW (Fig 1a).

4.3.3. *B. correcta*

B. correcta had a substantially smaller population than *B. dorsalis* and *Z. cucurbitae*. The population decreased as the weeks progressed, with the largest number reported in the 35th SMW (43 individuals and 4.3±1.49 FTW), after which the population declined. The population reached a small peak in the 40th SMW, with 16 individuals (1.6±0.65 FTW), before declining to 2 individuals (0.2±0.13 FTW) in the 44th SMW (Fig 1b).

4.3.4. *B. zonata*

B. zonata, as like *B. correcta*, had a diminishing population trend throughout the season. The peak in the population observed was in the 35th SMW with 34 individuals (3.4±1.53 FTW), followed by a significant decline in population, with no population recorded in the last four weeks (41st to 44th SMW) (Fig 1b).

4.3.5. *B. digressa*

Throughout the cropping season, *B. digressa* population displayed a mixed pattern of increase and decrease. On the 37th SMW, 1 individual was recorded (0.06±0.06 FTW), followed by a rapid peak in the population (18 individuals, 1.2±0.52 FTW) on the 40th SMW. The population gradually decreased to 1 (0.06±0.06 FTW) and 2 (0.13±0.09 FTW) individuals in the 43rd and 44th SMWs, respectively (Fig 1b).

4.3.6. *Z. tau*

Among the fruit fly species studied, *Z. tau* had the smallest population. No individuals were reported during the 35th to 39th SMWs. On the 40th SMW, 2 individuals (0.2±0.2 FTW) were collected, with a population trend growing to a maximum of 5 individuals (0.5±0.30 FTW) on 44th SMW (Fig 1b).

Table 3: Population variation in different fruit fly species along the Standard Meteorological Weeks (SMW) with total flies per trap catches per week (FTW±S.E.).

SMW	Different fruit fly species												
	B. dorsalis		***Z. cucurbitae***		***B. zonata***		***B. correcta***		***B. digressa***		***Z. tau***		**Total**
	Total	**FTW±S.E.**	**Total**	**FTW±S.E.**	**Total**	**FTW±S.E.**	**Total**	**FTW±S.E.**	**Total**	**FTW±S.E.**	**Total**	**FTW±S.E.**	
35	1606	160.6±40.40	18	1.8±0.67	34	3.4±1.53	43	4.3±1.49	0	0	0	0	1701
36	990	99±19.99	33	3.3±0.67	12	1.2±0.29	14	1.4±0.49	0	0	0	0	1049
37	2005	200.5±46.43	59	5.9±1.17	1	0.1±0.1	7	0.7±0.26	1	0.06±0.06	0	0	2073
38	794	79.4±21.88	98	9.8±3.33	1	0.1±0.1	4	0.4±0.13	1	0.06±0.06	0	0	898
39	679	67.9±19.78	169	16.9±5.77	2	0.2±0.2	1	0.1±0.1	1	0.06±0.06	0	0	852
40	783	78.3±19.83	134	13.4±3.37	2	0.2±0.2	16	1.6±0.65	18	1.2±0.52	2	0.2±0.2	955
41	416	41.6±6.72	205	20.5±3.71	0	0	4	0.4±0.1	9	0.6±0.23	2	0.2±0.13	636
42	220	22±4.46	200	20±3.98	0	0	1	0.1±0.1	5	0.33±0.15	1	0.1±0.1	427
43	132	13.2±3.46	378	37.8±8.28	0	0	1	0.1±0.1	1	0.06±0.06	3	0.3±0.3	515
44	42	4.2±1.33	367	36.7±10.65	0	0	2	0.2±0.13	2	0.13±0.09	5	0.5±0.30	418
Total	7667		1661		52		93		38		13		9524

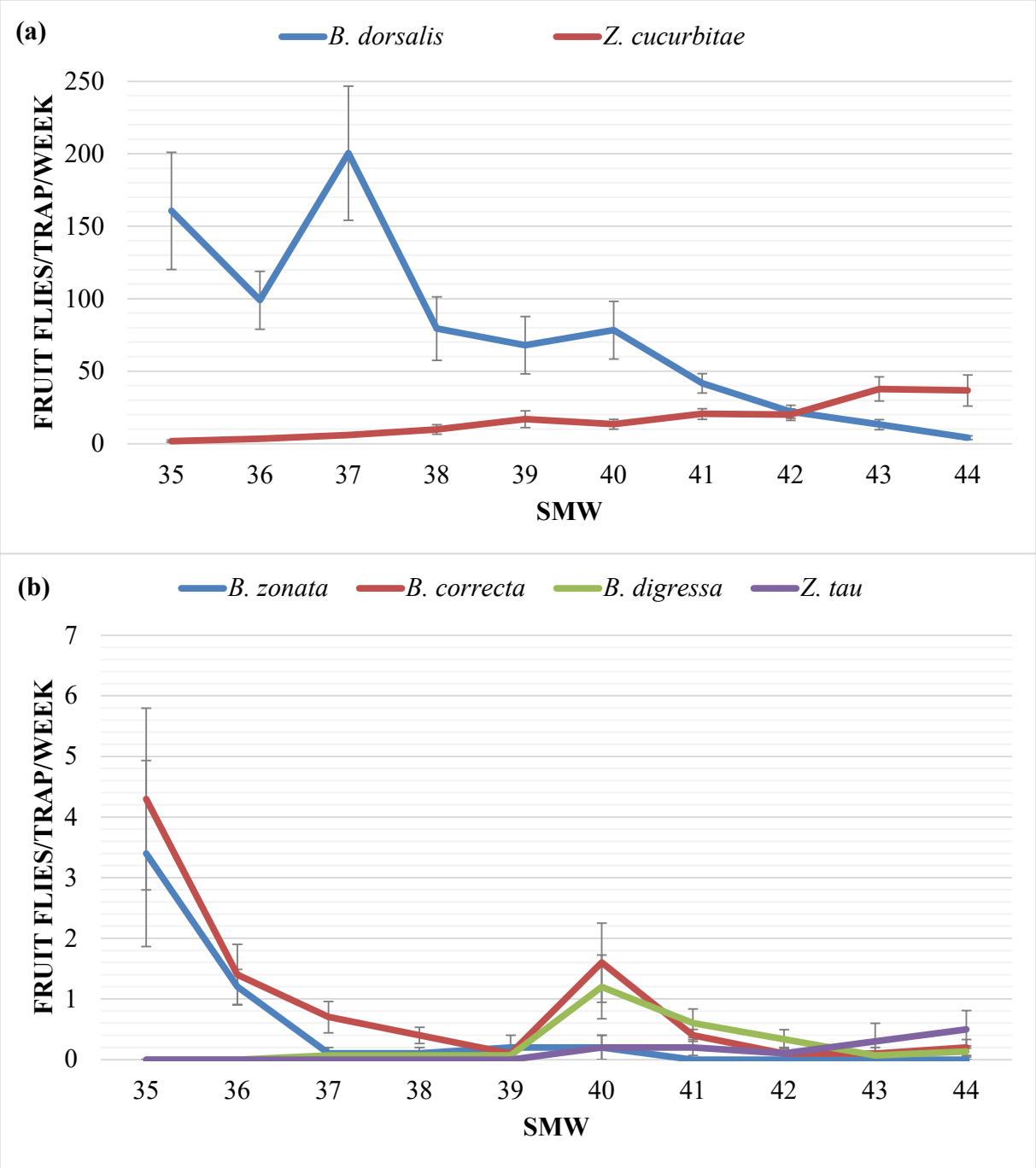

Figure 1: Population dynamics of fruit flies/trap/week (±S.E.) collected in cucurbit ecosystem during *kharif* season 2021. (a) *B. dorsalis* and *Z. cucurbitae*, (b) *B. zonata, B. correcta, B. digressa* and *Z. tau*.

4.4. Correlation between population of different fruit fly species and weather parameters

4.4.1. *B. dorsalis*

B. dorsalis population was positively correlated with maximum temperature (Tmax) (r=0.209), minimum temperature (Tmin) (r=0.498), and evening relative humidity (RHe) (r=0.504) throughout the research period, with a significant positive correlation between fruit fly population and rainfall (RF) (r=0.775). Morning relative humidity (RHm) was adversely connected with the population (r=-0.284), while sunlight hours (SSH) had a substantial negative correlation (r=-0.810) (Table 4). The weather parameters under combined study influenced the population of *B. dorsalis* by 96% (Table 5).

4.4.2. *Z. cucurbitae*

The population of *Z. cucurbitae* was shown to have a positive correlation with RHm (r=0.233), as well as a significant positive correlation with SSH (r=0.893). Tmax (r=-0.464) and RF (r=-0.527) were adversely correlated with the population, with a significant negative correlation with Tmin (r=-0.784) and RHe (r=-0.769) (Table 4). When studied combined, all the weather parameters showed a 99% influence on the population (Table 5).

4.4.3. *B. correcta*

Tmax (r=0.485), Tmin (r=0.291), RHe (r=0.101) and RF (r=0.128), all had positive correlations with *B. correcta* population. RHm (r=-0.434) and SSH (r=-0.332) had a negative correlation with the population (Table 4). All the abiotic factors showed 85% impact on the population (Table 5).

4.4.4. *B. zonata*

B. zonata population established a positive correlation with Tmax (r=0.547), Tmin (r=0.257), RHe (r=0.101) and RF (r=0.050) whilst RHm (r=-0.582) and SSH (r=-0.274) have negative correlations (Table 4). The weather parameters showed 84% influence on the population of *B. zonata* (Table 5).

4.4.5. *B. digressa*

B. digressa population had a positive correlation with Tmax (r=0.026), Tmin (r=0.191), RHm (r=0.007) and SSH (r=0.059), but had a negative correlation with RHe (r=-0.041) and RF (r=-0.118) (Table 4). The weather parameters here showed only 53% influence on the population of the fruit fly species (Table 5).

4.4.6. *Z. tau*

The population of *Z. tau* exhibited a positive correlation with RHm (r=0.142) and a significant positive correlation with SSH (r=0.891) throughout the research period. However, it showed a negative correlation with Tmax (r=-0.487), RF (r=-0.511) and significant negative correlation with Tmin (r=-0.859) and RHe (r=-0.880) (Table 4). The overall impact on the population of *Z. tau* due to weather parameters was 95% (Table 5).

Table 4: Correlation between weather parameters and different fruit fly species.

Fruit fly species	Correlation coefficient (r)					
	Maximum temp (Tmax)	Minimum temp (Tmin)	Relative Humidity (morning) (RHm)	Relative Humidity (evening) (RHe)	Sunshine hours (SSH)	Rainfall (RF)
B. dorsalis	0.209	0.498	-0.284	0.504	-0.810*	0.775*
Z. cucurbitae	-0.464	-0.784*	0.233	-0.769*	0.893*	-0.527
B. zonata	0.547	0.257	-0.582	0.101	-0.274	0.050
B. correcta	0.485	0.291	-0.434	0.101	-0.332	0.128
Z. tau	-0.487	-0.859*	0.142	-0.880*	0.891*	-0.511
B. digressa	0.026	0.191	0.007	-0.041	0.059	-0.118

***=significant at 5%, values in parentheses are p-values.**

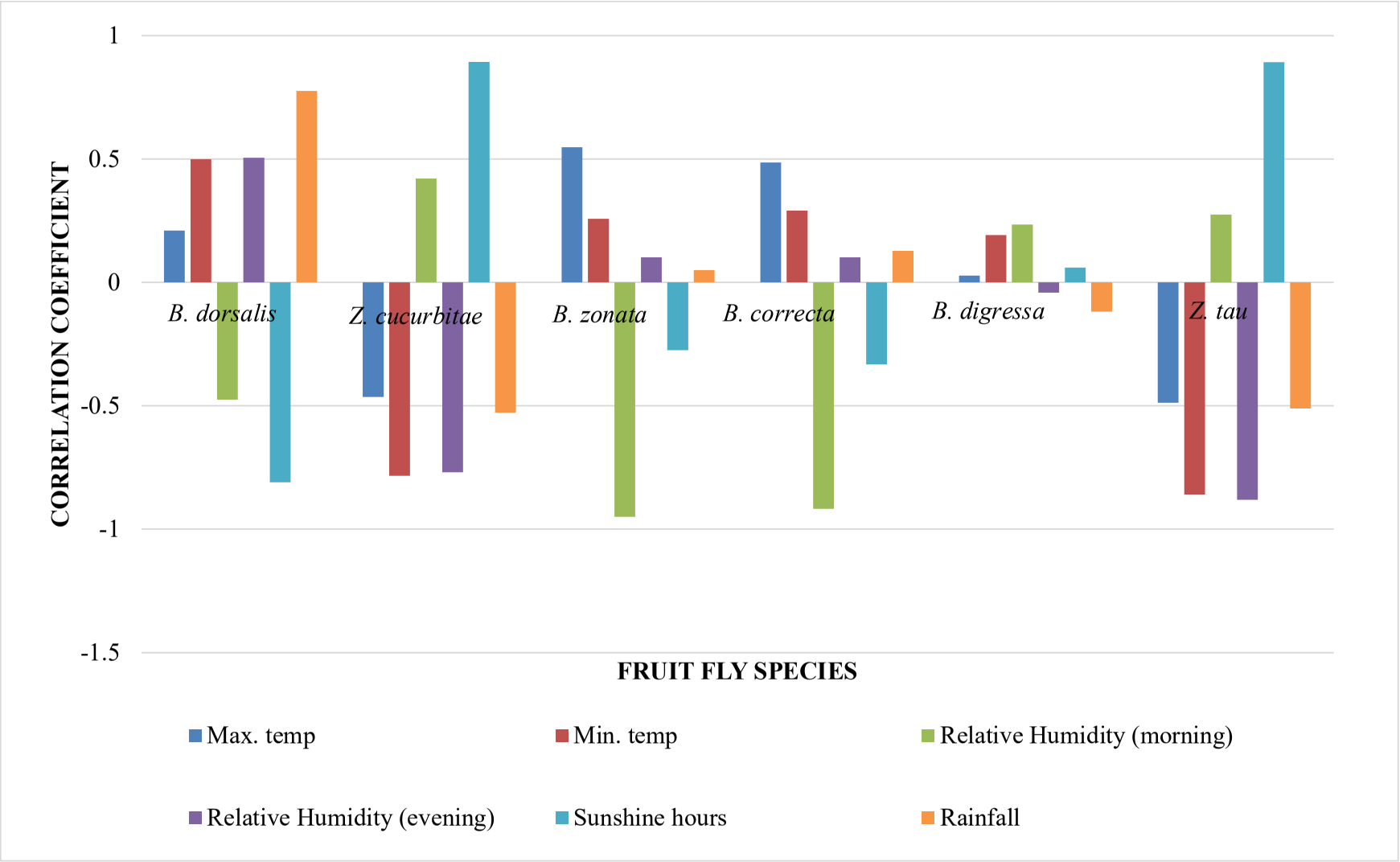

Figure 2. Graphical representation of correlation between fruit fly species and weather parameters.

Table 5: Multiple regression equations for population of different fruit fly species with weather parameters.

Fruit fly species	Multiple regression equation	R^2 value	F-value
B. dorsalis	$Y=18128.39+132.05X_1-134.34X_2-182.72X_3+8.31X_4-382.38X_5+4.43X_6$	0.96	9.78
Z. cucurbitae	$Y=-1546.79-82.18X_1+39.23X_26.65X_3+10.22X_4+320.67X_5+2.23X_6$	0.99	47.48
B. zonata	$Y=195.66+2.77X_1-1.85X_2-0.73X_3-0.79X_4-17.10X_5-0.16X_6$	0.84	1.75
B. correcta	$Y=255.19+1.26X_1-0.89X_{2+}0.35X_3-1.65X_4-27.28X_5-0.25X_6$	0.85	2.02
Z. tau	$Y=11.62-0.42X_1+0.03X_2+0.18X_3-0.14X_4-0.62X_5-0.01X_6$	0.95	6.95
B. digressa	$Y=-80.43-2.71X_1+3.13X_2+2.51X_3-1.09X_4-7.82X_5-0.10X_6$	0.53	0.39
Total population	$Y=16963.65+50.76X_1-94.71X_2-173.75X_3+14.85.09X_4-114.54X_5+6.13X_6$	0.95	7.58

X_1=Maximum temperature, X_2=Minimum temperature, X_3=Relative humidity (morning), X_4=Relative humidity (evening), X_5=Sunshine hours, X_6=Rainfall

4.5. Fruit fly trapping efficiencies of ME/CL in comparison to ME and CL

ME was shown to be sensitive to *B. dorsalis, B. correcta,* and *B. zonata*, whereas CL was found to be responsive to *Z. cucurbitae* and *Z. tau*. *B. digressa* was found to be attracted to both ME and CL (Table 2).

4.5.1. Trapping efficiency of fruit flies responsive to methyl eugenol in ME/CL vs ME traps

The population of *B. dorsalis* was significantly higher in ME traps (108.28±30.65) than in ME/CL traps (43.4±10.72) according to data on mean fruit flies per trap per week among ME responsive fruit flies (Fig 3a). *B. zonata*, *B. correcta* and *B. digressa* populations were likewise greater (although not significantly) in ME traps (0.74±0.49, 1.6±0.75 and 0.48±0.29) than in ME/CL traps (0.3±0.19, 0.22±0.10 and 0.26±0.10) respectively (Table 8).

Table 6: ME responsive fruit flies in ME traps as per Standard Meteorological Weeks (SMW) along with weekly means, standard deviation (S.D.) and standard error (S. E.) (A = *B. dorsalis,* B = *B. zonata,* C = *B. correcta* and D = *B. digressa*).

SMW	Fruit flies collected in Methyl eugenol traps (ME)																				Avg. (mean of 5 treatments)	S. D.	S. E.
	ME1				ME2				ME3				ME4				ME5						
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D			
35	200	3	3	0	90	1	6	0	428	17	14	0	204	1	9	0	117	3	7	0	220.6	142.41	63.69
36	114	2	1	0	145	2	2	0	119	0	1	0	59	1	1	0	245	2	5	0	139.8	70.34	31.46
37	234	1	1	0	225	0	1	0	354	0	2	0	335	0	2	0	417	0	1	0	314.6	82.05	36.69
38	97	0	0	0	57	0	1	0	117	0	0	0	147	0	1	0	204	0	0	0	124.8	55.01	24.60
39	70	2	0	0	87	0	0	0	107	0	0	1	70	0	1	0	207	0	0	0	109	56.79	25.39
40	101	2	6	3	44	0	3	1	130	0	1	4	201	0	4	7	80	0	1	0	117.6	62.10	27.77
41	45	0	1	2	43	0	2	2	46	0	0	0	66	0	0	0	13	0	0	1	44.2	18.79	8.40
42	41	0	0	1	34	0	0	0	5	0	0	0	26	0	0	0	13	0	1	0	24.2	14.90	6.66
43	19	0	0	0	6	0	1	0	3	0	0	0	23	0	0	1	8	0	0	0	12.2	8.87	3.96
44	1	0	0	0	9	0	0	0	2	0	0	0	4	0	0	0	2	0	1	0	3.8	3.11	1.39

Table 7: ME responsive fruit flies in ME/CL traps as per Standard Meteorological Weeks (SMW) along with weekly means, standard deviation (S.D.) and standard error (S. E.) (A = *B. dorsalis,* B = *B. zonata,* C = *B. correcta* and D = *B. digressa*).

SMW	Fruit flies collected in Methyl eugenol traps (ME)																				Avg. (mean of 5 treatments)	S. D.	S. E.
	ME/CL1				ME/CL2				ME/CL3				ME/CL4				ME/CL5						
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D			
35	13	1	0	0	27	1	0	0	108	3	0	0	304	2	0	0	114	2	4	0	115.8	116.49	52.10
36	20	2	3	0	77	0	1	0	51	0	0	0	97	1	0	0	63	2	0	0	63.4	27.57	12.33
37	28	0	0	0	30	0	0	0	51	0	0	0	262	0	0	0	67	0	0	0	87.6	98.80	44.18
38	1	0	0	0	4	0	0	1	10	0	0	0	124	1	0	0	33	0	0	0	34.6	51.92	23.22
39	3	0	0	0	0	0	0	0	6	0	0	0	90	0	0	0	39	0	0	0	27.6	38.26	17.11
40	17	0	1	0	28	0	0	0	9	0	0	2	121	0	0	0	22	0	0	1	40.2	45.60	20.39
41	53	0	1	2	67	0	0	0	8	0	0	0	56	0	0	0	19	0	0	0	41.2	25.97	11.61
42	26	0	0	2	26	0	0	0	4	0	0	1	39	0	0	0	6	0	0	1	21.0	14.57	6.51
43	15	0	0	0	17	0	0	0	1	0	0	0	36	0	0	0	4	0	0	0	14.6	13.79	6.16
44	10	0	0	1	0	0	0	0	11	0	1	0	0	0	0	0	3	0	0	0	5.2	5.89	2.63

Table 8: ME responsive fruit fly species response to ME and ME/CL combination ([a]=Mean flies/trap/week, *=significant at 5%).

Species	ME[a] ±S.E.	ME/CL[a]±S.E.	t-value[b]	p-value
B. dorsalis	108.28±30.65	43.4±10.72	2.96	0.0159*
B. correcta	1.6±0.75	0.22±0.10	2.02	0.0730
B. zonata	0.74±0.49	0.3±0.19	1.40	0.1944
B. digressa	0.48±0.29	0.26±0.10	0.86	0.4085

4.5.2. Trapping efficiency of fruit flies responsive to cue-lure in ME/CL vs CL traps

The population of *Z. cucurbitae* was significantly higher in CL traps (20.7±5.40) than in ME/CL traps (13.91±2.72) (Fig 3b). The population of *Z. tau* was likewise observed to be greater in CL traps (0.18±0.04) than in ME/CL traps (0.08±0.09) (although not significantly). In ME/CL traps, however, the population of *B. digressa* was found to be larger than in CL traps (Table 11).

Table 9: CL responsive fruit flies in CL traps as per Standard Meteorological Weeks (SMW) along with weekly means, standard deviation (S.D.) and standard error (S. E.) (E = *Z. cucurbitae,* F = *Z. tau* and D = *B. digressa*).

SMW	Fruit flies collected in Cue-Lure traps (CL)															Avg. (mean of 5 treatments)	S. D.	S. E.
	CL1			CL2			CL3			CL4			CL5					
	E	F	D	E	F	D	E	F	D	E	F	D	E	F	D			
35	0	0	0	0	0	0	7	0	0	3	0	0	2	0	0	2.4	2.88	1.28
36	1	0	0	0	0	0	4	0	0	4	0	0	4	0	0	2.6	1.94	0.87
37	5	0	0	5	0	0	9	0	0	10	0	0	11	0	1	8.2	3.11	1.39
38	3	0	0	2	0	0	13	0	0	8	0	0	27	0	0	10.6	10.16	4.54
39	2	0	0	8	0	0	12	0	0	10	0	0	53	0	0	17.0	20.46	9.15
40	7	0	0	16	0	0	32	0	0	13	0	0	30	2	0	20.0	11.42	5.10
41	13	0	0	14	0	0	44	1	0	20	0	0	16	0	0	21.6	13.35	5.97
42	8	0	0	15	0	0	21	0	0	14	0	0	13	0	0	14.2	4.65	2.08
43	31	0	0	42	0	0	25	0	0	29	0	0	10	0	0	27.4	11.58	5.18
44	27	0	0	35	0	0	14	0	0	14	1	0	33	0	0	24.8	9.85	4.40

Table 10: CL responsive fruit flies in ME/CL traps as per Standard Meteorological Weeks (SMW) along with weekly means, standard deviation (S.D.) and standard error (S. E.) (E = *Z. cucurbitae,* F = *Z. tau* and D = *B. digressa*).

SMW	Fruit flies collected in Cue-Lure traps (CL)															Avg. (mean of 5 treatments)	S. D.	S. E.
	ME/CL1			ME/CL2			ME/CL3			ME/CL4			ME/CL5					
	E	F	D	E	F	D	E	F	D	E	F	D	E	F	D			
35	0	0	0	1	0	0	1	0	0	1	0	0	3	0	0	0.8	0.44	0.20
36	1	0	0	1	0	0	6	0	0	5	0	0	5	0	0	3.6	2.40	1.07
37	2	0	0	1	0	0	4	0	0	8	0	0	1	0	0	4.6	3.28	1.46
38	1	0	0	2	0	1	2	0	0	29	0	0	5	0	0	12.8	14.80	6.62
39	2	0	0	0	0	0	4	0	0	41	0	0	26	0	0	17.6	21.40	9.57
40	10	0	0	7	0	0	9	0	2	31	0	0	5	0	1	18.0	11.95	5.34
41	7	0	2	31	1	2	11	0	0	33	0	0	15	0	0	24.0	12.80	5.72
42	43	1	2	19	0	0	16	0	1	42	0	0	8	0	1	33.2	13.98	6.25
43	77	0	0	27	0	0	17	0	0	91	3	0	24	0	0	61.8	37.15	16.61
44	91	3	1	0	0	0	102	0	0	29	1	0	15	0	0	51.4	44.77	20.02

Table 11: CL responsive fruit fly species response to CL and ME/CL combination ([a]=Mean flies/trap/week, *=significant at 5%).

Species	CL[a] ±S.E.	ME/CL[a]±S.E.	t-value[b]	p-value
Z. cucurbitae	20.7±5.40	13.91±2.72	2.51	0.03308*
Z. tau	0.18±0.04	0.08±0.09	1.04	0.3221
B. digressa	0.02±0.02	0.26±0.10	2.09	0.0659

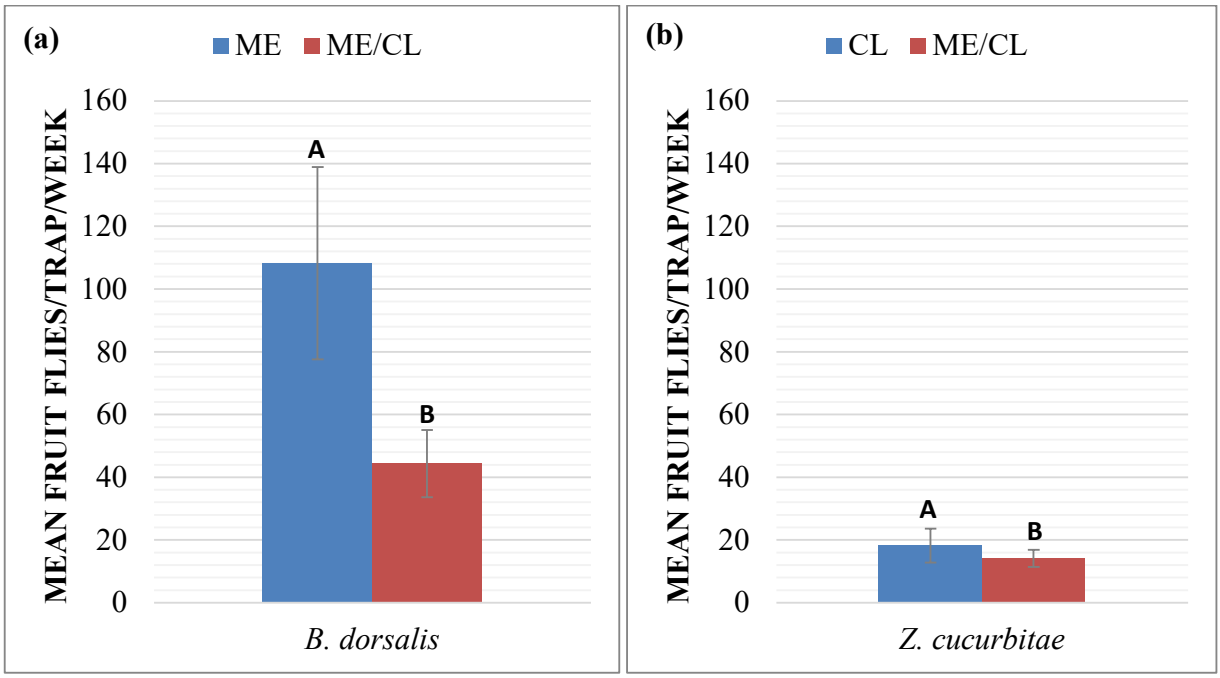

Figure 3. Mean weekly trap catches of fruit flies (±SE). (a) *B. dorsalis* to ME and ME/CL combination. (b) *Z. cucurbitae* to CL and ME/CL combination (Different capital letters indicate significantly different from each other).

4.6. Biodiversity measures of fruit fly species in cucurbit ecosystem

During our study period, from the 35th to the 44th SMWs, a total of 9524 fruit flies (of six species) were collected in various parapheromone traps. The Shannon-Weiner diversity index (H), Pielou's evenness index (E) and Margalef's richness index (R) were used to calculate biodiversity estimations, with values of 0.583, 0.326, and 0.545, respectively (Table 12).

Table 12: Biodiversity measures of fruit fly species in cucurbit ecosystem using Shannon-Weiner diversity index (H), Pielou's evenness index (E) and Margalef's richness index (R).

Total individuals	Number of species	Shannon-Wiener diversity index (H)	Pielou's evenness index (E)	Margalef's richness index (R)
9524	6	0.583	0.326	0.545

4.7. Fruit fly emergence from infested cucurbits in rearing chambers

Fruit flies emerged from infested fruits in rearing chambers from 40th to 42nd SMWs. A total of 67 fruit flies emerged from the 100 infested bitter gourd fruits stored in the rearing chambers (Fig 4a), all of which were *Z. cucurbitae*, with a sex ratio of 1:1.91 MF (males to female). A total of 320 fruit flies emerged from 100 infested cucumbers (Fig 4b), including *Z. cucurbitae* (195 individuals) and *Z. tau* (125 individuals) having a sex ratio of 1.14:1 MF and 1.05:1 MF, respectively (Table 13).

Table 13: Different fruit fly species emerged from infested cucurbits.

Fruit fly species emerged	Total number of individuals emerged			
	Bitter gourd		Cucumber	
	Males (M)	Females (F)	Males (M)	Females (F)
Z. cucurbitae	32	35	104	91
Z. tau	0	0	64	61
B. dorsalis	0	0	0	0
B. correcta	0	0	0	0
B. zonata	0	0	0	0
B. digressa	0	0	0	0

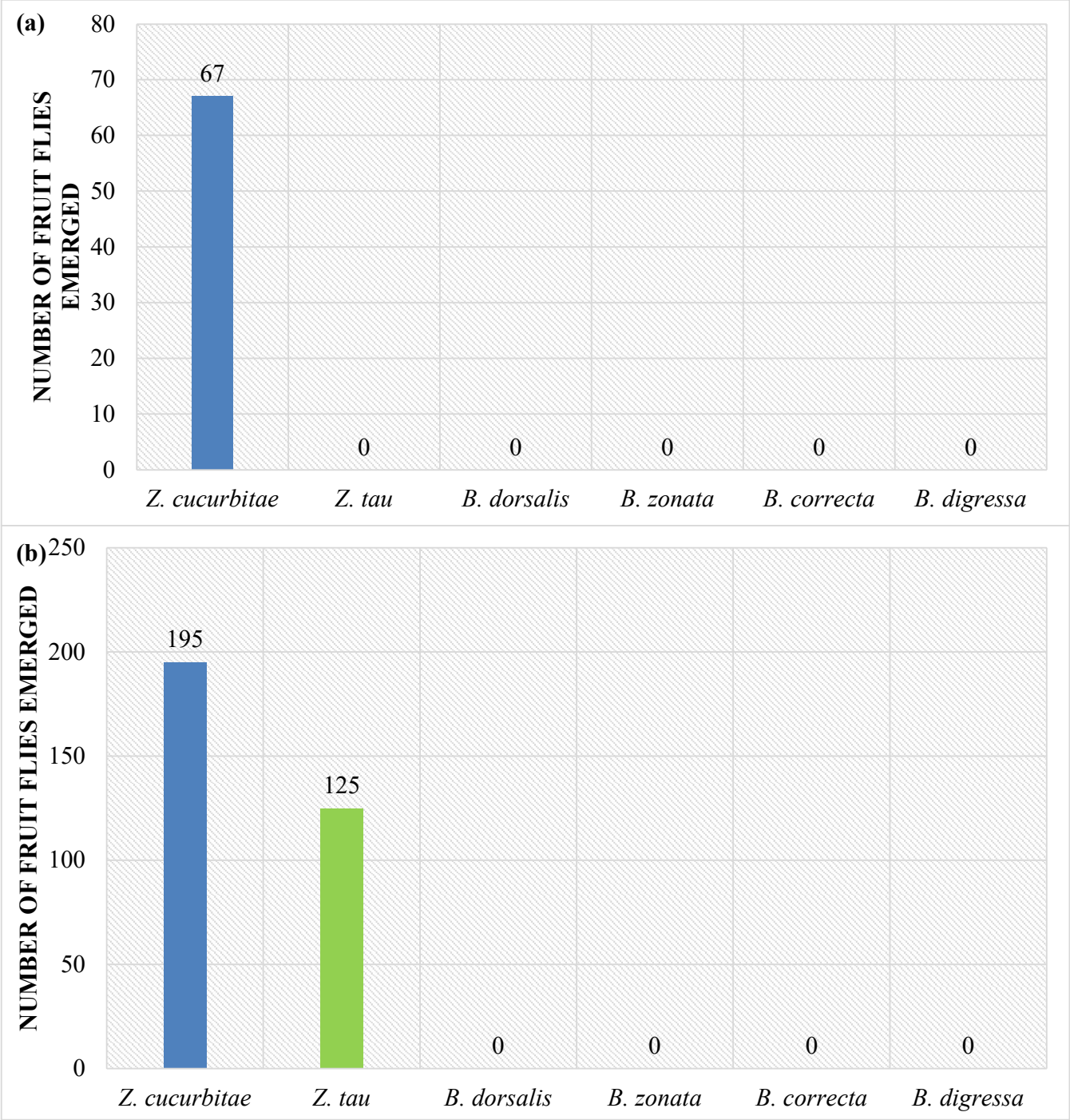

Figure 4. Fruit flies emerged from infested cucurbits. (a) bitter gourd and (b) cucumber.

4.8. Phylogenetic analysis of fruit flies

4.8.1. Pairwise genetic distance

A pairwise genetic distance between different fruit fly species was calculated using Molecular Evolutionary Genetic Analysis (MEGA 6) with *Nilaparvata lugens* (NCBI accession number: MN560658) used as an outgroup. Excluding out-groups, the pairwise genetic distance between the six species of fruit flies was in the range of 0.052-0.197. (5.2-19.7%). As per the results, *B. correcta* was found to be the most closely related to *B. zonata* and the least to *Z. tau* (with a genetic distance of 7.40% and 18.30%, respectively). *B. dorsalis* was equally closely related to *B. zonata* (9.30%) and *B. correcta* (9.30%) with the least close relation to *Z. cucurbitae* (18.30%). *B. zonata* was observed to be the closest with *B. correcta* (7.40%) and the least with *Z. cucurbitae* (18.80%). The closest relative of *B. digressa* was *B. zonata* (10.60%) with *Z. cucurbitae* being the most unrelated one (19.70%). *Z. tau* was the closest with *Z. cucurbitae* (5.20%) and the least with *B. digressa* (18.60%). *Z. cucurbitae* was the closest with *Z. tau* (5.20%) and the least with *B. digressa* (19.70%) (Table 14).

Table 14: Pairwise genetic distance between different fruit fly species under genus *Bactrocera* and *Zeugodacus*, with *Nilaparvata lugens* as an outgroup.

	B. correcta	***B. dorsalis***	***B. zonata***	***B. digressa***	***Z. tau***	***Z. cucurbitae***	***Nilaparvata lugens***
B. correcta		0.093	0.074	0.114	0.183	0.176	0.328
B. dorsalis			0.093	0.124	0.176	0.183	0.368
B. zonata				0.106	0.183	0.188	0.337
B. digressa					0.186	0.197	0.318
Z. tau						0.052	0.332
Z. cucurbitae							0.337
Nilaparvata lugens							

4.8.2. Maximum-likelihood tree

A maximum-likelihood tree was created using Molecular Evolutionary Genetic Analysis (MEGA 6) software with a bootstrap replication of 2000 and *Nilaparvata lugens* (NCBI accession number: MN560658) as an outgroup, the Kimura-2-parameter model was used. With the exception of *B. digressa*, which was shown to be attracted to both the lures was arranged right in between the ME (above) and CL (below) responsive fruit fly species. There were clear clades of fruit fly species reported to be responsive to ME and CL (with fruits and vegetable crops as major hosts, respectively). Fruit flies drawn to CL (*Z. cucurbitae* and *Z. tau*) and ME (*B. dorsalis, B. zonata* and *B. correcta*) had a 99% bootstrap value. *B. dorsalis* and *B. correcta* (both with a bootstrap value of 68%) were found to be monophyletic to their respective pairings, as are *Z. cucurbitae* and *Z. tau* (both with a bootstrap value of 99%) (Fig 5).

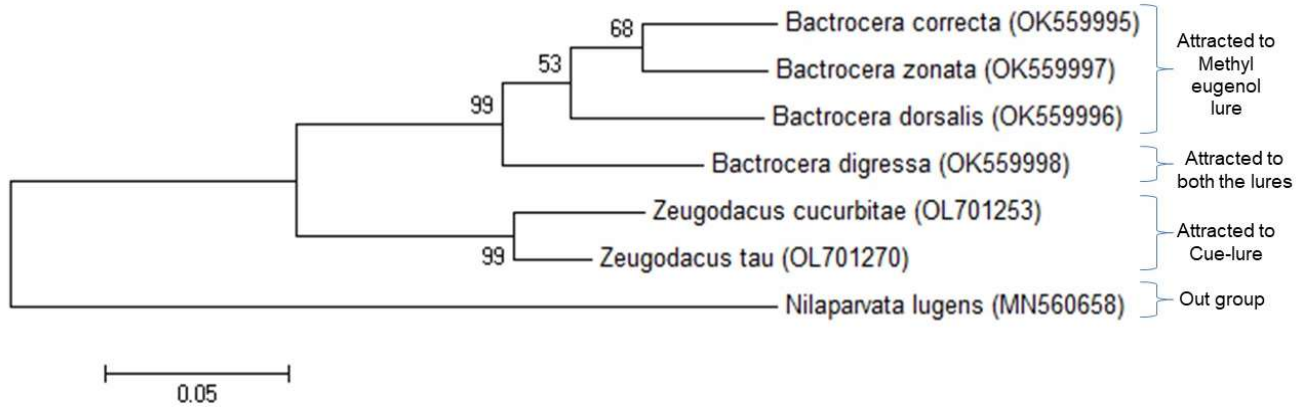

Figure 5. Evolutionary relationship between *Bactrocera* and *Zeugodacus* fruit fly species inferred by maximum-likelihood tree. Numbers on the nodes indicates bootstrap value and 0.05 tree length indicate 5 nucleotide substitutions per 100. The tree with the highest log likelihood (-2214.5202) is shown evolutionary analysis conducted in MEGA 6.

4.9. *Wolbachia* infection in fruit flies

Wolbachia infections were confirmed in four of the six fruit fly species examined, including *B. dorsalis, Z. tau, B. digressa,* and *B. correcta* (Table 15). Positive PCR-results were found in all three specimens of each of the above fruit fly species tested (Plate 13).

Table 15: Fruit flies observed in cucurbit ecosystem and their infection status with *Wolbachia*. + indicates infection with *Wolbachia* and vice versa.

Fruit fly species	*Wolbachia* infection
B. dorsalis	+
B. zonata	-
B. correcta	+
Z. cucurbitae	-
Z. tau	+
B. digressa	+

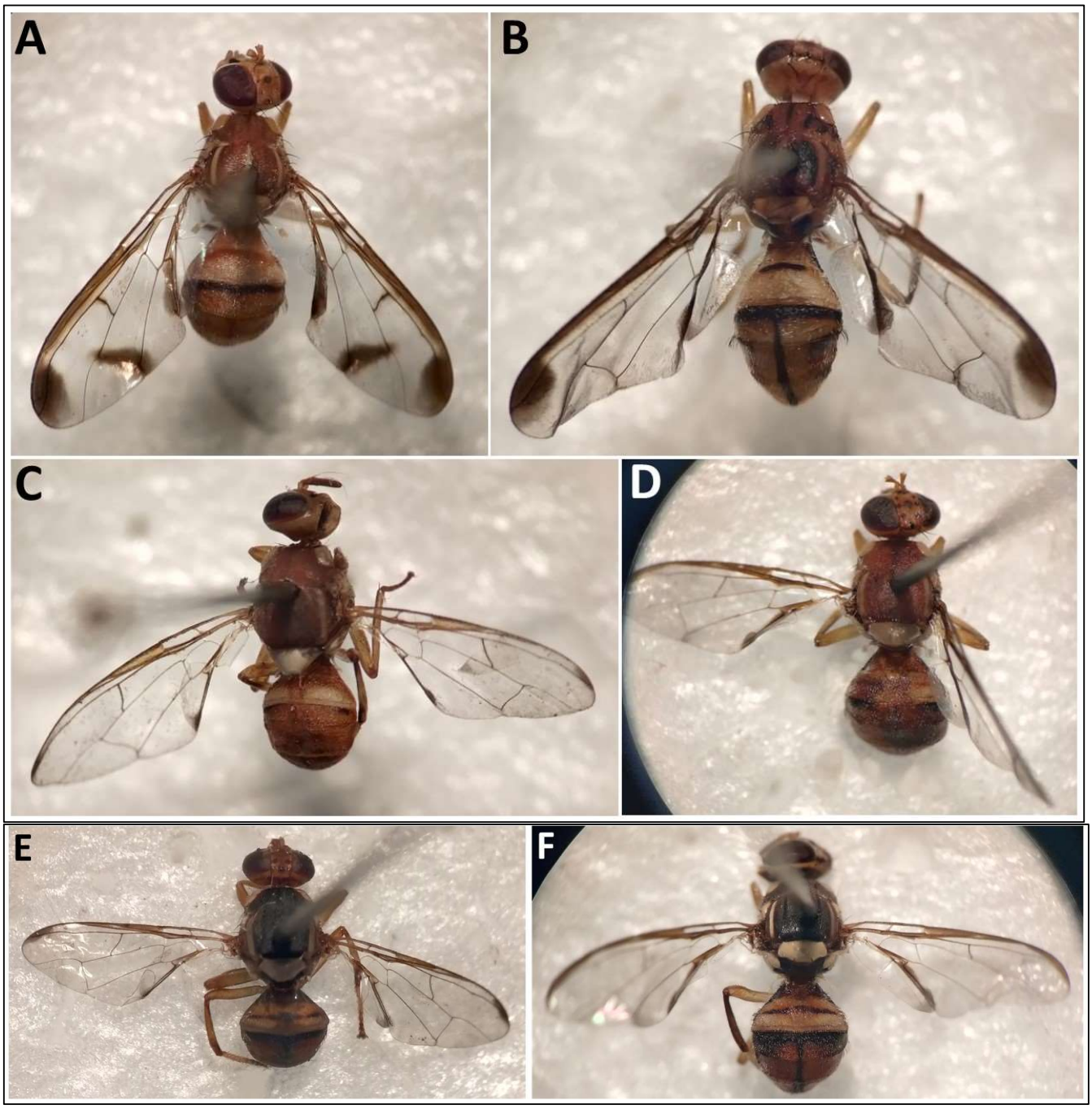

Plate: 11 Fruit fly species collected from parapheromone traps installed in cucurbit fields, observed under stereoscope. (A) *Z. cucurbitae,* (B) *Z. tau,* (C) *B. zonata,* (D) *B. digressa,* (E) *B. correcta* and (F) *B. dorsalis*

Plate: 12 Adults of *Z. cucurbitae* emerged from infested cucurbits in insect rearing chamber identified as males (left and centre) and females (right) based on the presence of pointed ovipositor.

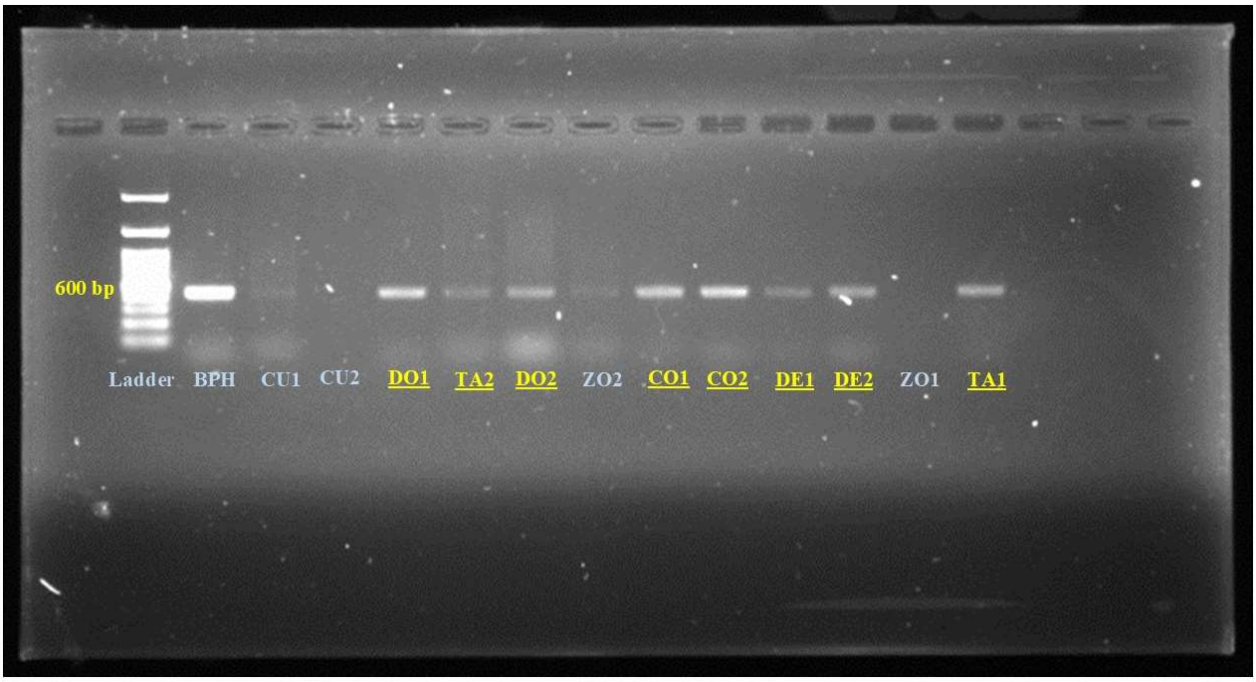

Plate: 13 *Wolbachia* infection in different species of fruit flies (BPH: Brown Plant hopper as a standard reference), DO-*B. dorsalis*, CO-*B. correcta*, DE-*B. digressa*, ZO-*B. zonata*, TA-*Z. tau*, CU-*Z. cucurbitae*.

Chapter V

DISCUSSION

The salient findings of present investigation on **"Study of population dynamics and molecular characterization of fruit flies associated with cucurbits"** during *kharif* of 2021 from 35th SMW to 44th SMW are discussed below:

5.1. Identification of fruit flies

Fruit flies were monitored in the cucurbit ecosystem for 10 weeks using methyl eugenol (ME), cue-lure (CL) and a combination of both the lures (ME/CL), revealed six species of fruit flies: *Bactrocera dorsalis*, *B. zonata, B. correcta, B. digressa, Zeugodacus cucurbitae* and *Z. tau*. Taxonomic literature (David and Ramani, 2011; Leblanc *et al*., 2019; Nair *et al*., 2018) and DNA barcoding utilizing the COI gene were used to authenticate their identities (Table 2). Taxonomic identification of distinct species within the genus *Bactrocera* is particularly difficult utilizing existing literature (Schutze *et al*., 2015) due to cryptic species and varying colour morphology within the species (Drew and Hancock, 1994; Schutze *et al*., 2015). Some major sources of conflict are evolution of species, presence of sibling species and intraspecific variation. Fruit flies are a serious pest of fruits and vegetables, and the current identification not only helped us recognize different fruit fly species, but it also led us to attempt to bridge the gap and update the material, as fruit flies require precise management practices.

5.2. Population dynamics of fruit flies associated with cucurbit ecosystem

5.2.1. *B. dorsalis*

The 'oriental fruit fly,' also known as *B. dorsalis*, is a major pest of fruit crops, with mango as its primary host (Amur *et al*., 2022). During the experimental period, the population of *B. dorsalis* was observed to be reducing from the 35th SMW (160.6±40.40 FTW) to the 44th SMW (4.2±1.33 FTW). According to the National Mango Database, Department of Biotechnology, Government of India, most mango varieties blossom from the first week of January to the end of March in Uttar Pradesh, India, with fruit set beginning in March and lasting until the first week of August. The absence of a mango crop throughout the experimental period explains why the population fell during the course of the investigation. The population peaked in

the 37th SMW (200.5±46.43 FTW), which is somewhat in line with Nandre and Shukla (2014), who reported that the population peaked in the second to third week of September. The population of *B. dorsalis* in the study area followed a similar trend as described by Chen and Ye (2007), with the lowest population seen in the month of November. The population coincided with the ripening cum harvesting of the mango crop.

5.2.2. *Z. cucurbitae*

Z. cucurbitae is also known as the 'melon fruit fly' because of its vast affinity for vegetable crops belonging to the Cucurbitaceae family (Obenewa, 2017). As a result of this preference, the population of *Z. cucurbitae* increased throughout the cropping period, from flowering through harvesting of cucurbits during the present study. The 35th SMW had the lowest population (1.8±0.67 FTW), gradually increased and peaked during the 43rd SMW (37.8±8.28 FTW). The population fluctuation followed a similar pattern to that seen by Raghuvanshi *et al*. (2012), Nair and Pal (2020), and Patel and Das (2021), where the peak of the *Z. cucurbitae* population occurred during the 43rd SMW coinciding with the peak cropping season of cucurbits and fruits were about to ripe.

5.2.3. *B. correcta*

B. correcta, often known as the 'guava fruit fly,' feeds on tropical fruit crops and is considered one of India's most serious fruit fly species (Jalaluddin *et al*., 1999). It has the same host crops as *B. dorsalis* (Gu *et al*., 2019), which could explain why the populations of both species have followed a similar pattern. The peak in the population was observed in the 35th SMW (4.3±1.49 FTW) after which the population subsequently declined. These findings were comparable to those of Liu *et al*. (2019), who saw a peak in the *B. correcta* population in August, and Begam *et al*. (2022), who observed a fall in the population in the late winter season.

5.2.4. *B. zonata*

B. zonata, popularly known as the 'peach fruit fly,' prefers a broad variety of fruit crops, including peach and mango (Saha *et al*., 2014). The population fluctuation of *B. zonata* was likewise comparable to that of *B. dorsalis* (Das *et al*., 2017b), indicating that it, like *B. correcta*

and *B. dorsalis*, is associated with fruit crops as primary hosts. The population was only detected for six weeks (35th to 40th SMW), after which no individuals were collected. On the 35th SMW, the highest number of individuals were trapped (3.4±1.53 FTW), following which the population began to drop. The results obtained are in accordance with Khan and Naveed (2017), stating the months of May to September to be the most active period of *B. zonata* along with the highest population observed in the month of August. According to the findings of Das *et al*. (2017b), no individuals of *B. zonata* were found throughout the late *kharif* season, including the months of October and November.

5.2.5. *B. digressa*

B. digressa is a new range record from Varanasi, Uttar Pradesh, India (Arya *et al*., 2022a), and nothing much is known about its population dynamics. The host crops include *Alangium salviifolium* (Family: Alangiaceae) (Nair *et al*., 2018) and *Alangium lamarkii* (David and Ramani, 2011) popularly known as Ankol, which are both present in Uttar Pradesh (Kumar and Gupta, 2020) and might have been found near the study experiment. A very small number of individuals were collected to establish a firm estimation of population dynamics, but the population fluctuation was still very different than other fruit fly species collected during the present study. The first individual collected was on the 37th SMW with a sudden peak in population observed on the 40th SMW (1.2±0.52 FTW) after which the population reduced back almost to its original number for the entire study period. Ankol is a tiny deciduous tree, shrub or straggler native to India, with a fruiting period ranging from mid-April to early August (David *et al*., 2010) or May to June (Jadeja *et al*., 2010). This meant that the population was surviving on a host that we didn't know about. Fruit flies have a flying range of 7-8 km on average, disregarding the effects of wind velocity and direction (Chen *et al*., 2015), suggesting that the unknown host may be present within a 10 km radius of the experimental location, creating a possibility for further research.

5.2.6. *Z. tau*

Z. tau, known as the 'pumpkin fruit fly,' is a major cucurbit pest found all throughout India (Kitthawee and Julsirikul, 2019). *Z. tau* followed a similar pattern of population expansion as *Z. cucurbitae* due to the host's preference for cucurbits; however, the initial population was

collected on the 40th SMW (0.2±0.2 FTW) until the 44th SMW, and it was quite tiny in number. The highest population recorded was on the 44th SMW (0.5±0.30 FTW). The present results are in accordance with Nair *et al*. (2020) stating that a peak in population was observed in the early winters, coinciding with the cucurbit maturity period. However, as observed likewise by Pramanik *et al*. (2020), the population of *Z. tau* obtained was substantially smaller than that of *B. dorsalis* and *Z. cucurbitae*. With the late harvesting of cucurbits, the population of *Z. tau* was predicted to grow even more, which may have had significant effects.

5.3. Correlation between population of different fruit fly species and weather parameters

The mean number of fruit flies during the experimental period was observed to be in a significant positive correlation with RF (r=0.757), as observed by Win *et al*. (2014) and Vayssières *et al*. (2009), who stated RF to be the most important factor influencing the population of fruit flies. Temperature and RH significantly influenced the population of fruit flies responsive to CL (*Z. cucurbitae* and *Z. tau*) (Gnanvossou *et al*., 2017). The observed population was in positive correlation with Tmin (r=0.235) and RHe (r=0.264) as per the findings of Ravikumar and Viraktamath (2006) with emphasis on fruit flies associated with mango orchards (*B. dorsalis, B. correcta* and *B. zonata*). Fruit fly population established a significant negative correlation with SSH (r=-0.718), as reported by Sawai *et al*. (2019) and to some extent with Win *et al*. (2014), reporting a negative but non-significant correlation. Tmax (r=-0.043) and RHm (r=-0.486) were both negatively correlated with the overall population of fruit flies, as supported by Wazir *et al*. (2019) and Nandre and Shukla (2014), respectively. The correlation of the individual fruit fly species population with the abiotic factors obtained is discussed herewith.

5.3.1. *B. dorsalis*

The findings are consistent with those of Bana *et al*. (2017) and Nandre and Shukla (2014), who found a positive correlation between the population of *B. dorsalis* and Tmin and RHe. The population demonstrated a significant negative correlation with SSH during the cropping season, as per proven by Chen and Ye (2007) and Susanto *et al*. (2022). According to Kannan and Rao (2006), Das *et al*. (2017b) and Kaur *et al*. (2020), the population demonstrated a negative correlation with RHm. According to Das *et al*. (2017b), the results demonstrated a

positive association with Tmax and a significant positive correlation with RF was proven by Patel *et al*. (2019).

5.3.2. *Z. cucurbitae*

The population of *Z. cucurbitae* exhibited a significant positive correlation with SSH, which supported the findings of Nair and Pal (2020). A positive correlation with the RHm was found, which in accordance with Rao and Ghike (2016) and Abhilash *et al*. (2017 A negative correlation was observed with RF as confirmed by Devi and Mehta (2015) and Sawai *et al*. (2019), as well as with Tmax, as proven by Rao and Ghike (2016) and Wazir *et al*. (2019). However, Ganie *et al*. (2013) and Abhilash *et al*. (2017) found a significant negative correlation between fruit fly population and Tmin and RHe, respectively.

5.3.3. *B. correcta*

As reported earlier by Jalaluddin *et al*. (1999) and Rao and Ghike (2016), no weather parameter showed a significant relationship with the population of *B. correcta*. The population was in positive correlation with RF, which is in accordance with the results of Neeraja (2010) and Begam *et al*. (2022). Tmin showed a positive correlation with the population, as supported by Deepa *et al*. (2009) and Begam *et al*. (2022). Kumar *et al*. (1997) discovered that the *B. correcta* population had a positive correlation with both Tmax and Tmin, which was similar to the results obtained. However, the population variation with SSH showed a negative correlation, as per the findings of Singh and Sharma (2012). The population was also in negative correlation with the RHm, as per the results by Neeraja (2010) but in a positive correction with RHe, supported by the research findings of Umesh *et al*. (2022).

5.3.4. *B. zonata*

A weak positive correlation was established between the population of *B. zonata* and Tmax and Tmin, as reported by Draz (2016) and Khan and Naveed (2017). The population established a negative correlation with RHm and a positive correlation with RF, which was supported by the findings of Das *et al*. (2017b). Sanjeev *et al*. (2008) discovered a positive correlation between fruit fly population and RHe, which is consistent with our findings. The results of SSH with the population of *B. zonata* were ambiguous to the research findings of

Sharma *et al*. (2015) and Khan and Naveed (2017), reporting a positive and no correlation of the population with SSH, respectively. The low population in the trap catches of fruit flies could be a possible reason for this.

5.3.5. *Z. tau*

The results obtained are in accordance with the findings of Hossain *et al*. (2019), who reported a negative correlation between the *Z. tau* population and Tmax, Tmin and RF, observing peaks in cooler months of the year. However, our results are contrary to Nair *et al*. (2020), establishing a significantly positive correlation of the population with Tmax, Tmin, RF and RH, mainly due to low trap catches of the population in the cropping season. Our findings are in association with Sawai *et al*. (2019), stating a positive correlation with RHm and an inverse correlation with RHe. The results reported a significant positive correlation with SSH, which was similar to the population fluctuation of *Z. cucurbitae* with abiotic factors (Nair and Pal, 2020).

5.3.6. *B. digressa*

B. digressa population variation was new to the climatic conditions of Varanasi, Uttar Pradesh, India. Because of the limited population collection in trap captures, the population does not fluctuate considerably with any of the abiotic parameters. As per the research findings of Arya *et al*. (2022a), the population variation revealed a specific pattern that was determined to be different from all other detected species. *B. digressa*, like B. *dorsalis, B. zonata*, and *B. correcta*, demonstrated a positive correlation with Tmax and Tmin. The population, like *Z. cucurbitae* and *Z. tau*, exhibited a positive connection with RHm and SSH, and a negative correlation with RHe and RF, with meteorological conditions having only a 53% influence on the population.

5.4. Fruit fly trapping efficiencies of ME/CL in comparison to ME and CL

Z. cucurbitae and *Z. tau* are responsive to CL (Nair and Pal, 2020; Arya *et al*., 2022b), whereas *B. dorsalis, B. correcta,* and *B. zonata* are responsive to ME (Kumar *et al*., 2018; Bajaj and Singh, 2018). *B. digressa* is reported to be responsive to CL (Leblanc *et al*., 2019), but as per our findings, was equally responsive to ME and CL. So, for mass trapping programmes, there will be a separate requirement for ME and CL traps in order to manage fruit flies associated with any given crop ecosystem. This can turn out to be laborious and expensive (Royer and Mayer,

2018). In order to check the possibility of combining different lures in a single trap, the present experimental findings showed a significantly higher number of individuals of *B. dorsalis* and *Z. cucurbitae* in individual lure traps of ME and CL, respectively, than compared with the population collected in ME/CL traps. The population of other fruit fly species was also higher (but not significant) in their respective lure traps compared with the ME/CL trap. For *B. digressa*, the trap catches were higher in ME than compared with ME/CL and lower in CL than compared with ME/CL.

The results obtained are in accordance with Royer and Mayer (2018), who reported that 24 out of 27 fruit fly species in Australia and 13 of 16 species in Papua New Guinea under the genus *Bactrocera* were found to be significantly less attracted to ME/CL traps than compared with individual traps. According to a similar study in Malaysia by Tan and Lee (1982), Dorsalure (a combination of ME and CL, with a higher proportion of ME) trapped fewer individuals of *B. dorsalis* as well as *Z. cucurbitae* than individual ME and CL traps. As per a study by Tan (1983), the trap catches of *B. dorsalis* and *B. umbrosa* were significantly higher in ME and CL, respectively, than compared with ME/CL mixed in three different ratios (v/v) in a single trap in separate wicks. In a study by Wee and Shelly (2013), the trapping efficiency of *Z. cucurbitae* was also found to be significantly higher in CL traps as compared to ME/Raspberry ketone (RK: a synthetic analogue to CL) wafers. The experimental findings are also supported by Hooper (1978), Shelly *et al*. (2004) and Vargas *et al*. (2000), which show that combining CL (either in a separate wick or mixed) with ME in a single trap significantly reduces the trap catches of *B. dorsalis*.

However, there are some studies that have shown combining different lures in a single trap to be as effective as individual lures for trapping fruit flies. According to research conducted in Hawaii, Mallet multi-crystalline (ML) wafers (2.8g ME and 1.9g RK) and Mallet Trimedlure, ME and RK (TMR) wafers (4.4g ME, 2.3g RK and 2.3g Trimedlure) were equally attractive to *B. dorsalis* as compared to individual ME lures (Leblanc *et al*., 2011; Shelly *et al*., 2012), whereas liquid mixed ME/CL (50:50) and Mallet TMR wafers were found to be equally attractive as CL to *Z. cucurbitae* (Vargas *et al*., 2000; Shelly *et al*., 2012).

The possible reason why combining different lures had an inhibitory reaction in response to the fruit fly trap catches can be the result of environmental factors. Some studies have shown

tropical environments to exert a more inhibitory effect on lure mixtures (Tan and Lee, 1982; Tan, 1983; Wee and Shelly, 2013) than compared to subtropical environments (Vargas *et al*., 2000; Leblanc *et al*., 2011; Shelly *et al*., 2012). The climatic conditions in Varanasi, Uttar Pradesh, are tropical monsoon (Singh *et al*., 2018), which could explain why the inhibitory effect was dominant. The volatility of individual lures in the tropical regions exerted a greater inhibitory effect (Royer and Mayer, 2018), thus reducing the trap catches of fruit fly species.

Unlike most of the chemicals, parapheromone also undergoes chemical decomposition under various environmental conditions when applied or installed in fields. Doolittle *et al*. (1970) identified 4-(p-hydroxyphenyl)-2-butanone as a degraded product of CL that, when mixed with pure CL or used as a parapheromone in traps, has no negative effect on melon fruit fly trap catches. This result was further supported by Keiser *et al*. (1973), where 100% degraded product when installed in the bait wick along with insecticide (25% lindane and 40% chlordane dust at the bottom of the traps) was equally effective as an insect attractant as was 100% commercially manufactured CL, installed in the field for 42 weeks. However, later the degraded product of CL was identified as raspberry ketone (RK) (Oliver *et al*., 2004), and CL was established as an acetate derivative of RK (Jang *et al*., 2007). This supported the fact that parapheromone traps can be installed in the field for a much longer time without monthly/weekly recharging the wicks with the lure, which could rather be highly economic.

5.5. Biodiversity measures of fruit fly species in cucurbit ecosystem

The diversity indices of different species of fruit flies associated with the cucurbit ecosystem were represented in Table 12. The data clearly indicated the population to be less diverse. The Shannon and Weiner index (H) measures the abundance and evenness of species in a community (Shannon and Weiner, 1949). In our study, the index was found to be 0.58, demonstrating the population to be less diverse, with only a limited number of species found to be associated with the cucurbit ecosystem in the experimental area. However, the results are more or less in accordance with a study conducted by Ganie *et al*. (2013) in Kashmir, India, where the fruit fly diversity index ranged from 0.25-0.51, reported to be less diverse. Species diversity was affected by a higher population of individuals of *B. dorsalis*, as found to be similar to the findings of Gnanvossou *et al*. (2017). Also, genetically closer plants are unlikely to support a wider diversity of Tephritidae species (Raga *et al*., 2017).

The evenness index (E) is a measure of a species distribution in an environment (Pielou, 1966). The index value of one implies that the distribution of species is uniform (Amala and Shivalingaswamy, 2018). However, the value of E obtained from the experiment was 0.32, implying that the fruit fly population distribution is relentlessly non-uniform. This could be the result of variable densities of the wine in fields, the maturation periods of different fruit and vegetable crops in the area not coinciding with one another and dominance of the ecosystem by a single species (*B. dorsalis,* accounting for 80.50% of the total individuals collected of all species) in trap catches. The other reason for the lower value is the occurrence of one or two species at a frequency greater than 75% (Silva *et al*., 2021).

The richness index (R) is influenced by the sample size, with a higher index resulting in a larger population size (Melo, 2008). According to Margalef (1972), the value rarely exceeds 4.5 and is found to be in a range of 1.5 to 3.5 for most of the species. The value of R obtained was low (0.54) in the experiment as only six fruit fly species were collected during our study. The lower value results in the dominance of a single or few taxonomical groups (Begon *et al*., 1990).

5.6. Fruit fly emergence from infested cucurbits in rearing chambers

Only two species of fruit fly (*Z. cucurbitae* and *Z. tau*) adults emerged from the infested cucurbit fruits. Although six species were observed to be associated with the cucurbit ecosystem, only *Z. cucurbitae* (67 adults) emerged from infested bitter gourd, *Momordica charantia,* while *Z. cucurbitae* (195) and *Z. tau* (125) emerged from infested cucumber, *Cucumis sativus*. The present study supports the cucumber as the preferred host of fruit flies, which is supported by the research findings of Vayssières *et al*. (2007) and Mwatawala *et al*. (2010). The result also proves that *Z. cucurbitae* is the most dominant infester of cucurbits, also claimed by Singh (2003), Mwatawala *et al.* (2009), and Kambura (2016), followed by *Z. tau* (Baimai *et al*., 2000; Jaleel *et al*., 2018; Vasudha *et al*., 2019) as compared with the other infesting species as per adult emergence. Although in this study, *B. dorsalis* is the dominant species, proving it to be a wild population infesting other horticultural crops in the locality. It is a polyphagous pest having a wider host range with a major preference for fruit crops (Ren *et al*., 2008; Rattanapun *et al*., 2009; Koswanudin *et al*., 2018). Similarly, *B. correcta and B. zonata* have been reported to infest cucurbits (Kunprom *et al*., 2015; Sohail *et al*., 2015;), but their main hosts are fruits, not cucurbits (Liu *et al*., 2013; Ma *et al*., 2012; Sarwar *et al*., 2013; Zingore *et al*., 2020).

Fruit fly adult females are the major source of infestation in fruits (Allwood, 1997). The level of infestation can also be judged by the number of females, which usually have a higher life expectancy than males (Miyatake, 2021). The sex ratio can provide a decent estimate of the average female reproductive state of the population (Houston, 1981). Female emergence of *Z. cucurbitae* was higher in bitter gourd, *Momordica charantia* (35 out of 32), with a lower MF sex ratio. However, the female emergence was lower in both *Z. cucurbitae* (91 out of 104) and *Z. tau* (61 out of 64) with a higher MF sex ratio (1.14:1 and 1.05:1, respectively) in cucumber, *Cucumis sativus*. To some extent, this supports the idea that infestation potential was higher in bitter gourd, but higher number of individuals of fruit fly were found infesting cucumber. Bitter gourd was found to be the most susceptible host for *Z. cucurbitae* however, cucumber supported the population of both *Z. cucurbitae* and *Z. tau*.

5.7. Molecular characterization of fruit fly

Cytochrome oxidase one (COI) mitochondrial gene has become a universal barcode for insect identification (Lin *et al*. 2015; Twinkle *et al*. 2020), mainly due to higher interspecific diversity and less intraspecific divergence helps to distinguish species (Pentinsaari *et al*. 2014; Srinivasa *et al*., 2020). COI genes have proven to effectively identify morphologically indistinguishable fruit fly species (Karimi and Darsouei, 2014; Manger *et al*., 2017), excluding the cryptic species under the genus *Bactrocera* (Kunprom and Pramual, 2019). The identification of fruit fly species in our study was achieved by Nucleotide Basic Local Alignment Search Tool (BLASTn) analysis of the COI gene sequences. The pairwise genetic distance and maximum likelihood analysis were done using the following sequences with *Nilaparvata lugens* (NCBI accession number: MN560658) as an outgroup.

5.7.1. Pairwise genetic distance

Molecular Evolutionary Genetic Analysis (MEGA 6) software with Kimura-2-parameter was used to calculate the pairwise genetic distance between six fruit fly species collected in the present study (Table 14). A minimum of 3% divergence at COI level is required between the groups to be designated as separate species (Hebert *et al*., 2004b; Whinnett *et al*., 2005). The genetic distance between four fruit flies under the genus *Bactrocera* (*B. dorsalis, B. zonata, B. correcta* and *B. digressa*) ranged from 0.074 to 0.124 (7.4-12.4%) while that of *Z. cucurbitae*

and *Z. tau* was 0.052 (5.2%) under the genus *Zeugodacus.* Among all the fruit fly species, the pairwise genetic distance between *Z. cucurbitae* and *Z. tau* was found to be the lowest (5.2%). The results obtained were in agreement with Jamnongluk *et al*. (2003) and Manger *et al*. (2017), stating *Z. cucurbitae* and *Z. tau* to be closely related under genus the *Zeugodacus*. *B. correcta* was closely related to *B. zonata* (genetic distance of 7.4%) and with *B. dorsalis* (9.3%), which was in accordance with the research findings of Liu *et al*. (2011), establishing a close relationship (8.9%) between *B. correcta* and *B. zonata*, and with *B. correcta* and *B. dorsalis* (9.2%) respectively. Of the limited fruit fly species collected in the study, *B. digressa* was the most closely related to *B. zonata* (10.6%), supported by Arya *et al*. (2022a). The outgroup *N. lugens* was belong to a different genus, which was confirmed by its genetic distance with the other fruit fly species, which ranged from 0.318 to 0.368 (31.8-36.8%).

5.7.2. Maximum-likelihood tree

A maximum-likelihood (ML) tree was constructed based on Kimura-2-parameter model with 2000 bootstrap value in MEGA 6 software using *N. lugens* as an outgroup (Figure 5). The ML tree constructed showed two separate clades of fruit fly species infesting major fruits (*B. dorsalis, B. correcta* and *B. zonata*), responsive to ME and vegetable crops (*Z. cucurbitae* and *Z. tau*), responsive to CL. However, *B. digressa* formed a separate clade, which was found to be responsive to both ME and CL, was placed at the root of ME responsive fruit fly species. The results obtained were supported by Manger *et al*. (2007), showing a clear separation of *Bactrocera* and *Zeugodacus* genus infesting fruits and vegetables, respectively. *B. zonata* was monophyletic with *B. correcta* whereas, *Z. cucurbitae* was with *Z. tau,* with a bootstrap support of 68% and 99%, respectively. The average nucleotide composition of fruit flies is Adenine and Thymine (AT) (28.38:35.06) biased, which is 63.48%, a common feature of COI mitochondrial genes of arthropods (Muraji and Nakahara, 2001; Srinivasa *et al*., 2020).

5.8. *Wolbachia* infection in fruit flies

Wolbachia is a gram negative, obligatory intracellular bacterium which is maternally inheritable and is one of the most predominant parasites amongst Arthropods, especially Tephritids (Jamnongluk *et al*., 2002). It is now widely used in pest management programmes under sterile insect technique (SIT) and incompatible insect technique (IIT) (Mateos *et al*.,

2020). In the present study, *Wolbachia* infection was checked in six fruit fly species, among which four of them were found to be infected (*B. dorsalis, Z. tau, B. correcta* and *B. digressa*) (Table 15 and Plate 13). The results are supported by Sun *et al*. (2014), Liu *et al*. (2006) and Kunprom *et al*. (2015), who previously reported *Wolbachia* infection in *B. dorsalis, Z. tau* and *B. correcta*, respectively. However, the infection was for the first time reported in *B. digressa* (Arya *et al*., 2022a), which may be an important finding for future studies. If infestation by the fruit fly species in the major fruit and vegetable host crops exceeds the economic threshold in the major geographical region, the species examined with a positive *Wolbachia* infection test can be used in IIT and/or SIT programmes.

Chapter VI

SUMMARY AND CONCLUSION

The undersigned experiment **"Study of population dynamics and molecular characterization of fruit flies associated with cucurbits"** was conducted on a farmer's field during *kharif* of 2021 from 35th SMW to 44th SMW. The results of the investigation are summarized below.

6.1. Identification of fruit flies

Morphological and molecular techniques was used to identify the fruit fly species associated with the cucurbit ecosystem, collected in ME and CL based traps. The identities of six fruit fly species were confirmed, which were: *B. dorsalis, B. correcta, B. zonata, B. digressa, Z. cucurbitae* and *Z. tau*. The morphological identification was based on the colour pattern on the thorax and the presence or absence of postsutural vittae on the thoracic tergum; the presence/absence/arrangement of spots on the wings; and the colour and marking pattern on the abdominal tergum. For further confirmation, the COI mitochondrial gene from each fruit fly species was amplified for DNA barcoding. The obtained sequences were checked and matched with the NCBI database of existing gene sequences. Morphological characters were very much distinct to identify them and COI gene found to be best to delineate the species of fruit flies.

6.2. Population dynamics of fruit flies associated with cucurbit ecosystem

The population dynamics of fruit flies were analysed on the basis of weekly trap catches of fruit flies in ME, CL and ME/CL traps. The variation in the population of *B. dorsalis, B. correcta* and *B. zonata* followed a similar pattern, where it subsequently decreased throughout the study period. The incidence of *B. dorsalis* was high during the 37th SMW and of both *B. correcta* and *B. zonata* in the 35th SMW. The population fluctuation of *Z. cucurbitae* and *Z. tau* also followed a similar pattern, where the population gradually increased from the beginning throughout the experimental period with the highest incidence during the 43rd and 44th SMW, respectively. However, the individuals collected of *B. digressa* and *Z. tau* were not enough to establish a firm population fluctuation throughout the study period.

The statistical analysis of the population, where the number of individuals collected were correlated with weather parameters, revealed that the overall population was significantly positively influenced by RF and negatively with the SSH. Tmin and RHe showed a negative correlation, whereas Tmax and RHm showed a positive correlation with the population variation. The most important climatic elements determining the occurrence of fruit flies were found to be SSH and RF, which must be taken into account when developing fruit fly monitoring and mass trapping programmes.

6.3. Fruit fly trapping efficiencies of ME/CL in comparison to ME and CL

Through the population dynamics study, *B. dorsalis* and *Z. cucurbitae* proved to be the major pest species during the study period, whose populations were found to be significantly higher in ME and CL traps than in ME/CL traps, respectively. The trap catches of *B. correcta, B. zonata*, and *B. digressa* in ME and *Z. tau* in CL were similarly higher than in ME/CL traps. The findings support the hypothesis that combining ME and CL in a single trap minimizes fruit fly trap captures responsive to individual lures, which can further lead to an ineffective pest management programmes. Using mix lure traps in a pest monitoring programme, on the other hand, can have greater efficiency. Fruit flies associated with cucurbits are responsive to CL and the same must be applied in field for pest management purposes.

6.5. Biodiversity measures and fruit fly emergence from infested cucurbits in rearing chambers

The biodiversity indices computed to analyse the fruit fly diversity stated than the population studied was less diverse, non-uniformly distributed and was dominated by few species (mostly *B. dorsalis* and *Z. cucurbitae*). This concluded that only limited species of fruit flies were found to be associated or infesting cucurbits in the study region. The management practices for future situation should be limited to the given species.

Only *Z. cucurbitae* and *Z. tau* were discovered to emerge from infested bitter gourd (*Momordica charantia*) and cucumber (*Cucumis sativus*) fruits collected and observed in rearing cages, indicating that their infestation was verified. According to our research, bitter gourd was the more susceptible host for *Z. cucurbitae* whereas, cucumber was found to support the population of both *Z. cucurbitae* and *Z. tau*. Further analysis revealed that *B. dorsalis*, *B. zonata*,

and *B. correcta* are ME-responsive fruit fly species, were found attracted to and collected in ME and ME/CL traps, infest fruit crops (perhaps mango and guava, which were identified nearby the experimental region). However, no strong evidence of the relationship of *B. digressa* with the aforementioned host crops has been found.

6.6. Pairwise genetic distance and maximum-likelihood analysis of fruit fly species

For phylogenetic analysis of fruit fly species gathered in the research, MEGA 6 software was used with a Kimura-2-parameter model, 2000 bootstrap replications and *N. lugens* as an outgroup. According to the lowest pairwise genetic distance, *B. zonata* was the most closely related to *B. correcta*, and *Z. cucurbiate* was the most closely related to *Z. tau*. It was also discovered that *B. dorsalis* is related to *B. zonata* and *B. correcta*. *B. zonata* was the closest species to *B. digressa*. The maximum likelihood tree constructed showed two separate clades of fruit flies infesting fruits (genus *Bactrocera*) and vegetables (genus *Zeugodacus*), found responsive to ME and CL, respectively. *B. zonata* and *B. correcta* were monophyletic, as were *Z. cucurbitae* and *Z. tau. B. digressa* was placed in a separate clade and found to be responsive to both ME and CL. The results of phylogenetic analysis explained that closely related fruit fly species follow a similar population fluctuation pattern, are attracted to a common lure and have common host crops. Related species or species belonging to a common clade can even be managed by common practices.

6.7. *Wolbachia* infection in fruit flies

Four fruit fly species (*B. dorsalis, Z. tau, B. correcta* and *B. digressa*) were found positive for *Wolbachia* infection. However, the extensive characterization of current strain is required, which could be used to improve SIT and IIT, as part of area-wide integrated pest management (AW-IPM) strategies for insect pest population reduction.

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A guide to prepare Cue-Lure for *Bactrocera cucurbitae* (Coquillett) management in cucurbits

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Varanasi (Uttar Pradesh, India) considered to be one of the most prominent vegetable belts of the country (Roy *et al*., 2017). Among the vegetables cultivate here, cucurbits (Fig.1) cover the major share in area and production. Among pest and disease complexes infesting cucurbits, majority of damage is caused by fruit fly complex (Bhowmik and Saha, 2017). Melon fruit fly *Bactrocera cucurbitae* (Coquillett) (Tephritidae: Diptera) reported to damage 81 host plants (Shivangi and Swami, 2017) and it considered to be the most serious one. It can cause a yield loss of about 30-100% in cucurbitaceous vegetables depending upon the season and other factors (Dhillon *et al.,* 2005).

Fig. 1. Cucurbit crops grown in Varanasi, Uttar Pradesh. (a) & (b) Sponge gourd (c) Bitter gourd (d) Bottle gourd (e) Round gourd (f) Ridge gourd

Damage symptoms of *B. cucurbitae*

Adult females lay eggs in soft and tender fruits by puncturing fruit skin with their sharp ovipositor. Infested fruits show brown resinous deposit in the oviposit area, maggots on hatching feed on internal pulp content of the fruit. Damaged fruits also fall off rapidly due to secondary infection by bacteria and fungi. Fruits drop prematurely which are also unfit for consumption. Along with above distorted and malformed fruits are also observed (Fig. 2).

Life cycle of *B. cucurbitae*

Adult female lays eggs in soft and tender fruit tissue. The eggs hatch into maggots in 2-5 days and larval duration ranges from 2-5 days. Matured larvae undergo pupation in soil at a depth of 0.5-15cm. Pupal period ranges from 9-13 days and adult longevity ranges from 13-15 days.

Management of fruit fly, *B. cucurbitae* using pheromone traps

Fruit flies on small and large scale can be managed by pheromone traps. Parapheromones are synthetically

Fig. 2. Fruit fly damaging symptoms in cucurbit crops. (a) Bitter gourd (b) Sponge gourd

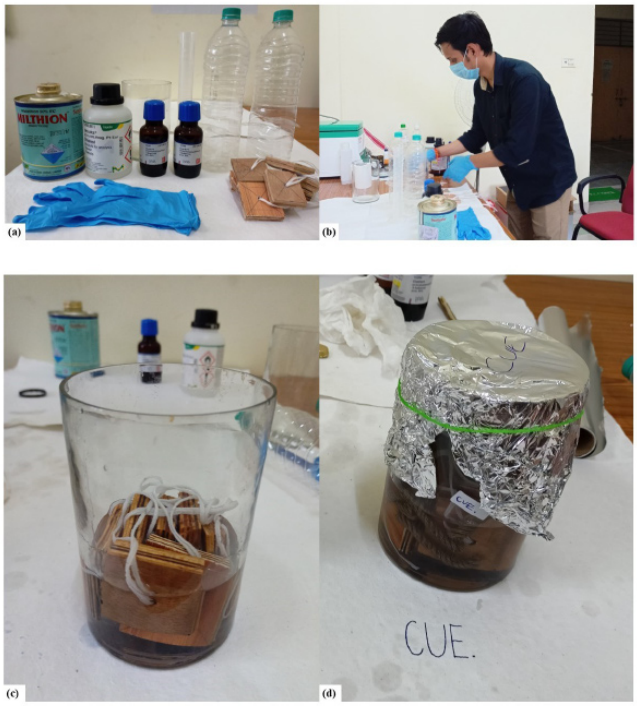

Fig. 3. Preparation of cue-lure baited trap with insecticide. (a) Chemicals used for making cue-lure traps (b) Preparation of lure (c) Plywood pieces dipped in cue-lure (d) Dipped plywood pieces covered with aluminum foil.

produced chemical compounds, that have pheromone like action similar to female insect sex pheromones which is released to attract males. The Cue-Lure is a parapheromone which attract males of *B. cucurbitae* (Shelly *et al.,* 2004). The Cue-Lure can be used for monitoring and mass trapping of male flies and baiting with pesticide will helps to kill them immediately.

Preparation of Cue-Lure baited traps

Mix ethyl alcohol, Cue-Lure [4-(p-acetoxyphenyl)-2-butanone] and Malathion 50EC (Insecticide) in the ratio of 6:4:2 in a glass container. Add plywood pieces of size 2″×2″×1″ (l×b×h) into above prepared mixture. Soak the plywood pieces in the Cue-Lure for 24-48 hrs (Fig. 3).

Preparation of low-cost trap bottles

Use transparent water bottle. Turn it upside down and make 2 window holes of 3″×1″ (l×b) from the top 1/3rd of the bottle. Tie the Cue-lure treated wooden blocks in the upper portion of bottle with thread or wire (Fig. 4).

Fig. 4. Installation of cue-lure baited traps in cucurbits field. (a) & (b) Installing low cost trap bottle, 1-2 m above the ground (c) & (d) Installed cue-lure traps in the field.

Usage and recommendation of Cue-Lure

Ten to twelve lure traps per acre are recommended. Traps must be placed from onset of flowering to harvesting (for wide area management) or throughout the year (for monitoring). Change lures in every 30-40 days and examine traps in every 15 days (Fig.

Fig. 5. Male *B. cucurbitae* flies collected in Cue-lure bait traps.

5). Unscrew the bottle cap to collect and discard the collected fruit flies safely.

Caution: Always wear mask and gloves while preparing the Cue-lure and their installation in field to get protection from pesticide. Mix the ethyl alcohol, Cue- Lure and insecticide with a thick stick, wash it properly and discard safely.

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DNA barcoding of fruit flies associated with cucurbit ecosystem and combination of Cue-Lure and Methyl Eugenol in trap is not effective for mass trapping of responsive fruit flies

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Abstract Fruit flies are key pests of vegetable crops, particularly cucurbits. Depending on the fruit fly species, the parapheromone, cue-lure (CL) or methyl eugenol (ME) is used in traps for monitoring and mass trapping. Many species of fruit flies belong to the genus *Bactrocera and Zeugodacus* which infest cucurbits and are responsive to CL and ME. Preparation and application of individual lures of CL and ME is uneconomical and laborious. Therefore, the present investigation was undertaken to document fruit flies associated with the cucurbit ecosystem, their diversity using CL and ME baited traps and to study the feasibility of combination of CL and ME in the trap for mass trapping of responsive fruit flies. Six species of fruit flies were found to be associated with the cucurbit ecosystem, viz., *Zeugodacus cucurbitae*, *Z. tau*, *Bactrocera dorsalis*, *B. zonata*, *B. digressa* and *B. correcta*. These species were identified using taxonomic keys and DNA barcoding. *Bactrocera digressa* was recorded for the first time from Uttar Pradesh (India) and it is also attractive to both CL and ME. The mitochondrial cytochrome oxidase I barcodes of *B. digressa* are novel to India. Though six species were collected in the lures, it was *Z. cucurbitae* and *Z. tau* that actually infested cucurbits, ascertained from fruit fly emergence from infested cucurbits, indicating the seriousness of pests. The population of *Z. cucurbitae* and *B. dorsalis* was found to be more in CL ($p=0.03308$) and ME ($p=0.0159$) respectively than in the ME/CL combination. The study thus indicated that the combined use of lures, CL and ME in single trap significantly reduces the trap catches of responsive fruit flies. The phylogenetic analysis revealed two distinct clades, i.e., one for fruit flies responsive to CL and another one for ME. Our findings are anticipated to have significant implications for pest identification, monitoring and management.

Keywords Methyl eugenol · Cue-Lure · Fruit fly · Cucurbit · DNA barcoding · Phylogenetic tree

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Introduction

Among the different vegetables, cucurbits are widely cultivated in fields and predominantly in household

gardens in India (Chakravarty et al., 2019). They are primarily creepers with high moisture content and excellent nutritional value, making them popular hosts for insects and pathogens. Out of the various biotic and abiotic factors diminishing crop growth and escalating economic losses, fruit fly infestation is a major reason (Deguine et al., 2015) for attaining qualitative marketable yields. Various species of fruit flies infest cucurbitaceous vegetables in India such as the melon fruit fly, *Zeugodacus cucurbitae* Coquillett (Mwatawala et al., 2015), the pumpkin fruit fly, *Z. tau* Walker (Nair & Pal, 2020), the striped fruit fly, *Z. scutellaris* Bezzi (Prabhakar et al., 2007), the three-stripped fruit fly, *Z. diversus* Coquillett (Royer et al., 2018), the peach fruit fly, *Bactrocera zonata* Saunders (Sohail et al., 2015), the oriental fruit fly, *B. dorsalis* Hendel (Darboe & Osekre, 2021) and the guava fruit fly, *B. correcta* Bezzi (Kunprom et al., 2015) along with minor pest species including *Z. caudatus* Fabricius (Nair et al., 2021), *Z. cilifera* Hendel (Nair et al., 2017), *Dacus longicornis* Wiedemann (Nair et al., 2017), *D. ciliates* Lowe (Vayssières et al., 2008), *Pliomelaena udhampurensis* Agarwal and Kapoor (Prabhakar et al., 2012) and *Dioxyna sororcula* (Wiedemann) (Prabhakar et al., 2012). Of all the infesting fruit fly species *Z. cucurbitae* Coquillett is the most serious one (Nair & Pal, 2020). The internally feeding maggots being both frugivorous and florivorous, are the active feeding stage of fruit flies and are very strenuous to manage by insecticides (Dhillon et al., 2005). On the other hand, the demand for insecticide-free fresh vegetables is being encouraged by utilization of environmental-friendly methods of pest management (Khan et al., 2015). Cue-lure {4-(p-acetoxyphenyl)-2-butanone)} (hereafter CL) and methyl-eugenol (4-allyl-1,2-dimethoxybenzene) (hereafter ME) are widely used parapheromone attractants to manage fruit fly population via male annihilation technique (MAT) (Vargas et al., 2014).

The fruit flies associated with the cucurbit ecosystem were established by either MAT technique (Ganie et al., 2013; Wazir et al., 2019) or fruit fly emergence from infested cucurbits (Gaddanakeri & Rolania, 2020; Rajpoot et al., 2002), and sometimes from both the methods (Nair et al., 2017; Prabhakar et al., 2012). However, MAT technique has severe limitations, as it attracts all the fruit flies responsive to the lure even the fruit flies not infesting cucurbits, leads to ambiguous results (Lux et al., 2003). Hence, observing adult emergence from contaminated fruits is critical for establishing a strong fruit fly-host connection. Several species of fruit flies which infest cucurbits are responsive to either CL or ME but not to both the lures (Shelly, 2017). Combining ME and CL lures into a single trap will reduce the cost of fruit fly monitoring and control because preparation and installation of individual lures is laborious, highly expensive and needs utmost care. However, previous results are much contrasting, indicating both positive and negative reports. For example, combining ME and CL in same trap reduce the number trap catches of fruit flies responsive to either CL or ME, then that in individual lures (Shelly & Kurashima, 2016; Royer et al., 2018; Wee & Shelly, 2013). However, combination lures have been found to be equally attractive to CL responsive flies (LeBlanc et al., 2011; Wee & Shelly, 2013) and even trap catches were found to be more in combined lures than that in individual lures (Shelly et al., 2004).

Cytochrome oxidase subunit I (COI) of the mitochondria is an extensively used marker for identification and establishing phylogenetic relationships between different species of invertebrates (Hebert et al., 2004) because of maternal inheritance, high rate of evolution, high copy numbers, and having higher interspecific diversity (Twinkle et al., 2020). *Wolbachia* is a maternally inherited, intracellular endosymbiotic bacteria known to infect an estimated 65% of species of insects (Hilgenboecker et al., 2008). It alters the host biology in diverse ways such as feminization, parthenogenesis, male killing and egg-sperm incompatibility (Werren et al., 2008). Of the 87 Tephritid species studied, nearly 66% were found to be infected with *Wolbachia* (Conte et al., 2019; Devescovi et al., 2019). Ascertaining the *Wolbachia* infection in fruit flies will have implications in genetic pest control such as incompatible insect technique (IIT). IIT depends on the maternally transmitted *Wolbachia,* in which males released are infected with *Wolbachia* results in sterile mating with wild females (Zheng et al., 2019), which are infected with different strain of *Wolbachia* leads to cytoplasmic incompatibility (Mateos et al., 2020). However, their infection in fruit flies associated with cucurbits has both positive (Asimakis et al., 2019) and negative reports (Manger et al., 2018) from India.

In the Indian context, the effect of combination lures on responsive fruit fly catches has not been

evaluated. Hence, this study was conducted to determine whether combination of CL and ME in the same trap would increase attractancy to responsive fruit flies, side by side samples of collected fruit flies were barcoded and their infection with *Wolbachia* was also checked.

Material and methods

The present investigation was conducted in a farmer's field (0.75 ha), Ramna village, Varanasi, Uttar Pradesh, India (25.3176^0 N, 82.9739^0 E). CL {4-(p-acetoxyphenyl)-2-butanone} and ME (4-allyl-1,2-dimethoxybenzene) were purchased from Sisco Research Laboratories, Mumbai, India. The CL and ME lures were prepared by mixing them individually with ethyl alcohol and Malathion 50EC in a 6:4:2 ratio (Arya et al., 2022; Mariadoss, 2020). Wooden blocks (5 × 5 × 2 cm) were dipped in the mentioned lures for 24–36 h, followed by the hanging of the lure in plastic water bottles (1 L) having window holes (2 × 5 cm) on the top 1/3rd of the bottle (Fig. supplementary). The combination lure (ME/CL) were prepared by hanging wooden blocks soaked with ME and CL side by side in the same trap bottle. To test the efficiency of attractancy of fruit flies to CL, ME and ME/CL, five replications of individual lures (ME and CL) and combination (ME/CL) were installed at a 10 m distance in bitter gourd, *Momordica charantia* from the 35th standard meteorological week (SMW) to the 44th SMW in a randomized complete block design (RCBD). Therefore, the experiment consists of three treatments (CL, ME and ME/CL) with five replications for each. The experimental area was also surrounded by other cucurbits such as bottle gourd (*Lagenaria siceraria* Molina Standl.), pumpkin (*Cucurbita maxima* Duchesne.), sweet gourd (*Momordica cochinchinensis* Spreng.), ridge gourd (*Luffa acutangula* Mill.), cucumber (*Cucumis sativus* L.), pointed gourd (*Trichosanthes dioica* Roxb.) and snake gourd (*Trichosanthes cucumerina* L.). The traps were regularly monitored and replaced every 30 days, with fruit flies collected on a weekly basis. To study the population dynamics of each fruit fly species, the males collected were identified and their population was counted on a weekly basis. The weekly population of each species is an average of 10 traps (five samples from individual lures and another five from combination lures). To identify the fruit flies that are actually infesting cucurbits, 100 infested bitter gourd and cucumber fruits were collected from the field and kept in an insect chamber (1 × 0.25 × 0.25 m^3) with soil as base material for pupation and for checking the emergence of fruit flies.

DNA extraction, PCR and sequencing

The DNA was extracted from the hind legs of fruit flies by following (Srinivasa et al., 2020). The genomic DNA was kept at -20 °C until it was used in the polymerase chain reaction (PCR). The PCR reaction mixture of 25 µl consisted of 12.5 µl of PCR master mix (EmeraldAMP GT PCRMaster Mix, Takara), 9.5 µl of nuclease-free water, 2 µl of template DNA and 0.5 µl of each primer. LCO1490 (5′-GGTCAACAAATCATAAGAATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) primers were used to amplify the mitochondrial cytochrome oxidase I barcode region (Folmer et al., 1994). The thermal cyclic conditions are as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 47 °C for 40 s, and extension at 72 °C for 40 s, followed by final extension at 72 °C for 8 min. The amplified PCR products were resolved on a 1% agarose gel and then visualized under UV using Image Lab software Version 6.1 (Bio Rad). Furthermore, the samples were sequenced in both directions at Eurofins Genomics India Pvt. Ltd. (Bengaluru, India). The obtained sequences were checked for stop codons using expasy translate (Gasteiger et al., 2003), trimmed and aligned using Bio edit 7.2.5 (Hall, 1999). The consensus sequence of fruit flies was deposited at NCBI to get accession numbers.

Phylogenetic analysis

The consensus sequences of fruit flies were aligned using ClustalW and a phylogenetic maximum likelihood tree was constructed with 2000 bootstrap values and the Kimura-2-parameter method using MEGA 6 (Tamura et al., 2013) with *Nilaparvata lugens* as an out group. The pairwise genetic distance between fruit flies and the out group was calculated using the MEGA 6 programme and the Kimura-2-parameter method (Kimura, 1980).

Wolbachia infection in fruit flies

From all the identified fruit flies, *Wolbachia* infection was checked using the wsp81-F and wsp691-R primers (Braig et al., 1998) in 3 specimens for each species. For PCR composition and thermocyclic conditions, we followed Braig et al. (1998). The amplified PCR products were resolved on a 1% agarose gel and then visualized under UV using Image Lab software Version 6.1 (Bio Rad).

Statistical analysis

The effect of combining lures (ME and CL) in the same trap on the attraction of responsive fruit flies in CL, ME and ME/CL combination was analysed by comparing the mean of fruit flies/trap/week data of individual species by Paired t-test. Species diversity of fruit flies associated with cucurbit ecosystem was calculated using the formulae given by Shannon and Weiner (1949), based on function: $H = -\Sigma pi \log_{10} pi$, where $pi = Ni/N$, Ni = total number of individuals in a species and N = total number of individuals in all species (Table 1).

Results

Identification of fruit flies

The fruit flies were identified using the literature by David and Ramani (2011), Leblanc et al. (2019), Nair et al. (2018) and by DNA barcoding. Six species of fruit flies were observed in the cucurbit ecosystem and their identities were confirmed by DNA barcoding, namely: *B. dorsalis* (NCBI accession number: OK559996), *Z. cucurbitae* (OL701253), *B. zonata* (OK559997), *Z. tau* (OL701270), *B. correcta* (OK559995) and *B. digressa* (OK559998) (Table 2). Among them, *Z. cucurbitae* and *Z. tau* were attracted to CL, while *B. dorsalis, B. zonata and B. correcta* were attracted to ME, but *B. digressa* was attracted to both the lures (Table 1).

Trapping efficiency of ME/CL vs CL and ME

Mean fruit flies/trap/week of *B. dorsalis* (108.28 ± 30.65, $p = 0.0159$) and *Z. cucurbitae* (20.70 ± 5.40, $p = 0.03308$) were significantly higher in ME and CL than in the ME/CL (43.4 ± 10.72 and 13.91 ± 2.72) respectively. Whereas the populations of the other four fruit flies (*B. zonata, Z. tau, B. correcta* and *B. digressa*) were < 1% trap catches with insignificant trapping efficiency (Table 3 and 4).

Population dynamics of *B. dorsalis* and *Z. cucurbitae*

The population of *B. dorsalis* was higher during the 35th SMW (160.6 ± 40.40), peaked in the the 37th SMW (200.5 ± 46.43), and then declined during 44th SMW (4.2 ± 1.33), whereas *Z. cucurbitae* population was lower during the 35th SMW (1.8 ± 0.67), which gradually increased, and peaked in the 44th SMW (36.7 ± 10.65) (Fig. 1A). The population of other fruit flies was negligible and varied among the weeks (Fig. 1B).

Table 1 Fruit fly species associated with cucurbit ecosystem along with per cent total trap catches. ME-Methyl eugenol and CL- Cue-lure

Fruit fly species	Para pheromone lure	Percent of total trap catches
Bactrocera dorsalis	ME	80.50%
Bactrocera zonata	ME	0.54%
Bactrocera correcta	ME	0.97%
Zeugodacus cucurbitae	CL	17.44%
Zeugodacus tau	CL	0.13%
Bactrocera digressa	CL and ME	0.39%

Table 2 NCBI Accession no of fruit flies observed in cucurbit ecosystem and their infection status with Wolbachia. + indicates infection with *Wolbachia* and vice versa

Fruit fly species	NCBI Accession No	*Wolbachia* infection
Bactrocera dorsalis	OK559996	+
Bactrocera zonata	OK559997	-
Bactrocera correcta	OK559995	+
Zeugodacus cucurbitae	OL701253	-
Zeugodacus tau	OL701270	+
Bactrocera digressa	OK559998	+

Table 3 ME-responsive fruit flies response to ME and ME/CL combination ([a] = Mean flies/trap/week, * = significant at 5%

Species	ME[a] ± S.E	ME/CL[a] ± S.E	t-value[b]	*p*-value
B. dorsalis	108.28 ± 30.65	43.4 ± 10.72	2.96	0.0159*
B. correcta	1.6 ± 0.75	0.22 ± 0.10	2.02	0.0730
B. zonata	0.74 ± 0.49	0.3 ± 0.19	1.40	0.1944
B. digressa	0.48 ± 0.29	0.26 ± 0.10	0.86	0.4085

Table 4 CL-responsive fruit flies response to CL and ME/CL combination ([a] = Mean flies/trap/week, * = significant at 5%

Species	CL[a] ± S.E	ME/CL[a] ± S.E	t-value[b]	*p*-value
Z. cucurbitae	20.7 ± 5.40	13.91 ± 2.72	2.51	0.03308*
Z. tau	0.18 ± 0.04	0.08 ± 0.09	1.04	0.3221
B. digressa	0.02 ± 0.02	0.26 ± 0.10	2.09	0.0659

Species diversity and fruit flies emerged from infested cucurbits

The total fruit flies collected in the study were 9524, among which *B. dorsalis* (7667), *Z. cucurbitae* (1661), *B. correcta* (93), *B. zonata* (52), *B. digressa* (38) and *Z. tau* (13) accounted for 80.50%, 17.44%, 0.97%, 0.54%, 0.39% and 0.13% of the total fruit flies collected, respectively (Table 1). The

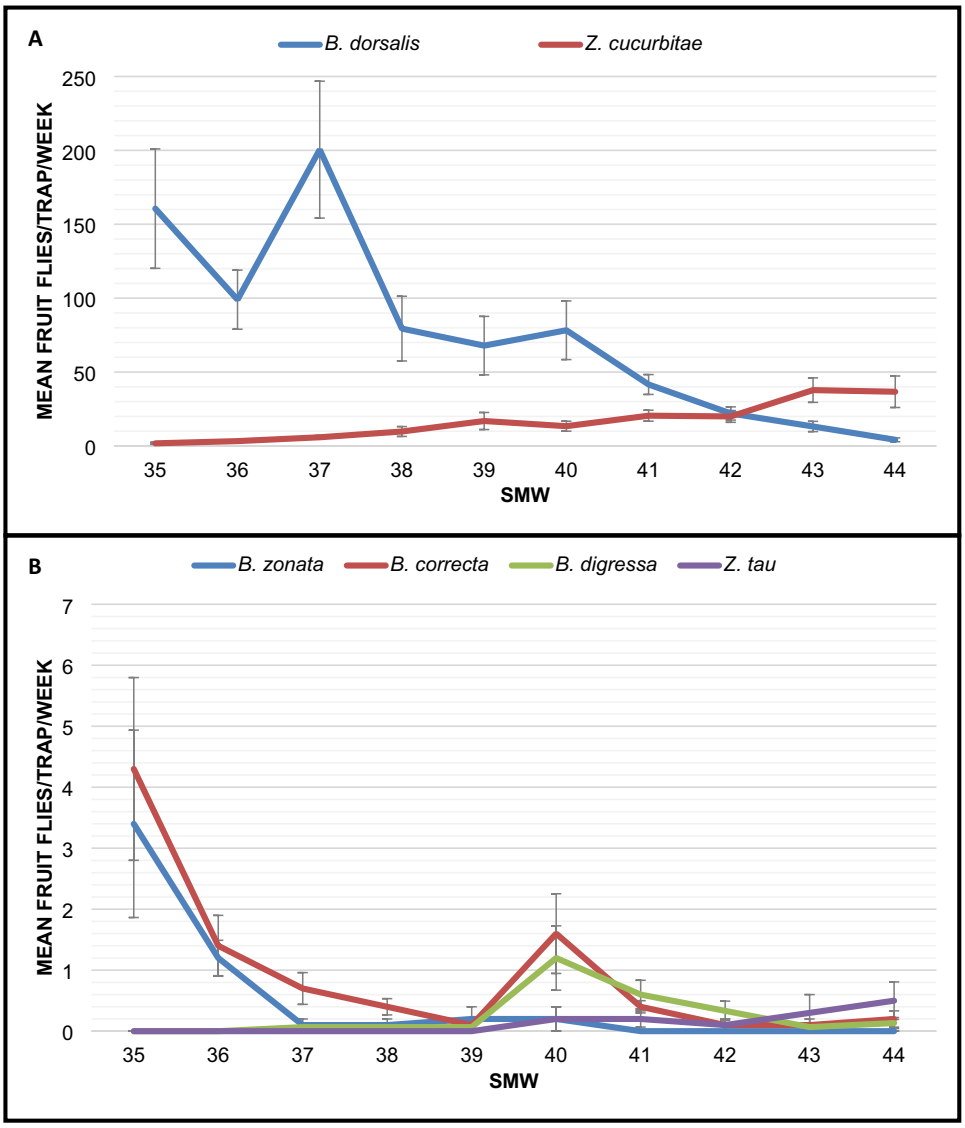

Fig. 1 Population dynamics of different species of fruit flies (mean fruit flies/trap/week ± S.E.) collected in bitter gourd (*Momordica charantia*) ecosystem during kharif season 2021. **(A)** *B. dorsalis* and *Z. cucurbitae*, **(B)** *B. zonata, B. correcta, B. digressa* and *Z. tau*

Shannon-Weiner diversity index (H) of fruit flies is 0.583. Only 67 individuals of *Z. cucurbitae* emerged with a sex ratio of 1:1.91 {Male:Female (M:F)} from 100 infested bitter gourds, whereas 195 *Z. cucurbitae* and 125 *Z. tau* emerged with the sex ratios of 1.14:1 (M:F) and 1.05:1 (M:F), respectively, from 100 infested cucumbers.

DNA barcoding, phylogenetic analysis and *Wolbachia* infection

The nucleotide basic local alignment search (BLASTn) analysis of COI sequences produced in the study revealed six species of fruit flies, namely: *B. dorsalis*, *Z. cucurbitae*, *Z. tau*, *B. zonata*, *B. digressa* and *B. correcta* from the cucurbit ecosystem. All the identified species showed 99–100% identity with reported sequences in the National Centre for Biotechnology Information (NCBI) GenBank. All the analysed sequences were submitted to NCBI and accession numbers were obtained (Table 2). *Bactrocera digressa* is a new range record from Uttar Pradesh, India. The pairwise genetic distance between six species of fruit flies, excluding out-groups, is in the range of 0.052–0.197 (5.2–19.7%). The minimum genetic distance (0.052) was observed between *Z. tau* and *Z. cucurbitae* as compared to other fruit fly species (Table 5). The evolutionary relationship of all the six species was established using the maximum-likelihood method of analysis using the Kimura-2-parameter method with 2000 bootstrap replications (Figs. 2 and 3). The maximum likelihood (ML) phylogenetic tree inferred clear clades for those fruit flies attracted to CL, which are major pests of cucurbits (*Z. cucurbitae* and *Z. tau*) with a 99% bootstrap value and ME, which infests fruits including mango and guava

Table 5 Pairwise genetic distance between different species of fruit flies in comparison with out-group *Nilaparvata lugens*

	B. correcta	*B. dorsalis*	*B. zonata*	*B. digressa*	*Z. tau*	*Z. cucurbitae*	*Nilaparvata lugens* (MN560658)
B. correcta	000	0.093	0.074	0.114	0.183	0.176	0.328
B. dorsalis			0.093	0.124	0.176	0.183	0.368
B. zonata				0.106	0.183	0.188	0.337
B. digressa					0.186	0.197	0.318
Z. tau						0.052	0.332
Z. cucurbitae							0.337
Nilaparvata lugens (MN560658)							

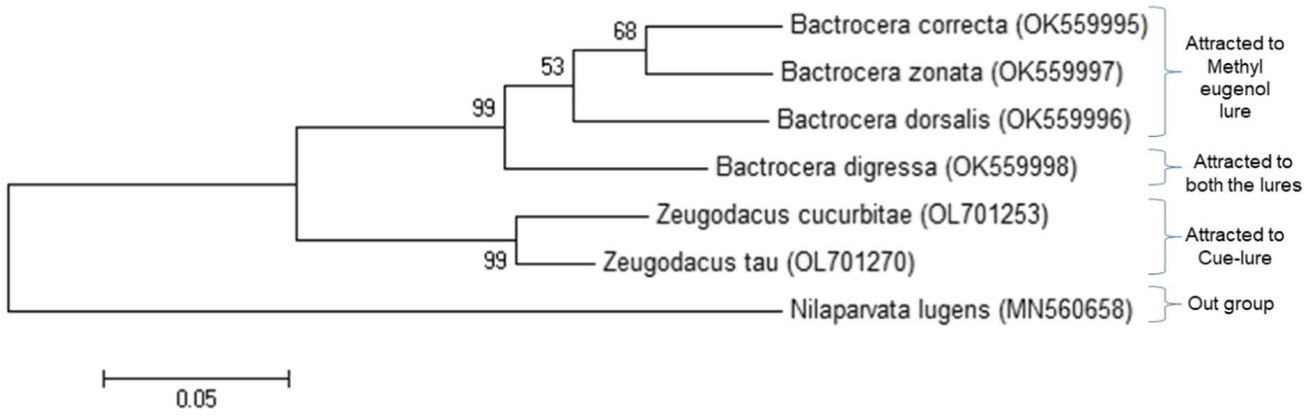

Fig. 2 Evolutionary relationship between Fruit flies inferred by Maximum likelihood tree. Numbers on the nodes indicates bootstrap value and 0.05 tree length indicate 5 nucleotide substitutions per 100. The tree with the highest log likelihood (-2214.5202) is shown and evolutionary analysis conducted in MEGA 6

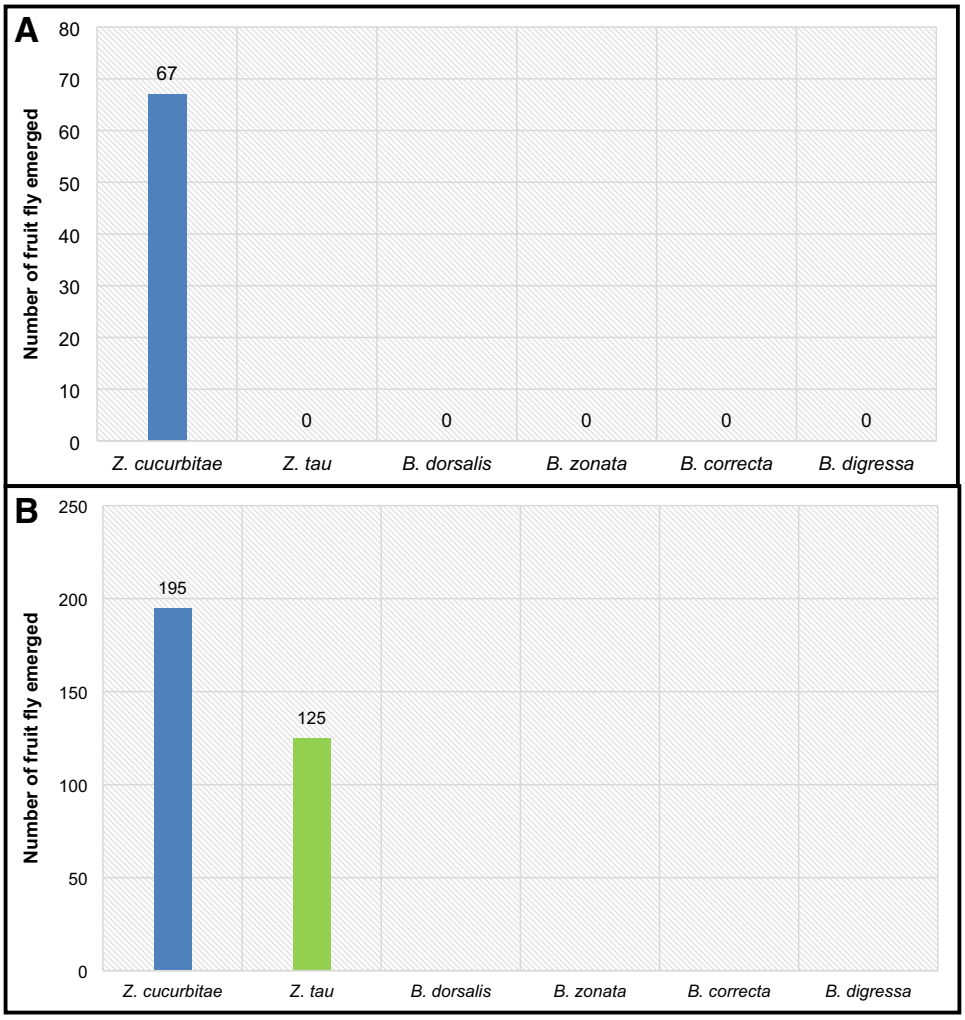

Fig. 3 Fruit flies emerged from infested cucurbits. **(A)** bitter gourd (*Momordica charantia*) **(B)** cucumber (*Cucumis sativus*)

(*B. dorsalis, B. zonata* and *B. correcta*), again with a 99% bootstrap value. However, one exception is *B. digressa* which is attracted to both the lures (ME and CL). This is also the first report of the attraction of *B. digressa* to ME. *Wolbachia* infection is confirmed in four species of fruit flies out of six tested, namely: *B. dorsalis, Z. tau, B. digressa* and *B. correcta* (Table 2 and Fig. 4).

Discussion

There are six species of fruit flies namely: *B. dorsalis, Z. cucurbitae*, *B. zonata, Z. tau, B. correcta* and *B. digressa,* found in cucurbit ecosystem which were identified by taxonomic literature (David & Ramani, 2011; Leblanc et al., 2019; Nair et al., 2018) and by DNA barcoding. With the help of available literature, the identification of different species of fruit flies has been difficult (Schutze et al., 2015), because of cryptic species and varied colour morphology within the species (Drew & Hancock, 1994; Schutze et al., 2015). Fruit fly infestation is a serious problem for agriculture. To address it, many countries are devoting significant resources and capital to fruit fly trapping programmes that employ separate traps for CL and ME. Combining different lures in a single trap not only sounds economic but might also increase the efficacy of fruit fly trap catches (Royer & Mayer, 2018). But contrary to the assumptions, the study showed the trap catches of fruit flies in combination lures (ME/CL) were less as compared with the population captured under individual traps of ME and CL. The populations of *B. dorsalis* (108.28 ± 30.65, $p = 0.0159$) and *Z. cucurbitae* (20.7 ± 5.40, $p = 0.03308$) collected were significantly higher in individual lure traps than in ME/CL (43.4 ± 10.72 and 13.91 ± 2.72), respectively. While the other four species have shown no

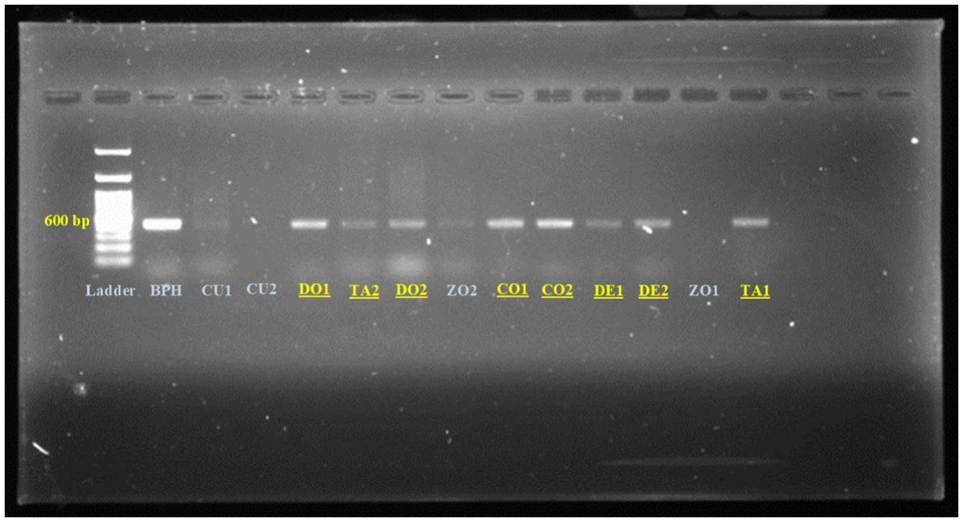

Fig. 4 *Wolbachia* infection in different species of fruit flies (BPH: Brown Plant hopper as a standard reference, DO-*B. dorsalis*, CO-*B. correcta*, DE-*B. digressa*, Zo-*B. zonata*, TA-*Z. tau*, CU-*Z. cucurbitae*

significant difference, mainly because of the very low population per trap catch in the cucurbit ecosystem (Table 3 and 4). Thus, the study implies that usage of individual lures is better than combination to trap the fruit flies responsive to particular lures.

Our results are in agreement with previous research findings that ME alone attracts more *B. dorsalis* than the ME/CL combination (Shelly et al., 2004; Tan, 1983; Vargas et al., 2000) vis-à-vis CL to *Z. cucurbitae* than the combination of ME/CL (Royer et al., 2018; Wee & Shelly, 2013). However, for monitoring of responsive fruit flies combined lures are effective as it reduces the cost of lure preparation and application. Any reduction in sensitivity of lures due to combining multiple lures can be overcome by increasing density of traps (Stringer et al., 2019). Due to the dominance of a single species, *B. dorsalis* (80.50%) and less population collection of other species in the trap catches, the fruit fly population in the cucurbit ecosystem was found to be less diverse (H = 0.583), proving the presence of a limited number of fruit fly species associated with the cucurbit ecosystem in the study location. But the more we know about the abundance of insects, the greater is the possibility of the presence of other species too. Further research on species redistribution in the region is required. Though, six species of fruit flies observed in cucurbit ecosystem only *Z. cucurbitae* (67 adults) emerged from 100 infested bitter gourd, *Momordica charantia* while from 100 infested cucumber, *Cucumis sativus, Z. cucurbitae* (195) and *Z. tau* (125) emerged (Fig. 3). The result proves the claim by Mwatawala et al. (2009), Singh (2003) and Kambura (2016) that *Z. cucurbitae* is the most dominant infester of cucurbits, followed by *Z. tau* (Baimai et al., 2000; Jaleel et al., 2018; Vasudha et al., 2019) than other infesting species as per adult emergence analysis. Although *B. dorsalis* is a dominant species in the study, it may be a wild population infesting other fruit crops in the locality, as it being a polyphagous pest having a wider host range and mainly prefers fruits (Koswanudin et al., 2018; Rattanapun et al., 2009; Ren et al., 2008). Similarly, *B. zonata* and *B. correcta* have been reported to infest cucurbits (Kunprom et al., 2015; Sohail et al., 2015), but their main hosts are fruits, not cucurbits (Liu et al., 2013; Ma et al., 2012; Sarwar et al., 2013; Zingore et al., 2020). *B. digressa* is primarily reported on *Alangium salviifolium* but its incidence on cucurbits is unknown. Apart from *Z. cucurbitae* and *Z. tau*, fruit flies such as *Z. scutellaris* (Prabhakar et al., 2007), *Z. diversus* (Royer et al., 2018) *Z. caudatus Fabricius* (Nair et al., 2021), *Z. cilifera* (Nair et al., 2017), *Dacus longicornis* (Nair et al., 2017), *D. ciliates* (Vayssières et al., 2008), *Pliomelaena udhampurensis* (Prabhakar et al., 2012) and *Dioxyna sororcula* (Prabhakar et al., 2012) infest cucurbits but they are currently minor pests (Nair et al., 2017) and restricted to small pockets (Prabhakar et al., 2012) of Indian subcontinent.

Population dynamics studies are an effective pest management strategy (Vargas et al., 2012). A proper management approach can be implemented with the knowledge of seasonal incidence in the population. The population of *B. dorsalis* was higher during the beginning of the study (35-37th SMW) and later declined, following the 44th SMW. This must be due

to fruit flies emerging from ripened fruits of mango after harvesting and their population declining due to non-availability of host crops (Ndlela et al., 2016; Singh et al., 2013) (Fig. 1A). A similar but opposite trend was observed in the increasing population of *Z. cucurbitae* when the growing population coincided with the ripening of bitter gourd (*Momordica charantia* L.) and other cucurbits, which was found in accordance with Raghuvanshi et al. (2012) and Patel and Das (2021), where the peak of *Z. cucurbitae* population was observed in the 43rd SMW (Fig. 1A). The population of other fruit flies was negligible and varied among the weeks (Fig. 1B).

COI has become a universal barcode for identification of insects (Lin et al. 2015; Twinkle et al., 2020) due to less intraspecific divergence and higher interspecific diversity helps to clearly delineate the species (Pentinsaari et al., 2014). Nuclear encoding of mitochondrial genes (NUMTs) and poor amplification from non-DNA friendly preservation leads to overestimation of species. On the other hand, COI genes have been shown to effectively identify morphologically distinct species of fruit flies (Manger et al., 2018; Karimi & Darsouei, 2014), but not cryptic fruit fly species (Kunprom & Pramual, 2019). Six fruit fly species were found in our study and were identified by BLASTn analysis of COI sequences. The DNA barcodes generated in our study clearly indicated six species with higher intraspecific divergence (Table 5). The lowest pairwise genetic distance was found between *Z. tau* and *Z. cucurbitae*. Our results are in agreement with Manger et al. (2018) and Jamnongluk et al. (2003). Previously, *B. digressa* was reported from southern {Karnataka (David & Ramani, 2011), Tamil Nadu (Radhakrishnan, 1999) and Telangana (Latha & Sathyanarayana, 2016)} and eastern {Sikkim (Nair et al., 2018)} Indian states. It is the first report from north India (Uttar Pradesh) and its economic importance and pest status still unknown (Hancock, 2015). Earlier reports indicate it is attracted to CL (David & Ramani, 2011), but in our collection, it was also found in ME in an equal proportion to CL, but the population in both the lures were negligible (Table 3). The ML tree constructed using *Nilaparvata lugens* as an outgroup clearly formed two clades, i.e., one containing CL responsive and major cucurbit fruit flies *(Z. cucurbiate* and *Z. tau*) and another of ME responsive and fruit infesting fruit flies (*B. dorsalis, B. zonata* and *B. correcta*). However, *B. digressa* was found to be responsive to both ME and CL, separated between the clade of ME and CL but placed at the root of ME responsive fruit flies. Our findings are consistent with those of Manger et al. (2018), who described the fruit flies of north-eastern India, and Asokan et al. (2011), who used COI barcodes to infer the phylogeny of fruit flies. As expected, the average nucleotide composition of fruit flies is AT (28.38: 35.06) biased i.e., 63.48%, which is a common feature of COI genes of arthropods (Srinivasa et al., 2020). *Wolbachia* is a maternally inheritable, obligatory intracellular bacteria known to be one of the most ubiquitous parasites amongst Tephritids (Jamnongluk et al., 2002). In this study, we examined six fruit fly species for infection with *Wolbachia.* Four out of the six species were found to be infected with the endosymbiont (Table 2 and Fig. 4), which were: *B. dorsalis, Z. tau, B. correcta* and *B. digressa* and the results are in agreement with Sun et al. (2014), Liu et al. (2006) and Kunprom et al. (2015). However, *Wolbachia* infection has not yet been reported earlier in *B. digressa* proving it to be an important finding for future studies. The sterile mating occurs when the mating between *Wolbachia* infected male with wild female having infection with different strain of *Wolbachia* or non-infection (Cytoplasmic incompatibility) and its exploitation in pest control called Incompatible Insect technique (Mateos et al., 2020). The IIT was first applied for the successful control of mosquitoes (Ross et al., 2019). The knowledge of *Wolbachia* infection in fruit flies obtained in this study will help in designing IIT pest management strategy in fruit flies.

Conclusion

Six species of fruit flies were found to be associated with the cucurbit ecosystem, but based on fruit fly emergence studies from infected fruits, it was proved that *Z. cucurbitae* and *Z. tau* are the major species infesting cucurbits in the study location. Combining ME and CL in a single trap will impact negatively on the trap catches of responsive fruit flies and may eventually lead to failed control programmes. Hence, we recommend the use of single lures for mass trapping of responsive fruit flies but for monitoring purpose combined lures preferred. Fruit flies infesting cucurbits are responsive to CL, therefore same must be installed in cucurbits from flowering till harvesting to manage them sustainably.

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Author contributions VA conducted experiments, SN and SVSR designed experiment, data analysis, CPS, ST, TS and PD did statistical analysis and helped in designing of experiment. All the authors contributed in writing manuscript. All authors have read and approved the manuscript.

Declarations

Conflict of interest Authors declare no conflict of interest.

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