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Chapter - 1

DNA Barcoding as a Tool to Confirm Invasive Banana Skipper *Erionota Torus* (Evans) From Malabar Region of Kerala

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Abstract

It is now broadly accepted that invasive species are a major cause of global biodiversity loss. Invasion of banana leaf roller *Erionota spp*. has been noticed in banana cultivating areas of Malabar regions of Kerala which forms a part of Western Ghat, India. Many researchers proposed that banana leaf roller *E.thrax* and *E. torus* occur together in Kerala and still others consider them as single species. Currently there is no published information regarding whether, one or both the species of *Erionota*, the invasive pest of banana coexist during the seasonal outbreak in several areas in Malabar region of Kerala. Moreover unless the taxonomic identity of the pest is correctly determined the correct implementation of the biological control programme will be difficult. Also the associated parasitoids their host range and host preference including whether the parasitoids attack both the species of the host *Erionota* with equal preference or showed higher preference for one or the other species is also important.The Knowledge of the host choice by the parasitoids is also most crucial information for the successful implementation of the effective biological control programmes against this pest. Moreover the early larval stages of *E. thrax* and *E. torus* that attack banana leaves cannot be distinguished easily from each other because of their morphological similarity. Thus, the taxonomical identification of species becomes all the more difficult. This chapter is aimed to identify and confirm this invasive *Erionota* species from Malabar region of Kerala with molecular techniques of DNA barcoding.In this technique 650 base pair long DNA of mitochondrial cytochrome oxidase I gene was amplified and sequenced from specimens collected from selected localities of Malabar region of Kerala,India. The partial DNA sequence of mitochondrial

cytochrome oxidase I gene of the banana skipper proved the existence of *E.torus* species in the pest infested areas.The work also proved that all the parasitoid species collected during the course of study from Malabar region of Kerala where that of *E.torus*.The chapter also discuss the invasiveness and seasonal fluctuations of the pest. The larval instars damage banana leaves by rolling and feeding on the leaves of almost all cultivars of banana plantain causing severe damage. This lowers the banana yields by delaying the fruit maturity and reducing the bunch size.The damage to the leaves also prevent the traditional use of leaves. The pest attack was more during monsoon and post monsoon seasons, ranging from 10 to 40% causing 50% loss of plant leaf area. Pre flowered plant showed a higher infestation accounting to 37%, and damaged leaves in pre-flowered, flowered and bunched plants corresponds to 5± 1, 3.8± 0.4 and 2.5± 0, respectively (n=100).

Keywords: Invasion, *Erionota torus, Erionota thrax,* DNA barcoding, COI, *Musa* spp.

1. Introduction

Biodiversity is the variety and variability of life on earth at all its levels, from genes to ecosystems, and the ecological and evolutionary processes that sustain it [1]. The major causes of decline in biodiversity include habitat loss and its degradation, pollution of the environment, overexploitation of the resources, and due to invasive species. Invasive species are generally documented as a major cause of global biodiversity loss, and as a result, public interest in the subject has risen in recent decades [2]. Biological invasions are becoming more common as a result of increased transportation and foreign trade, with potentially negative implications for ecosystem services [3]. Invasive species are those that have established and spread in a new geographic range [4]. Invasion of *Erionota s*p. in different banana cultivating plots of Kerala had been reported [5].

The banana is one of the most important fruit crops in the world, and it is widely grown in tropical countries [6]. It is a favorite fruit of all classes of people and has good export potential due to its year round availability, affordability, and varietal variety, and taste, nutritive and medicinal value. Almost all parts of the banana plant are valuable with nutritive, commercial, religious and ecological values. Banana is the cheapest sources of nutrients for a major part population and is a richest source of carbohydrates, minerals and vitamins. In traditional medicine, the banana plant sap is used to treat a wide variety of disorders, including leprosy, hysteria, fever, digestive disorders, hemorrhage, epilepsy, hemorrhoids, and insect bites [7]. The banana leaves are is a regular income sources of many people in South India.

It is widely used as a substitute for dinner plates and wrapping materials for specialized food product. In India, many of the social and religious ceremonies require whole banana tree, apart from leaves and fruits. Musa spp. are widely distributed in the tropical region, from 175^0 E to 150^0 W longitude and from 30^0 N to 23^0 S latitude. It is widely distributed in India, Sri Lanka, and Bangladesh, south and southeast China, Myanmar, Laos, Vietnam, Cambodia, Thailand, Malaysia, Indonesia, Philippines, New Guinea and in the hundreds of islands of south and Southeast Asia and west tropical Pacific Ocean [9]. India is the largest producer of banana in the world and also in Asia, and contributes 22.15 percent to global production from 7.4 % area (2009) followed by China and Philippines [10]. In India the major banana cultivating states are Andhra Pradesh, Assam, Bihar, Gujarat, Karnataka, Kerala, Tamil Nadu, Madhya Pradesh, Maharashtra, Orissa and West Bengal [11]. According to NHB, Kerala is one of the major banana producing states in India and production in Kerala was 375.9 thousand MT in 2002-03, the total production in India standing at 13304.4 thousand MT. Nendran variety has 50% share of total banana export from Kerala. Among the 470 insects and mites recorded globally on banana as major and minor pests, 250 feed on the foliage [12]. During September -October 2013, epidemic buildup of leaf roller pest *Erionota* was reported from banana plantations at several places in Kerala v*iz.*, Peechi, Palghat and Nilambur [13]. A survey conducted in southern districts of Kerala exposed thepresence of the banana leaf roller *Erionota* in all most all important banana varieties.The infestation percentage was up to 40 and severe defoliation with mean leaves damaged per plant to 2.88.% [14]. *E. thrax and E. torus*are the chief defoliators of *banana* where they overlap in distribution in the mainland of South-East Asia and in the Southern Philippines and have not been distinguished in the economic literature [15]. Both *Erionotathrax* and *Erionota torus* were reported in different parts of Kerala [16]. *E.thrax* and *E.torus* have similar morphology often making their distinction by classical identification using morphological criteria difficult [17]. Since there is no reliable way to separate the early stages of *E. thrax*, *E. torus* and *E. acroleuca*morphologically,a careful study and documentation of reared material, supported by reared vouchers and barcoding of individuals, is needed [18] .DNA barcode based identification has been proposed as a molecular method for assigning individual specimens to known species [19]. DNA barcode involves DNA sequence analysis of a portion of themitochondrial gene cytochrome *c* oxidase subunit I (COI) of about 600-700 bp. The basic concept is that barcode variation between even closely related species will be substantially greater than that observed within species. The objective of this chapter is to assess the applicability of DNA

barcoding to identify *Erionota* spp. which cause serious damage to banana in Malabar region of Kerala, India.It also aims to explain the life cycle, invasiveness and seasonal fluctuation of this species.

2. DNA barcoding to confirm the presence of *Erionota torus* (Evans) in Malabar region of Kerala

Identification and taxonomic conformation of species based on morphological characters is often a difficult process that necessitates the use of highly experienced taxonomists. The morphology-based procedures take a long time and don't always result in species-level resolution [20]. Furthermore, even under the watchful eye of an expert taxonomist, phenotypic plasticity of species can lead to misidentifications [21]. Even for an experienced taxonomist, the sibling species often cannot be discriminated easily, which often cause hurdles in the implementation of successful biological control programmes.

1.1 Principle of DNA barcoding

DNA barcoding or gene barcoding involves the technique of amplifying a short segment of the mitochondrial cytochrome oxidase I (COI) gene with PCR, and further sequencing, and comparing it to a database of a known species available in the Gen Bank. The mitochondrial cytochrome c oxidase I (COI) gene sequence is very peculiar and unique for each species. The corner stone of DNA barcoding resides in this short approximately 658 base pair longcytochrome c oxidase I (COI) gene. The collected PCR amplified sequences are then compared to a similar reference species sequence available in the Gen Bank, to identify and classify them and for further to establish their phylogenetic lineage.

1.2 Steps in DNA barcoding

1.2.1 Collection of specimens

The larvae of butterflies of *Erionota* spp were collected from severely infested banana plantations at Payyanur located latitude 12.0972° N, longitude 75.1934° E and altitude of 19 MSL in Kannur district of Kerala. The emerged adults of the collected larvae were then preserved in the laboratory at -20^0 C for genomic DNA extraction. All the details of samples like sample ID, date of collection, location of collection were recorded. Due care was taken to prevent any damage to the wings and other taxonomic features.

1.2.2 The primer used for the study:

The various guidelines and methodology described by Sambrook and

Russell [22] and Green and Sambrook [23] were followed for the preparation of various reagents and buffers and also for the isolation, purification and extraction of mitochondrial DNA of *E torus* for DNA barcoding.The primer used for the study was that proposed by Folmer et al [24], a universal primer,which was initially developed for marine metazoans. This primer was successfully used for barcoding of several insect species including Lepidoptera and hence in the present study the same primer was used.The forward primer was named LCO1490 and reverse primer was named HCO2198. In the present DNA amplification LCO1490 was used with HCO2198 with the following nucleotide sequences LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG- 3' and HCO2198: 5' TAAACTTCAGGGTGACCAAAAAATCA- 3'

1.2.3 DNA preparation of *Erionota torus*(Evans):

The genomic DNA of *Erionota torus* was isolated from one of the leg muscles, using Macherey-Nagel NucleospinTissue' kit(Machery-Nagel, Duren, Germany) following the Manufacturer's protocol.

1.2.4 PCR amplification of the COI region:

PCR amplification was performed in the PCR reaction mixture consisted of 2 nanograms of genomic DNA in 1 µl, 1 µl each forward and reverse primers at a concentration of 10 µM, 2.5 µl of dNTPs (2 mM), 2.5 µl 10X reaction buffer, 0.20 µlTaq polymerase (5 U/µl) and 16.8 µl H_2O. The thermo cycling program consisted of 95 ^{0}C/3 minutes for initial denaturation followed by 45 cycles of 95 ^{0}C/10 seconds, 50 ^{0}C/45 seconds, 72 ^{0}C/45 seconds and with a final extension of 72^{0}C for 3 minutes. The PCR amplified product was column purified using Mo Bio Ultra Clean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California) as per the Manufacturer's instructions.

1.2.5 DNA sequencing and analysis:

The purified product was sequenced with forward and reverse primers using the Sanger's sequencing method at Sci Genome Labs, Cochin. The forward and reverse sequence was aligned and the consensus sequence was used for analysis. The phylogeny analysis was done using the NCBI nucleotide BLAST software (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) [25].

1.3 Phylogeny analysis of *Erionota torus*:

Phylogeny analysis of *Erionota torus* when carried out by the partial sequencing of the mitochondrial COI yielded a 630 bp product by PCR

amplification. GenBank deposition indicated the novel and first time records of *Erionota torus* in Malabar region of Kerala and was provided with accession numberKT783538.1. The phylogenetic tree was constructed by Fast Minimum Evolution method (Fig.1) and by neighbor joining method (Fig.2). Genbank analysis showed the sequence has 100% similarity with *E. torus* (GenBank accession number: KP299167.1), 98.10% similarity with GenBank accession number: KY019746.1 which is deposited as *Erionota thrax* and 95.73 % similarty with GenBank accession number: KY019745.1 which is deposited as *Erionota spp*. by Sahoo *et.al*. [26], are its nearest neighbors (Table.1). The phylogenetic tree constructed by Neighbor joining method using the partial nucleotide sequence of COI gene also indicates that both *Erionota thrax* and *Erionota torus* are monophyletic species. The early stages of *E. thrax and E. torus* are similar and they are common pests of banana. Classical taxonomic method using morphological features of the early stages of two species is difficult. Since there is no published information regarding whether, one or both the species of *Erionota* coexist during the occasional outbreak of banana skipper in Malabar region of Kerala. In order to introduce successful management programmes against this invasive pest, precise species-level knowledge would be extremely useful.

Table 1: Table showing query cover, percentage identification of related species with accession number

Scientific Name	Max Score	Total Score	Query Cover	Per. ident	Acc. Len	Accession No
Erionota torus	1216	1216	100%	100	658	KP299167.1
Erionota thrax	1118	1118	96%	98.1	1456	KY019746.1
Erionota sp.	1027	1027	96%	95.73	1449	KY019745.1
Conga iheringii	872	872	100%	90.58	658	MF547231.1
Nyctelius sp. nyctelius	872	872	100%	90.58	658	JF760932.1
Saliana triangularis	850	850	100%	89.98	658	KP849323.1
Megathymus streckeri	850	850	100%	89.97	658	HM914977.1
Wallengrenia otho sapuca	845	845	100%	89.82	658	MF546161.1
Hylephila phyleus	845	845	100%	89.82	658	MF545890.1
Amblyscirtes reversa	845	845	100%	89.82	658	JF855101.1
Tromba xanthura	845	845	100%	89.82	658	GU156369.1
Thracides phidon	845	845	100%	89.83	658	FJ769066.1
Agrotis gladiaria	839	839	100%	89.7	658	MF921113.1
Orphe gerasa	839	839	100%	89.67	658	KU239107.1
Limenitis sydyi	839	839	100%	89.68	1099	JX185862.1
Dardarina dardaris	839	839	100%	89.67	658	GU161460.1

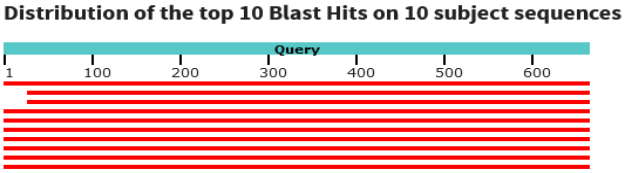

Fig 1: Distribution of the top 10 last hits on 10 subject sequences.

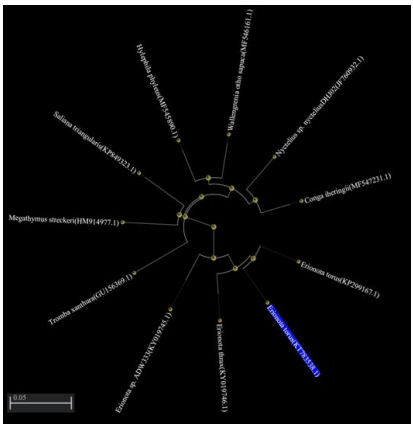

Fig 2: Evolutionary relationships of *Eionota torus*. The phylogenetic tree was constructed by fast minimum evolution method using the partial nucleotide sequence of CO I gene

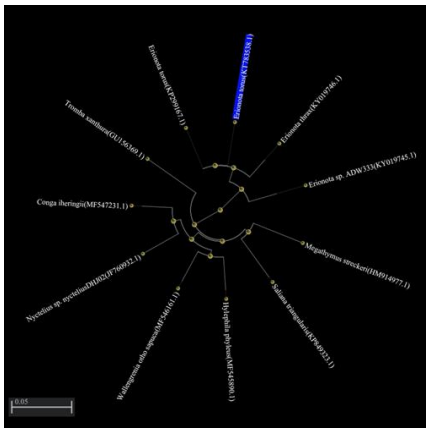

Fig 3: Evolutionary relationships of *Erionota torus*. The phylogenetic tree was constructed by neighbor joining method using the partial nucleotide sequence of COI gene

2. Invasiveness and seasonal fluctuation of *Erionota torus*

Invasion of banana leaf roller *Erionota torus* on cultivars was

observed with roving survey technique in the selected area. Adult butterflies lay small, eggs on the under sides of banana leaves and different larval instars (Fig. 3) damage banana plants by feeding on and rolling up the leaves [27]. From each plantation plot, ten plants at random were selected. A plant with a minimum of one leaf roll in a leaf was taken as infested. Total number of infested leaves per plants, number of leaf rolls per each leaf, length of fifth instar larval and pupal leaf nest and length of leaf blade used to construct leaf roll were observed. Leaf shelters selected at random were opened up and examined for the presence of larvae and pupae. Monthly temperature and humidity were also recorded. The life stages of the banana were categorized with appropriate modification as bunched plants (BP), flowering plants (FP) and pre flowered plants (PF) [28]. BP was considered as plants with new bunches up to when the bunches were harvested; FP was plants with newly emerged flowers up to when the female phase is over; PF were plants without flowers, 4-6 months old. Infestation % was calculated from hundred randomly selected plants from each plot.

Monsoon and post monsoon season is the favorable time for infestation and the population density attains its peak during late monsoon. During premonsoon season (summer) the infestation is scanty indicating a negative correlation of pest population with temperature and humidity (Fig.4). Preflowered plant showed high degree of infestation (36± 1%), in flowered plant it becomes 8± 1%, and that of bunched plant 3± 1% (Fig. 5); likewise, damaged leaves/ preflowered plant is 5± 1, 3.8± 0.6 leaves in flowered plants, and 2.5± 0.5 leaves in bunched plants (Fig. 6). A rigorously affected leaf had a grazed appearance, leaving only midrib (Fig. 6a-d).Many parasitoids and predators including, spiders, ants, and slugs and birds were noted in the field signifying the possibility of biotic control mechanism behind the pest population fluctuation. Egg parasitoid *Ooencyrtus* sp. (Hymenoptera: Encyrtidae), larval parasitoides like *Elasmusbrevicornis* Gahan (Hymenoptera: Eulophidae) and *Cotesiaerionotae* (Wilkinson) (Hymenoptera: Braconidae) and pupal parasitoids belonging to Tachnidae and Phoridae were observed. Of these, *E. brevicornis* is common in the study area. The presence of important fauna of parasitoids of the banana-skipper is an important asset in the perspective of the biological control. It would be necessary to continue this survey by studying to identify and describe the taxonomy of the unknown or undescribed species of parasitoids. This related work will also to provide additional data on the biodiversity of these important insects for the integrated pest management program.

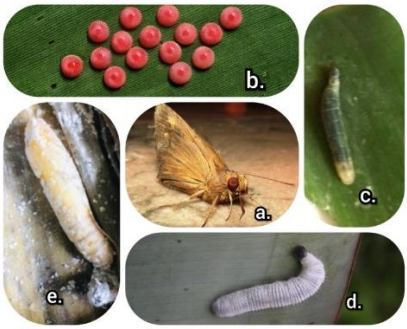

Fig 4: (a) Adult, (b) Eggs, (c) & (d) larval instars, (e) Pupa

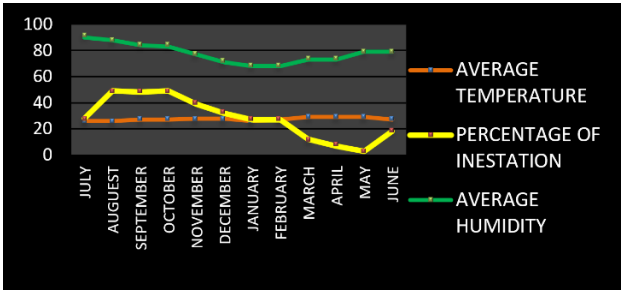

Fig. 5: Incidence of *E. torus* (2015-16)

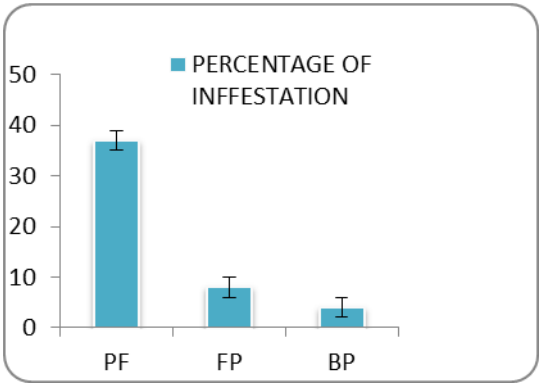

Fig 6: Incidence of *E. torus*. PF-Pre flowered plants, FP-Flowering plants,BP-Bunched plants.

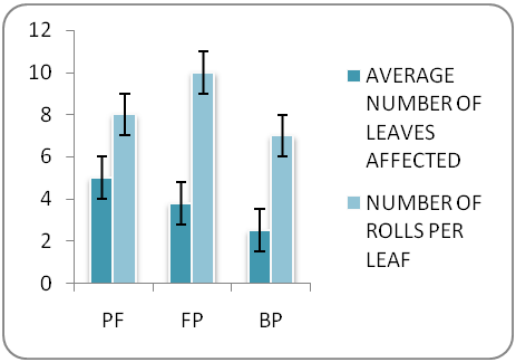

Fig 7: Leaves affected and fifth instar larval leaf roll of *E. torus* in PF-Pre flowered plants,FP-Flowering plants, BP- Bunched plants.

Fig 8: Infestation of Banana skipper *Erionota torus*

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Chapter - 2

Biochemical Basis of Insect Resistance in Plants: A Review

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Abstract

There exists a continuous battle between insects and plants for their existence and survival. During the course of evolution plants have developed several metabolites and also developed physical mechanisms to deter insects and thereby preventing phytophagy. Insects on the contrary developed and modified their biochemical pathways to detoxify the plant produced chemicals and secondary metabolites to resist the plant defence mechanisms**.**

Plant resistance is categorised into three main groups namely antibiosis, tolerance and non-preference [5]. Generally plants develop structural characteristics or produce secondary metabolites as a means of direct resistance against herbivores [9]. Insect attack followed by damage of tissues induces plant to produce volatile compounds, which attract predators and parasitoids of the insect and in turn provide indirect resistance by the attraction of natural enemies of the pest and as a result this will reduce the degree of damage [9-11]. Susceptible varieties of okra like *Pusa sawani* and AC 320 showed a higher content of reducing sugars and total sugars in comparison with resistant varieties [18]. Compounds like phenols, alkaloids, flavoniods, tannins, terpenes, aldehydes, ketones, and esters, nutrients like carbohydrates, reducing sugars, proteins, amino acids and lipids have a major role in offering a phytochemical response and natural defense mechanism against herbivore attack. This usually involves the activation of some enzymes leading to the increased formation of secondary metabolites and polymers at the site of insect attack.

Latex of Papaya tree (*Carica papaya*) contains a cysteine protease enzyme called papain. Papain is responsible for the growth inhibition of two

important pests, *Mamestra brassicae* and *Spodoptera litura* and lepidopteran larvae such as *Samia ricini*. Phenols and antioxidant enzymes like SOD, POX, CAT, PPO, AO and GR played a very important role in the defense against the infestation of leaf hopper in hybrid varieties of mango [46].

Keywords: Plant resistance, phytophagy, antibiosis, tolerance, papain, phenols, alkaloids, flavoniods, tannins, terpenes, reducing sugars, super oxide dismutase, phenylalanine ammonia lyase, peroxidase)

1. Resistance against insect pests

Broadly speaking, no plant is susceptible to the attack of all types of insects or in other words no insect is a pest of all plant species. A host plant is a species which is usually fed upon by an insect while non-hosts are those which are not fed. Resistance of a plant is the characters that equip a plant to tolerate, avoid or recover from insect attack [1]. It is the inherent plant property that help the plant to prevent the growth of insect species [2]. A complex defense mechanism consisting of chemical toxic agents, structural barriers or natural enemy attraction of the specific pest are usually involved in plant's response to pest attack [3]. Plant's response to insect attack usually produces toxic, repellent or anti-nutritional secondary metabolites and proteins or development of specialized morphological structures [4].

1.1 Types of Plant Resistance

Plant resistance was categorised into three main groups namely antibiosis, tolerance and non-preference [5]. However the term antixenosis was proposed instead of non-preference, in which the insect do not prefer the plant as its suitable host due to some morphological characters or the presence of some chemical compounds that act as a repellants [2]. Antibiosis was described as a type of plant resistance, in which the physiological characters of insects such as development, survival and fecundity are affected. Reduction in fecundity and fertility, life cycle disturbance and larval mortality are the main effects due to antibiosis [6-7]. In plant tolerance the attacked plant possess the ability to recover or withstand the pest attack and the insect is not adversely affected [7-8].

Generally plants develop structural characteristics or produce secondary metabolites as a means of direct resistance against herbivores [9]. Indirect plant defense mechanism also occur in which plant produce certain volatile compounds which trigger plant's interaction with the natural enemies of the pest which in turn decreases the number of pests [10]. *Meteoridea hutsoni* (Nixon) a primary parasitoid of *Opisina* get attracted and showed host searching and oviposition and behavior towards fresh uninfested cut coconut

leaves suggesting the possibility of certain specific substances in the host plant leaf which attracts the parasitoid [11]. This behaviour of the parasitoids protects the plants from severe pest attack. Insect attack followed by damage of tissues induces plant to produce volatile compounds, which attract predators and parasitoids of the insect and in turn provide indirect resistance by the attraction of natural enemies of the pest and as a result this will reduce the degree of damage [9-12]. Oviposition by the female insect also induces the release of volatile compounds [13].

1.2 The Role of Metabolites and Anti-Oxidant Enzymes in Plant Resistance.

There had been a proven relationship between plant's resistance to phytophagous insects and biochemical profiles of the plant. Compounds like phenols, alkaloids, flavoniods, tannins, terpenes, aldehydes, ketones, and esters, nutrients like carbohydrates, reducing sugars, proteins, amino acids and lipids have a major role in offering a phytochemical response and natural defense mechanism against herbivore attack. This usually involves the activation of some enzymes leading to the increased formation of secondary metabolites and polymers at the site of insect attack. This in turn affects the feeding, growth, reproductive capacity and larval development of the insect.

The phenolic compound, Gossypol was found to be more abundant in glanded varieties of cotton plants than glandless varieties which in turn offers the glanded variety a toxic effect against cotton ball worm and hence reduce its attack [14]. When the larvae of cotton ball worm and tobacco bud worm, were fed with artificial diet containing cotton plant pigments rich in phenols like gossypol and quercetin the larval mortality up to 70% was achieved with different concentrations [15]. A very significant negative correlation (r=-0.06) was found in a study to prove the relationship between total phenolic contents and resistance of wheat to leaf aphids (*Rhopalosiphum medis*) [16].

The infested and uninfested leaves of resistant *Abelmoschus esculentus* (L) variety, AE 22 and susceptible *Pusa sawani* demonstrated that the susceptible variety had a higher presence of organic acids and sugars [18]. Sugars play a very vital role in plant's defense against pests because the precursors for the synthesis of lignin, phenolics and callos are sugars [18]. Susceptible varieties of okra like *Pusa sawani* and AC 320 showed a higher content of reducing sugars and total sugars in comparison with resistant varieties [19].

In *Heliothis armigera* and *Spodoptera litura* there existed a tissue

preference for nutrition and reproduction, which was age correlated, mostly related to the presence of phenolic compounds such as gallic acid, pyrogallol, resorcinol, phloroglucinol, vanillic acid and protocatechuic acid. These compounds vary within different plant parts and different species also [20]. The resistance of maize plants towards *Sitophilus zeamais* and *Prostephanus truncatus* were characterized by a significant difference in the phenolic acid content of the plants. Resistant varieties like Sinaloa-2 and Sinaloa-35 exhibited a greater level of ferulic acid compared to the other susceptible varieties [21].

Cotton plants, *Gossypium hirsutum* L, infested with larvae of *Spodoptera exigua* Hubner and *Spodoptera littoralis* (Boisd.) showed a preference for undamaged young leaves on the control and previously damaged plants, demonstrated that the more susceptible variety has a higher content of reducing sugar (0.057%) than those of resistant lines (0.042%) [22].

In many plants generally a large number of proteins and enzymes are found in latex that exudes when leaves or stem are damaged. Latex of Papaya tree (*Carica papaya*) contains a cysteine protease enzyme called papain. Papain is responsible for the growth inhibition of two important pests, *Mamestra brassicae* and *Spodoptera litura* and lepidopteran larvae such as *Samia ricini*. Hence latex plays a very important role in providing a general defense against these pests. It was found that if the latex was washed off or leaves were painted, the defense ability against herbivorous insects was lost [23].

Volatile chemical signals were released by plants that were attacked by insects, which in turn attract natural enemies of the pest. In a study of feeding of *Spodoptera exigua* larvae on the leaves of cotton plant demonstrated that natural feeding of the larvae and its regurgitant released, enhanced the amount of plant volatiles than an artificial mechanical injury to the plant [24].

The infestation of mango gall fly (*Procontarinia matteiana*) on the leaves of mango cause considerable reduction of fruit yield and it was observed that three cultivars of mango plants like Heidi, Keitt and Sensation showed different degree of susceptibility to gall fly infestation and terpenes were found to play an important role in defense against these flies. Isolation by GC-FID and GC-MS analysis revealed that tarpenes like α- and β-pinene and camphene may be useful as biomarkers of susceptibility. The susceptibility or resistance of twenty other varieties were predicted by developing a orthogonal partial least square (O-PLS) model and prediction of 90% of cultivars were done accurately, which were further verified by

physical examination [25].

A study conducted in Pakistan showed that out of 12 varieties selected, 'Chaunsa' variety of mango was more susceptible to the attack of mango mealy bug, while 'Tukhmi' variety was found to be comparatively resistant. Biochemical analysis of leaves of these varieties confirmed that the susceptible 'Chaunsa' variety was observed maximum contents of carbohydrates, while the resistant 'Tukhmi' variety has minimum contents of carbohydrates and demonstrated a positive correlation with mango mealy bug population [26].

Poly phenol oxidase (PPO) and its role in plant defence mechanism has been well studied [27-29]. The way by which PPO affect herbivory of insects might be (a) the formation of quinones, which can alkylate certain essential amino acids and can decrease the nutritional quality of the leaves, (b) oxidative stress may be produced in the gut lumen of the insect because of the quinone's redox cycling and (c) as a result reactive oxygen (hydrogen peroxide) and quinones thus formed may be absorbed by the gut and have toxic side effects. In vitro studies by them also demonstrated that PPO can reduce protein quality also, if incubated with chlorogenic acid and dietary protein. Transgenic tomato plants with PPO over expression were used first to test the defense role of PPO, wherein it showed lesser lesions and enhanced resistance to pathogenic bacteria, *Pseudomanas syringae* [30], whereas tomato plants with suppressed PPO showed higher susceptibility with more lesions [31]. PPO and its role in pathogen defense was studied in pearl millet against downy mildew disease in which there was a correlation of increased PPO levels with improved pathogen resistance [32].

Studies in the root of tobacco, potato and carrots showed that an increase in iso-peroxidase activity in response to an injury [33]. Increase in peroxidase activity was observed in *Solonum dulcamera* in response to gall mite infestation [34] and in tobacco plants in response to wounding and infestation by tobacco mosaic virus [35].

The response of peroxidase, catalase and glutathione reductase in different tissues of maize seedlings (*Zea mays* L.) to chilling and acclimation, showed that chilling induced oxidative stress and induce an accumulation and increase of H_2O_2, while it was prevented by acclimation [36]. Acclimation or chilling induced none of the anti-oxidant enzymes to increase, while glutathione levels were found to be increased, which can cause the scavenging H_2O_2 in an increased manner. Induction of major peroxidase enzymes, two of which in the cell wall, indicating lignification and improving mechanical strength by elevating content of lignin.

Acclimation and responses to this demonstrate that in maize seedlings, chilling tolerance can be enhanced by these ways.

In tomato plant leaves the herbivore attack or mechanical injury activate the defensive gene and led to the synthesis of a polypeptide known as systemin. Systemin activate a cascade of signals, which is similar to the defensive mechanism in animals. The study shown that systemin was the key factor of defense against herbivores. Further studies revealed that mutant varieties of tomato plants deficient of this pathway were more susceptible to the attack of *Manduca sexta* larvae [37].

Barley plants infested with aphids induce the production of H_2O_2, indicating that this could be the starting of a complex cycle of events leading to the synthesis of components of defense mechanism, protecting the plant from further damage [38]. Insect feeding or wounding a plant tissue or stress induces the synthesis and release of various phytochemicals, in which reactive oxygen species (ROS) and intracellular calcium levels play an important role in activation of genes, regulation of transcription and phosphorylation related to that [39].

Cabbage plants (*Brassica olereacea*) infested by phloem sucking aphids (*Brevicoryne brassicae*), there was a change in the levels of compounds having anti-oxidant properties like ascorbic acid, glutathione, phenols and carotenoids due to the feeding by aphids. Specifically, the levels of ascorbic acid, polyphenol oxidase and polyphenol peroxidase were found to be increased, while antioxidant enzymes like ascorbate oxidase, ascorbate peroxidase and superoxide dismutase were found to be decreased [40].

The white fly (*Bemisia tabaci*) feeding induced fluctuations in enzymatic activities of catalase and peroxidase of nine susceptible and resistant black gram (*Vigna mungo* (L.) Hepper) varieties. The results showed that generally the infestation of white flies decreased the activity of catalase and increased peroxidase activity. Resistant genotypes like KU 99-20 and NDU 5-7 reported higher activities of these enzymes under stressed conditions induced by white fly, which may explain the bio protection and resistance against *B. tabaci* by black gram plants [41].

The defensive response of rice genotypes against rice leaf folder (*Cnaphalocrocis medinalis*) and the possibility of developing resistant varieties from these genotypes, 3 resistant genotypes (TKM6, PTB 33 and LFR831311), 2 susceptible genotypes (IR36 and TN1), one hybrid genotype (CORH1), one scented genotype (Pusa Basmati), one popular genotype (ADT36) and 2 wild genotypes (*Oryza rhizomatis* and *Oryza minuta*) were

selected and studied. The study showed that the infested resistant genotypes and wild genotypes showed a greater activity of phenylalanine ammonia and peroxidase enzymes, which were proven to play very important role against leaf folder infestation [42].

Sweet potato (*Ipomoea batata* (L.)) infested with tobacco cut worms (*Spodoptera litura*) were studied for antioxidant enzymatic activity. In healthy plants, activity of the enzymes like super oxide dismutase (SOD), phenylalanine ammonia lyase (PAL) and peroxidase (POX) were increased, while polyphenol oxidase (PPO) level was found to be decreased. In plants infested with *Spodoptera litura*, an increased activity of antioxidant enzymes were observed, which can be considered as plant's defense response against the pest [43].

The studies on the infestation of fungal pathogen, *Sclerotinia sclerotiorum* and its induction of oxidative stress in pea plants showed that microbes like *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Trichoderma harzianum* used in consortium and also singly for their capacity to give disease protection by correlating the changes in H_2O_2 and ascorbic acid production, anti-oxidant enzymes and lipid peroxidation showed that an increased production of H_2O_2 and ascorbic acid was observed after 24 hours and activity of ascorbate peroxidase was observed after 48 hours of infestation by *S.sclerotiorum*. Lipid peroxidation and enhanced activity of catalase, glutathione peroxidase and guaiacol peroxidase was observed after 72 hours. Hence it showed that, microorganism's interaction increases the protection from pathogen attack mediated oxidative stress by inducing the formation of antioxidant enzymes [44].

Studies in five ripe mango varieties (*Mangifera indica* L.) like Amrapali, Himsagor, Ashwina, Langra and Fazli, which are commercially important, revealed that Amrapali variety has the maximum amount of carbohydrate content of 3.53% while a higher content of ascorbic acid was exhibited by Ashwina (65.66 mg/100g) and Langra (59.17 mg/100 g) varieties. Lower ascorbic acid content of 10.83 mg/100g was shown by Himsagor and Fazli (12.5 mg/100g), showing a moderate correlation with anti-oxidant activity. This study further demonstrated that phenolic contents were higher in Langra variety, showing a vital role and strong positive correlation with anti-oxidant activity and thus Langra proves to be the best in this regard [45].

Studies on different degree of infestation and related biochemical changes in the defense mechanism of tea (*Camellia sinensis*) clones in

Dibrugarh district of Assam against *Helopeltis theivora* indicated that the leaves of infested clones such as TV23 and TV1, found to be highly susceptible while Tinali and S3A3 were found to be moderately susceptible. These clones showed an enhanced activity of enzymes such as polyphenol oxidase and peroxidase due to oxidative stress, which led to biochemical mediated defense mechanism against the pest [46].

The role of phenols and antioxidant enzymes of both old leaves and new flush of five hybrid mango varieties in the infestation of leaf hoppers were studied[46]. Different biochemical parameters and enzymatic activities of antioxidant enzymes like superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO), ascorbate oxidase (AO) and glutathione reductase (GR) of both infested and healthy leaves were analysed in the study. The result showed that the levels of phenols and antioxidant enzymes were increased in new flush than old leaves after pest attack. Out of five hybrids, 'Mallika' was found to be more tolerant to the pest with higher antioxidant activity, while 'Ratna' was more susceptible with lower enzyme activity. Thus it was concluded that phenols and antioxidant enzymes like SOD, POX, CAT, PPO, AO and GR played a very important role in the defense against the infestation of leaf hopper in these hybrid varieties of mango [47].

Studies in *Musa* cultivars against the infestation of *Odoiporus longicollis* showed that the susceptible cultivars have least presence of secondary metabolites like poly phenols, flavonoids and total phenols and the activity of enzymes related to the synthesis of these metabolites such as poly phenol oxidase (PPO), peroxidase (PO) and phenyl alanine ammonia lyase (PAL) were less. Further it was demonstrated that the resistant cultivars of *Musa* have a very high presence of phenols and flavonoids and the activities of PPO, PO and PAL were very high. These cultivars never got infestation by this pest [48-49].

The study conducted to find out various biochemical factors involved in mango plant's susceptibility or resistance to the attack of mango leaf cutting weevil, *Deporaus marginatus* (Pascoe) in five different mango varieties namely Neelum, Mundappa, Totapuri, Mercury and Chakkara. The estimation of various biochemicals, viz; alkaloids, phenols, flavoniods, carbohydrates and reducing sugars in the mature healthy, tender healthy and infested tender leaves were carried out. Anti-oxidant enzymes like superoxide dismutase, catalase, peroxidase, and glutathione reductase and ascorbate peroxidase were estimated quantitatively in mature healthy, tender healthy and tender infested leaves and showed that these enzymes have

direct role in the plant defence mechanisms [50].

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Chapter - 3

Breeding behavior and threats of survival of Red-wattled Lapwing (*Vanellus indicus)* in Udaipur District, Rajasthan, India

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Abstract

Red-wattled lapwing is a common resident bird of Indian subcontinent; they are inhabitants of agricultrual land, wetland, forests and also reides in urban habitats. Red-wattled lapwing generally breds from end of March to the end of July; incubation period nearly 28 to 30 days. Male and females both participate in nest construction, incubation and parental care of young ones. During study of two years from 2017 to 2019, maximum hatchling success rates were recorded in uncultivated land (76.43%) followed by around wetland (68.8%), agricultural land (65.31%) and minimum were recorded in human residential area and colonies (48.58%). Nestling success were highest observed in uncultivated land (55.21%) followed by agricultral land (43.06%), wetland (41.39%) and minimum nestling success were observed in human residential area and colonies (20.33%) due to anthropogenic distrbance, construction activity of roads and buildings, cattle movement, vehicles transportation, improper disposal of garbage and lack of suitable nesting site negatively influence hatchling and nestling success of Red-wattled lapwing. They are facing high risk of predation pressure due to natural predators like – House and Jungle crow, Ibis, Mangoose, eagle and kite, other predator feral dogs.

Keywords: Red-wattled lapwing, breeding, hatchling, nestling, habitat, threat

Introduction

Birds are very susceptible to alteration in natural habitat and acts as bioindicator species of changes among ecosystem due to expansion of urbanization, industrialization and pollutions (Clergeau *et al.,* 1998; Blair, 1999; Savard *et al.,* 2000; Francl and Schnell, 2002). Several studies have been done on the impact of urbanization on avain communities (Mills *et al.,* 1989; Jokimaki and Huhta, 2000; Cam *et al.,* 2000). Birds species highly effected due to urbanization. Urbanization also affects habitat, nesting and roosting sites of birds, they produce feeble offspring as compared to rural habitat (Shochat, 2004). Birds community is a significant implent in biodiversity conservation and identifying quality of ecosystem and conservation actions in human-dominated landscape (Kremen, 1992; Shafiq *et al.,* 1997). Large scale changes among natural habitat due to excessive anthropogenic pressure, an extension of urbanization and industrialization, development of road & highways leads to habitat fragmentation, degradation, cause adverse effect on biodiversity including birds (Khan *et al.,* 1993). Recent study shows that insectivore birds population are decling day by day due to excessive use of insecticide, pesticide and rodenticides (Parasharya *et al.,* 1994; Salek and Smilauer, 2002; Siriwardena, 2010). The Red-wattled lapwing (*Vanellus indicus*) belongs to family Charadiidae of the order Charadriiformes. The species are widely distributed in Iran, Iraq, the Arabian/ Persian gulf of all south Asia, including Pakisthan, Afghanisthan, Bangladesh, Nepal and India (Ali and Ripley, 2001; Birdlife international, 2009).

Red-wattled lapwing are an important bird of agricultral areas and inhabits around wetlands, colonies, rural and forests areas. The recognizable characters of this birds is the flesh red wattle in front of the eye and red ring around the eyes, male and female look alike (Madhava and Botejue, 2009). Males and female of Red-wattled lapwing can not be easily identified but male having a more noticeable and remarkable crest, facial wattles, sporting spurs (Piersma and Wiersma, 1996). The head, chest and front part of the neck are black in colour, the beak is also red with a black tip and the legs are long and yellow in colour (Mirza, 2007; Grimmett *et al.,* 2008). They produced loud call, usually seen in pair during the breeding season and small folks in non-breeding season nearby wetlands due to abundance and availability of food and water (Gupta *et al.,* 2011). Red-wattled lapwing breeds from February to August (Lok and Subaraj, 2009), Some study recroded breeding season from April to May month (Kragten and de Snoo, 2007), April to lasts June month (Khalil *et al.,* 2019); March to August (Vyas R, 1997). The Red-wattled lapwing usually foraging and prefer to live

in the open field areas of near wetlands, freshly irrigated croplands. It usually feed on caterpillars, insects, beetles, ants, vegetable residue and aquatic vegetations and aquatic insects (Grimmett *et al.,* 2008; Ali and Ripley, 2001). Therefore Red-wattled lapwing acts as a efficient natural biocontrol agent and controlling the insect and pest population in agricultral areas (Ali and Riply, 2001). They also found in cultivated land, mainly in Maize fields, plowered areas, gardens, grasses, and in marshy areas (Piersma and Wiersma, 1996). The shape and size of the egg is distinguishable species character, the egg is oval to a conical with one end round and another is pointed end (Al-obaidi and Al-shadeebi, 2002), both parents participate in egg incubation, parental care and incubation period is 28-30 days (Ali and Riply, 1998; Desai and Malhotra, 1976). Nidifugous chicks capable of leaving nest, develop in 28 to 30 days. Newly hatchling has fast-moving ability and follows parents for food searching , three to five months old young once have capable of flight (Piersma and Wiersma, 1996; Saxena and Saxena, 2013; Muralidhar and Barve, 2013).

Objective Of Study

The present study was designed to assess breeding biology, habitat, hatchling and nestling success, threats of Red-wattled lapwing survival in the various habitats of Udaipur district, Rajasthan, India.

Material And Methods

Line transcet, point count and nest cont methods were applied for observation of Red-wattled lapwing habitat, nesting behaviour, nests and threats of its survival in Udaipur district from 2017 to 2019. Habitat, nesting behaviour, hatchling and nestling success and threats were capture with the help of Nikon P900 and Nikon P1000 cameras. Observation and photograph were taken from appropiriate distance from nesting of Red-wattled lapwing without distrubed. Nest were spotted during foraginig, incubation, flying near nests. Frequent visit and continuous observation were made in 2-5 days before hatchling, after egg lying frequencies of observation were increased near hatchling dates. When nests were found unoccupied, the unoccupied nests observed carefully and observations were taken. Nest were considered successful when eggshell fragments were present in nests and at least one chick was alive; or by observing parent behavior as they become more aggressive if the nestling survives. Nesting failure has been considered if found empty before predict date or if there was evidence of predation like large eggshell portion and dead chicks (Galbraith H, 1998; Kaur, M and Khera KS, 2017).

Study Area

Udaipur is located between 24°34'16.5720"N latitude and 73°41'29.5584"E longitudes. It is about 598 meters above mean sea level. It has interconnecting perennial and several temporary water bodies, due to this Udaipur temperature ranges between maximum of 43.5°C and 6.3°C throughout the year. Study area were categorized into four major habitats – wetland, human residential area and colonies, agricultral and uncultivated land. Categoriztion were based on availability of vegetations or tree density, water bodies, constructions percentage and anthropogenic disturbances (Figure 1, 2, 3,4).

Result And Disscusion

Nesting And Breeding Behaviour Of Red-Wattled Lapwing

Pairing in Red-wattled lapwing generally starts in early bredding sesson. During pairing they performed characteristic and noticeable display behaviour and activity like – foraging, flying, feeding, roosts together (Figure 5,6). After the pairing, shows frequently courthship display behaviour like slow sound production with wings movements, beak movement upward. After the courtship display, frequent mating attempts were also sighted and it was frequently observed in early morning and it was continued up to the egg-laying.

Red-wattled nesting and breeding season starts from the end of March to end of July month. Nest construction activity strated in end of march in every breading season in early morning and evening. Male and female both participate in nest contruction and almost equal contribution were observed. Red-wattled lapwing usually constructs a nest on the ground by constructing slight depression on the ground surface (Figure 7). The female lapwing lays eggs in a bit of depression in an open area on the ground nearby wetlands and other habitats, encompassed by pebbles or bits of hard earth normally boarded with cattle dungs and goat stools (Saxena and Saxena, 2013). Nests boundaries boarded with the cattle dugs and soil, usually nest constructs in an open area (Figure 8). Male and female participate in nest construction, guarding of nests, incubation of eggs, parental care of young once throughout the breeding season(Figure 9,10). They perform territorial and antipredatory behaviour to produce loud voices and flying surrounding of nesting sites (Figure 11). Red-wattled lapwing is a noisy bird produces a loud sound every time, at even in night (Hayman *et al.*, 1986). Male and female both become aggressive against the predators, movement of humans and livestock nearby nests. Egg clutch size ranges from two to four eggs, but mostly four eggs clutch size is commonly observed in Red-wattle lapwing

(Figure 7, 8). Eggs were usually greenish-grey in colour and black spots (Figure 7,8). Newly hatched hatchlings of Red-wattled lapwing greyish in colur and young onces wings becomes more darker in colour (Figure 12, 13, 14, 15). Newly hatched hatchlings can run and have ability to conceal themselves in enviornment from predators and threats. They were active and use to immediately respond to alarming call of the parents, hidden in grasses and they sat down motionless (Kaur and Khera, 2011). Hatchling and nestling forage and feed nearby nests, they usually feed upon insects, caterpillars, beetles, ants vegetable remnant and debris, water vegetations and insects (Grimmett *et al.,* 2008; Ali and Ripley, 2001) (Figure 16, 17). Red-wattled lapwing acts as a natural biocontrol agent in cropland by foraging of insect population and abundance (Figure 18). After one week chances of hatchling survival increase due to fast-moving ability, escaping from predators and concealment in surrounding. They keeps the nest clean and tidy, eggshells were removed from nests by male and female.

Hatchling and nestling success rate of Red-wattled lapwing

Hatchling, nestling and overall breeding success were calculated by following formulas-

$$\textbf{Hatchling success rate calculated by} = \frac{\text{Number of hatchlings sucesss in all nest}}{\text{Total number of eggs laid in all nests}} \text{X100}$$

$$\textbf{Nestling success rate calculated by} = \frac{\text{Number of hatchlings survive in all nest}}{\text{Total number of eggs laid in all nests}} X\ 100$$

$$\textbf{Overall breeding success rate calculated by} = \frac{\text{Number of nestling survival in all nests}}{\text{Total number of egg laid in all nests}} \text{X100}$$

Overall hatchling success rate in particular habitat =

$$\frac{\text{Number of hatchling sucess in selected habitat}}{\text{Number of eggs laid in selected habitat}} \text{X100}$$

Overall nestling success rate in particular habitat=

$$\frac{\text{Number of nestling sucess in selected habitat}}{\text{Number of eggs laid in selected habitat}} \text{X100}$$

During the study (from 2017 to 2019), we observed total 310 Red-wattled lapwing nests from various habitats like- around wetlands(AW), agricultral land(AL), human residential area & colonies(HRA &C) and unculivated land (UCL). In total Red-wattled lapwing nests we observed 1163 eggs, out of these 388 eggs were found destroyed and predated by anthropogenic activity, cattle grazing and egg predation by natural predators including feral dogs. During the study, overall hatchling success was recorded 66.63% and nestling success was 42.04% in all habitats (Table 1, Graph 1). In 2017 year, maximum Red-wattled lapwing nests were recorded

in agricultral land (28) followed by uncultivated land (23), around wetland (19) and minimum nests were observed in human residential areas & colonies (16) (Table-1). Maximum hatchling success were recorded in uncultivated land (79.77%) followed by around wetland (75.34%), agricultral land (66.98%) and minimum hatchling success recorded in human residential areas & colonies (45.28%) of urban area. Maximum nestling success were observed in uncultivated land (59.55%) followed by agricultral land (48.11%), around wetland (47.94%) and minimum nestling success were observed in human residential areas & colonies(20.75%)(Graph 1). In 2018 year, maximum Red-wattled lapwing nests were found in agricultral land (33) followed by uncultivated land (28), around wetland (26) and minimum were found in human residential areas & colonies (13). In 2018, maximum hatchling success were recorded in uncultivated land (73.39%) followed by agricultral land (65.04%), around wetland (63.72%) and minimum hatchling success were found in human residential areas & colonies. In 2018, maximum nestling success were observed in uncultivated land (55.96%) followed by agricultral land (37.39), around wetland (35.29%) and minimum recorded in human residential area & colonies(Table 1)(Graph 1). In 2019, we found maximum Red-wattled lapwing nests in around wetland (44) followed by agricultral land (31), uncultivated land (26) and minimum nests were found in human residential areas & colonies (23). In 2019, maximum hatchling success recorded in uncultivated land (76.76%) followed by around wetland (69.04%), agricultral land (64.1%) and minimum hatchling success were observed in human residential areas & colonies (53.16%). In 2019, maximum nestling success were observed in uncultivated land (50.50%) followed by agricultral land (44.44%), around wetland (42.46%) and minimum nestling success were recorded in human residential areas & colonies (17.72%)(Table 1)(Graph 1).

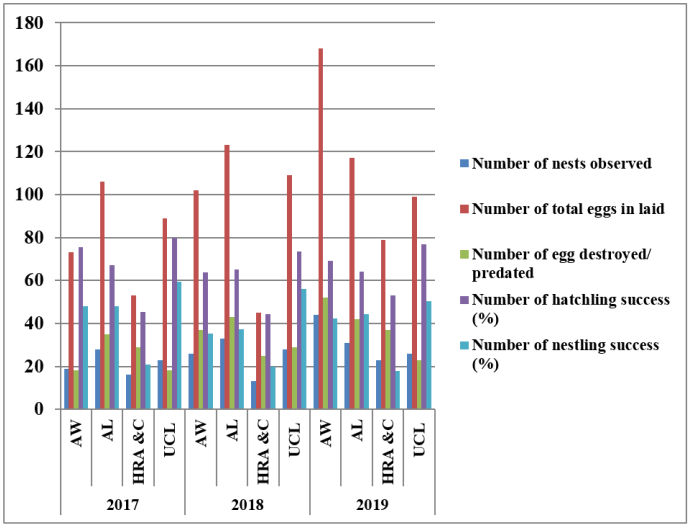
Overall hatchling and nestling success in different habitat

During the study from 2017 to 2019, we observed maximum hatchling success of Red-wattled lapwing were observed in uncultivated land (76.43%) followed by around wetland (68.8%), agricultral land (65.31%) and minimum were recorded in human residential area & colonies (48.58%); maximum nestling success were obsved in uncultivated land (55.21%) followed by agricultral land (43.06%), around wetland (41.39%) and minimum nestling success were obseved in human residential area & colonies (20.33%) (Graph-2). Studies indicate anthropogenic disturbance, cattle grazing, transportation negetively influnce hatchling and nestling success of Red-wattled lapwing in study area (Figure 19, 20,21).

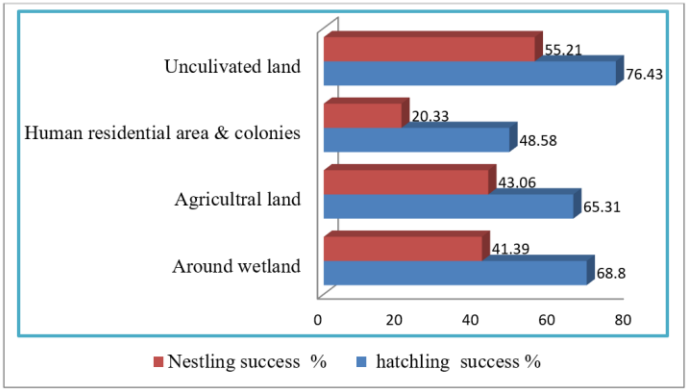
Table 1: Hatchling success and nestling success rate of Red-wattled lapwing in different habitat of Udaipur from 2017 to 2019

Habitat types	2017				2018				2019				Overall nests, eggs, hatchling success
	AW	AL	HRA &C	UCL	AW	AL	HRA &C	UCL	AW	AL	HRA &C	UCL	
Number of nests observed	19	28	16	23	26	33	13	28	44	31	23	26	310
Number of total eggs in laid	73	106	53	89	102	123	45	109	168	117	79	99	1163
Number of egg destroyed/ predated	18	35	29	18	37	43	25	29	52	42	37	23	388
Number of hatchling success (%)	75.34	66.98	45.28	79.77	63.72	65.04	44.44	73.39	69.04	64.10	53.16	76.76	66.63
Number of nestling success (%)	47.94	48.11	20.75	59.55	35.29	37.39	20	55.96	42.26	44.44	17.72	50.5	42.04

Note- AW- Around wetland, AL- Agricultral land, HRA &C- Human residential areas & colonies, UCL- Uncultivated land



Graph 1- Number of nests, eggs, hatchling success and nestling success of Red-wattled lapwing in different habitat



Graph 2- Hatchling success and nestling success rate of Red-wattled lapwing in different habitat of Udaipur from 2017 to 2019

Threats and Factor Affecting Hatcling and Nestling Survival-

Red-wattled lapwing is ground-nesting birds that are highly vulnerable to breeding failure due to destruction of nesting habitat, egg, hatchling and nestling die to predatory attack, anthropogenic distrubance and cattle grazing. Red-wattled lapwing nestling facing high degree of threats from predator attack namely- feral dogs, house and jungle crow, Ibis, mongoose and raptor species including egales, kites. Excessive anthropogenic pressure, transportation and movement of vehicles were also reducing breeding

success of Red-wattled lapwing due to road-vehicles mortality in study areas (Figure 22). At present rapid expasion of urbanization increase the variety of pollution, reduction in natural habitat becoming limiting factors for biodiversity survival in worldwide. Improper and unregulated disposal of household waste, industrial waste, plastics, fibers sheet and cloths are covers nests of Red-wattled lapwing also responsible for nesting failure in urban habitat(Figure 23, 24). Uncontrol movement of humans and cattles nearby water bodies also increase breeding failure in Red-wattled lapwing due to destruction of eggs before hatchling. During the rainy season, water logging in the ponds and agricultral land, the eggs of washed away, which can also reduces the hatchling and nestling success rate. Construction activity in an urban areas and around natural habitats also responsiable of declining breeding habitat of all birds species.

Fig 1: Wetland habitat of urban area Udaipur district

Fig 2: Harvested agricultural land of Udaipur district

Fig 3: Red-wattled lapwing and Yellow-wattled lapwing in uncultivated land

Fig 4: Human residential area and colony of urban area Udaipur

Fig 5: Courtship behavior display by the flapping of wings

Fig 6. Red-wattled lapwing breeding pair foraging in uncultivated land

Fig 7. Red-wattled lapwing eggs inside nests, nest border guarded by cow dung

Fig 8. Red-wattled lapwing eggs and one hatchling in the nest

Fig 9: Adult Red-wattled lapwing hide her nestling under the body part from escaping threats

Fig 10: Red-wattle lapwing incubating eggs near wetland habitat

Fig 11: Anti predatory behavior performed by Red-wattled lapwing against Red-napped black ibis

Fig 12: Red-wattled lapwing two days old hatchling in uncultivated land

Fig 13: Red-wattled lapwing three days old hatchling in wetland habitat

Fig14: Red-wattled lapwing four to five days old nestling in agricultural land

Fig 15: Red-wattled lapwing juvenile almost one to two month old

Fig 16: Red-wattled nestling forage and feeding in uncultivated area

Fig 17: Red-wattled lapwing sub adult forage in wetland habitat

Fig 18: Red-wattled lapwing foraging in agricultural habitat

Fig 19: Anthropogenic movement near wetland influenced Red-wattle lapwing nestling

Fig 20: uncontrolled and frequent cattle grazing around wetland reduce breeding success of Red-wattled lapwing

Fig 21: Figure21- Feral dogs and vehicle transportation increase risk of Red-wattled lapwing survival

Fig 22: Red-wattled lapwing road killing in a human residential area

Fig 23: Improper disposal of plastic waste hide Red wattle lapwing nest and eggs

Fig 24: Improper disposal of plastic and household waste reduced breeding success of Red-wattled lapwing

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Chapter - 4

Studies on Tropical Mulberry Varieties (L Series) of Regional Sericulture Research Station, Sahaspur, Dehradun, Uttarakhand

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Abstract:

Sericulture is one of the most potential agro- based industries in the world. This report analyses Studies on Tropical Varieties of Mulberry of L series (Local accessions collected from different places of Uttarakhand) at RSRS, Sahaspur, Dehradun (Uttarakhand). The report mainly focuses on the quantitative as well as qualitative characters of sixTropical Mulberry Varieties. This Sector expects high returns on low investment and is also productive and eco-friendly as it helps to conserve soil, trees and saving foreign exchange to the country.

To studies the quantitative and qualitative character of tropicalmulberry, as a global source for the development of natural products like fuel, timber, fodder, tannins, medicines and elegant queen of textiles i.e. silk.

The study is based on primary as well as secondary sources of information. Primary data is collected on the basis of observation and questionnaires. Secondary data are collected from various sources like published articles, journals, newspaper, departments of sericulture, Sericulture research station, central silk board, Sahaspur, Dehradun.

1. Introduction

Sericulture is an agro-based rural industry combining the features of agricultureand village industry. It is an age-old land-based practice in India with high employment potential and economic benefits to agrarian families. It is remarkable for its low investment and quick and high returns which make it an ideal industry or enterprise and fits well into the socio-economic fabric of India. It is also capable of providing continuous income to farmers as it is employment and income generating industry. It is rightly called as the

“kalpavriksha” or “Kamdhenu” of the poor farmers (Sharnagat *et al.*). Silk is the most elegant textile in the world with unparalleled grandeur, natural sheen and inherent affinity for dyes, high absorbance, light weight, soft touch, high durability and known as the “Queen of textiles” (Dandin *et al.,*). Employment and income generating industry, it is rightly called as the “Kalpvriksha” or “Kamdhenu” of the poor farmers Sericulture is an agro-industrial activity that involves mulberry (*Morus*spp) cultivation, silkworm rearing, and the collection of silk thread for the textile industry. The latter aspect has been highly exploited (Cifuentes *et al.,*). Sericulture is carried out mainly in rural and suburban zones. Since it is limited by the supply of mulberry leaves, a lot of plants are required near the breeding site. It is environmentally friendly because it does not generate any pollutant waste, smell or sound and can therefore coexist happily with already populated areas. Silkworm rearing is not recommended however for industrial or intensely agricultural zones that use agrochemicals, given that silkworms are seriously affected by the presence of toxic product (Pescio F, *et al.,*)

Silk is a natural fiber that is produced by the silkworm- the caterpillar larva of the domesticated silk moth *Bombyx mori*. The silk is a pasty secretion which is produced by the silk glands. The cultivation of silk from silkworms is a process known as silk farming, or sericulture. Silkworms were first discovered by the Chinese around 2,700 BC and for many centuries, the Chinese were the only civilization with the knowledge to make silk but eventually, the secrets of sericulture spread to other parts of the world. Here, we explore how silk is harvested and turned into silk threads.

Sericulture begins with knowing which silkworm will yield high quality. A silkworm, the caterpillar of the silk that is both strong and naturally lustrous. There are several species domesticated silkmoth *Bombyx mori*of silkworms which have been bred exclusively to make this type of silkbut of these, *Bombyx mori*is the most widely used species. In fact, there is evidence to suggest that *B.mori*was the species originally discovered by the Chinese during the Neolithic Age, when silk was first cultivated. But having the right type of silkworm is only the first part of the process of sericulture. Caterpillars eat continuously and silkworms are no different. The typeof food they eat will affect the kind of silk they produce so it is crucial to control the types of plants they eat to ensure that they have the right proteins needed when it comes time for them to spin their cocoons. For silk worms, this diet consists solely of mulberry leaves.

Each silk worm will eat mulberry leaves until they are about 10,000 times heavier than when they were hatched. They will grow and molt four times

before they are ready for the next phase of their lifecycle. It is at this point that these caterpillars are ready to spin their silk cocoon. To create their cocoon, the silkworms secrete a dense fluid composed of proteins. This fluid is secreted in one long, continuous strand that solidifies upon contact with air to create a single strand of silk that is wrapped around the cocoon thousands of times. A single strand can be up to 1200 yards long which is about the length of 12 football fields and only 10 micrometers (5aath of an inch) in diameter--about 1/15ththe width of a human hair. Next, this silk strand must be carefully harvested, a process that requires careful timing. If the silkworm undergoes metamorphosis and matures into a silk moth, it will escape the cocoon by secreting an acidic fluid which dissolves a hole in the cocoon, splitting the silken fiber into short fragments that cannot be reeled or used for silk yarn. Before this happens, a seri culturist must harvest the silk. To harvest the silk, the cocoon is immersed in boiling water. This process kills the silk worm pupae, but also frees the silk filaments from the tightly wound cocoon and readies them for reeling. From here, each strand is combined with strands from other cocoons to create a single thread of silk that can be used to create textiles. One thread contains up to 48 silk filaments which is then wound onto a reel, ready to be dyed and eventually used in the fabrics and threads used to create silk embroideries.

Sarees made from Tasar Silk

Silk loom in Varanasi

Surprisingly, the process of sericulture has not changed much over itsthousand-year history. Modern day sericulturists follow centuries of technique and wisdom in silk cultivation to make high quality silk that is naturally shiny and strong. Throughout history, attempts at sericulture have been made worldwide but China, the original discoverer of sericulture thousands of years ago, remains the world's leadingproducer of luxury and high quality silk.

1.1. Classification of mulberry silkworm species

The bulk of commercial silk produced in the world comes from this

variety. Mulberry silk comes from the silkworm *B.mori*, which feeds solely on the leaves of the Mulberry plant. These silkworms are completely domesticated and reared indoors. In India, the major Mulberry - silk - producing states are Karnataka, Andhra Pradesh, West Bengal, Tamil Nadu and Jammu and Kashmir, which together account for 92% of the country's total mulberry raw silk production. *B.Mori*, the domesticated silkworm, has been reared for over 2000 years. During this long history many mutations have occurred. The process of mutation has resulted in a combination of different genes in a large number of silkworm races. Silkworm races are classified on the basis of:

1. Place of origin;
2. Voltinism (the number of broods or generations produced in a year); and
3. Moulting (the number of times the caterpillar sheds its skin before
4. Starting to spin silk).

1.) Classification based on place of origin :

Based on the place of origin *B. mori* is classified into Chinese, Japanese, European and Indian races. These races can be distinguished from one another on the basis of morphological characters of egg, larva, cocoon, adult, biological characters like duration of life cycle diapauses characters, rubber of larval moults, and resistance to environmental factors and diseases, commercial letters of the cocoon like length of the filament, thickness of filament, percentage of deformed cocoons, etc.

- **Commercial Races:**

Each country in an attempt to improve the silk yield has developed anumber of hybrids. The commercially significant Indian races are:

a) SH6- Bivoltine race developed at RSRS Sahaspur Dehradun (marked silkworm, elongated cocoon, with faint constriction).

b) MB4D2- Bivoltine race developed at CSRSTI, Mysore (plain worms, constricted cocoons)

   - SH6 × NB4P2 is a flagship silkworm hybrid of North and North west India for the last 30 years.

c) Dun 6- Bivoltine race developed at RSRS Sahaspur Dehradun (plain silkworm, oval cocoon)

d) Dun 17- Bivoltine race developed at RSRS Sahaspur Dehradun (plain silkworm, oval cocoon)

e) Dun 18- Bivoltine race developed at RSRS Sahaspur Dehradun (marked silkworm, dumbbell cocoon)

f) Dun 22- Bivoltine race developed at RSRS Sahaspur Dehradun (marked silkworm, dumbbell cocoon)

- Dun 6 × Dun 22 was authorized by Race Authorization Committee of Central Silk Board in 2005 and Dun 17 × Dun 18 was authorized in 2008.

2.) Classification by voltinism:

Voltinism is a biological term referring to the number of broods or generations an organism may produce in a year, silkworm's areclassified in:

a) Univoltine (races having one generation a year),

b) b.Bivoltine (races having two generations a year) and

c) Multi orPolyvoltine (races having more than two generations a year).

➢ **Univoltines:**

These races have only one generation in a year. The larvae body sizeis large. The cocoon weight, cocoon shell weight, shell ratio andcocoon filament weight are high. The cocoon filament quality is good.

➢ **Bivoltines:**

These races have two generations in a year. The cocoon weight,cocoon shell weight, shell ratio and cocoon filament weight are less as compared to univoltine. Larvae are more robust and more uniformcompared to univoltines.

➢ **Multivoltines:**

These races have a year in two to more generations. The life cycle isshort. Laryae are robust and can withstand high temperature. Thecocoon size is small. The Cocoon weight, cocoon shell weight, shellratio and cocoon filament weight are lower compared to bivoltines,cocoon filament is fine.

3.) Classification based on moulting:

Based on moulting characteristics, the silkworms are classified in:

- Trimoulters;
- Tertamoulters;
- Pentamoulters;
- (Morerarely) bimoulters and hexamoulte
- Tetramoulters are mainly reared for commercial purposes.

Non - mulberry silk species:

A large number of species (400 - 500) used in the production of non - Mulberry silks, but only about 8 have commercially exploited in Asiaand Africa, mainly by tribal communities, the major varieties of nonmulberry silk are described below –

1	Tasar	Tasar (tussah) is coarse silk of copperish color, mainly usedfor furnishings and interiors. It is less lustrous than mulberry silk,but has its own feel and appeal. Tasar silk is produced by thesilkworm, *Antheraeamylitta* which thrives on food plants like Asanand Arjun.	  (A) Worm (B) Moth
2	Oak tasar	Oak tasar is a fine variety of tasar produced intemperate climate. In India oak tasar is produced by the silkworm*Antheraeaproyeli* J. This species feeds on oak, found inabundance in the sub-Himalayan belt of India, covering the statesof Manipur, Himachal Pradesh, Uttar Pradesh, Assam, Meghalayaand Jammu and Kashmir. China is the major internationalproducer of oak tasar. Chinese oak tasar is produced by thesilkworm *Antheraeapernyi*.	  (A) Worm (B) Moth
3	Eri	: Eri is also known as Endi or Errandi. Ery is a multivoltine silkspun from open ended cocoons, unlike other varieties of silk. Erisilk is the product of the domesticated silkworm, *Philosamiaricini* which feeds mainly on castor leaves, like tasar, the cocoon variesin color, size and softness. In India Eri iscultivated mainly in the north - eastern states and Assam. It is alsofound in Bihar, West Bengal and Orissa (Dhavalikar, 1962).	  (A) Worm (B) Moth
4	Muga	This golden yellow silk is the specialty of India and is thepride of Assam state. It is obtained from semi – domesticatedmultivoltine silkworm, *Antheraeaassamensis*. Muga culture is specific the state of Assam and is anintegral part of the tradition and culture of that state.	

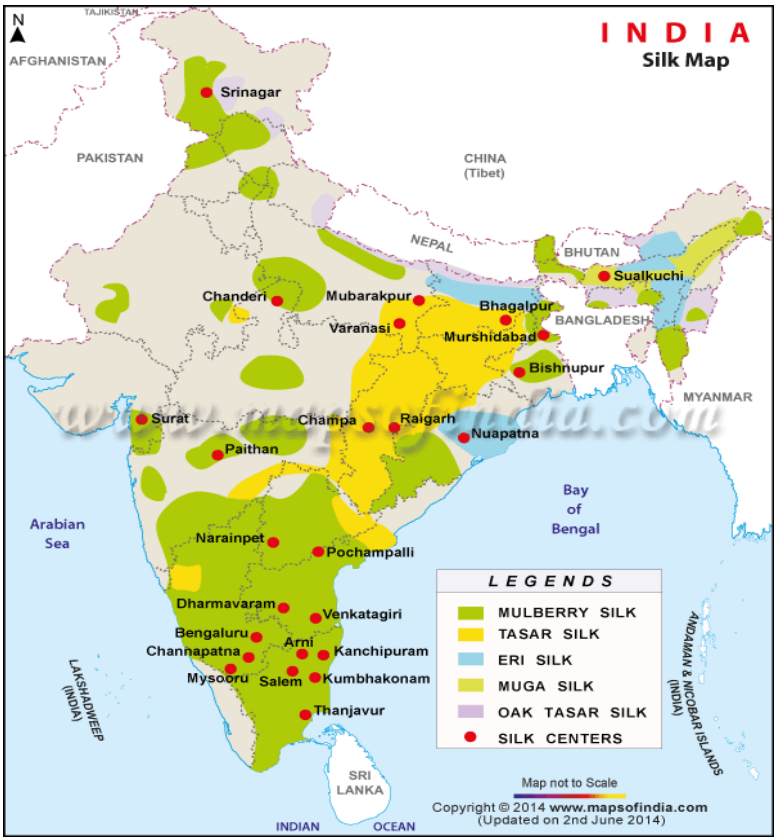
			 (A) Worm (B) Moth

1.2. sericulture industry in India

India continues to be the second largest producer of silk in the World. India has the unique distinction of being the only country producing all the five kinds of silk – *Mulberry, Eri, Muga*, Tropical *Tasar* and Temperate *Tasar*. Sericulture is an important labour-intensive and agro-based cottage industry, providing gainful occupation to around 7.25 million persons in rural and semiurban areas in India. Of these, a sizeable number of workers belong to the economically weaker sections of society. There is substantial involvement of women in this Industry.

In India, Sericulture is mostly a village-based industry providing employment opportunities to a large section of the population. Although Sericulture is considered as a subsidiary occupation, technological innovation has made it possible to take it up on an intensive scale capable of generating adequate income. It is also capable of providing continuous income to farmers. India is the second largest producer of silk in the world with an annual silk production of more than 21,000 M. Tons in 2010-11(provisional). Although, all the known varieties of silk, viz. *Mulberry, Eri, Muga* and *Tasar* are produced in India, Mulberry silk is the most popular variety. Mulberry silk alone contributes more than 80% of the Country's silk production. Silk and silk goods are very good foreign exchange earners. Export potential of this sector is promising as silk production in Japan is declining and that of China, the largest silk producer the World, it is stagnant. The present global scenario clearly indicates the enormous opportunities for the Indian Silk Industry.

For the development of silk industry in India, the Central Silk Board, a statutory body, is functioning under the administrative control of the Ministry of Textiles, Govt. of India with its Headquarters at Bengaluru.



Map showing production of silk by different states of India

The following are the important functions assigned to the Board.

a) Promoting the development of silk industry by such measures as it thinks fit.

b) Undertaking, assisting and encouraging scientific, technological and Economic Research.

c) Devising means for improved methods of mulberry cultivation, silkworm rearing, developing and distributing healthy silkworm seeds, improving methods of silk reeling, improving the quality and production of raw silk.

d) Improving the marketing of raw silk.

e) The collection and compilation of statistics relating to the sector.

f) Advising the Govt. of India an all matters relating to the development of silk industry including import and export of raw silk

1.3. Sericulture industry in uttarakhand

Uttarakhand has a long tradition and great history of practicing silk production. Sericulture is practiced in 12 out of 13 districts of Uttarakhand. It is believed that Captain Hutton introduced sericulture in the hills of Mussoorie in the year 1858 and Messers Lister & Company took up commercial production of silk in a village on Dehradun - Haridwar Road, which is later named as **"Resham Majari".**

Uttarakhand state is the only state which is producing all four kind of cocoon viz Mulberry, Oak tasar, Muga and Ericulture. But Uttarakhand also known as *"Bowl of Bivoltine silk in India* "because of its high quality Bivoltine cocoons of International grade.

Central Silk Board in the state provides good Research & Development (R&D), seed production and Post cocoon Technology (PCT) base. Research and Development in mulberry is supported by Regional Sericulture Research Station (RSRS), Sahaspur, Dehradun.Other extension centers related to RSRS are present at Kalsi (Dehradun), Haldwani (Nainital), Pathri (Haridwar), Pauri&Bageshwar.

For Post Cocoon Technology (PCT), Silk Technical Service Center (STSC) is present at Premnagar, Dehradun. Apart from this, Department of Sericulture (DOS).Uttarakhand is engaged with rearing of all four types of silk in Uttarakhand.

Women in Uttarakhand are generally engaged in agro-based or household based activities. Women constitute about 60% of work in sericulture sector. The major portion of income from sericulture is captured by the primary producers, i.e., the farmers who produce cocoons followed by the reelers, weavers and traders.

1.4. Introduction to Moriculture

Cultivation of mulberry plants is called moriculture. There are over 20 species of mulberry, of which four are common: *Morusalba, M. indica, M. serrata and M latifolia*. Mulberry is propagated either by seeds, root- grafts or stem cuttings, the last one being most common. Cuttings, 22-23 cm long with 3-4 buds each and pencil thick, are obtained from mature stem. These are planted directly in the field or first in nurseries to be transplanted later. After the plants have grown, pruning is carried out routinely which serves two purposes, induction of growth and sprouting of new shoots (Chinnaswamy *et al.* 1995).

Harvesting of leaves for feeding larva is done in three ways: leaf picking,

branch cutting and top shoot harvesting. In leaf picking, individual leaves are handpicked. In branch cutting method, entire branch with leaves are cut and offered to 3rd instar larva. In top shoot harvesting, the tops of shoots are clipped and given to the 4th & 5th instars. The yield and quality of leaf depend upon the agronomic practices for cultivation of mulberry trees, namely irrigation, application of fertilizers etc. It is estimated that 20,000 to 25,000 kg of leaves can be harvested per hectare per year under optimum conditions. It has also been estimated that to rear one box of 20,000 eggs, 600-650 kg of leaves are required for spring rearing and 500-550 kg for autumn rearing in Japan. In India, to rear 20,000 eggs the quantity of leaves required is about 350-400 kg (Choudhury, P.C. 1997).

Area cultivated with mulberry in India

1. Mulberry foliage is the only food for the silkworm *(Bombyxmori)* and is grown under varied climatic conditions ranging from temperate to tropics. Mulberry leaf is a major economic component in sericulture since the quality and quantity of leaf produced per unit area has a direct bearing on cocoon harvest. In India, most states have taken up sericulture as an important agro-industry with excellent results. The total acreage of mulberry in India is around 282,244 ha.
2. Species and varieties under cultivation in india

There are about 68 species of the genus *Morus*, the majority of them occur in Asia, especially in China (24 species) and Japan (19). Continental America is also rich in its Morus species. The genus is poorly represented in Africa, Europe and Middle East, and it is not present in Australia.

In India, there are many species of *Morus*, of which *Morusalba*, *M. indica*. *M. serrata* and *M. laevigata* grow wild in the Himalayas. Several varieties have been introduced belonging to M. multicaulis, *M. nigra*, *M. sinensis* and *M. phillippinensis*. Most of the Indian varieties of mulberry belong to *M. indica*.

Though mulberry cultivation is practiced in various climates, the major area is in tropical zone covering Karnataka, Andhra Pradesh and Tamil Nadu states, with about 90%. In the sub-tropical zone, West Bengal, Himachal Pradesh and north-eastern states have major areas under mulberry cultivation.

3. General Description

Mulberry is a fast growing deciduous woody perennial plant. It has a deep-root system. The leaves are simple, alternate, stipulate, petiolate, entire or lobed. Number of lobes varies from 1 to 5. Plants are generally dioecious.

Inflorescence is catkin with pendent or drooping peduncle bearing unisexual flowers. Inflorescence is always auxiliary. Male catkins are usually longer than the female catkins. Male flowers are loosely arranged and after shedding the pollen, the inflorescence dries and falls off. Number of parianth lobes are 4. Number of stamens are 4 and implexed in bud. Female inflorescence is usually short and the flowers are very compactly arranged. Number of parianth lobes are 4 and persistent. Ovary is one-celled and stigma is bifid. The chief pollinating agent in mulberry is wind. Fruit is a sorosis and the colour of the fruit is mainly violet black (Rangaswami *et al*. 1976)

Most of the species of the genus *Morus* and cultivated varieties are diploid having 28 chromosomes. However, triploids (2n=(3x)=42) are also extensively cultivated for their adaptability, vigorous growth and quality of leaves.

4. Climatic Requirement of Mulberry

Mulberry thrives under various climatic conditions ranging from temperate to tropic located north of equator between 28° N to 55°N latitude. The ideal range of temperature is from 24-28°C. It grows well in places with annual rainfall ranging from 600mm to 2500mm. In area with low rainfall, the growth is limited due to moisture stress resulting in low yields. On an average mulberry requires 85,000 gallons of water per hectare once every 10 days in case of loamy soils and 15 days in clayey soils. The atmospheric humidity in the range of 65-80% is ideally suited for mulberry growth. Sunshine is one of the important factors controlling growth and leaf quality. In tropics, mulberry grows with a sunshine range of 9.0 to 13.00 hours a day. Mulberry can be cultivated from sea level up to an elevation of 1000 m above mean sea level (Sastry *et al*. 1984)

5. Soil Condition

Mulberry flourishes well in soils which are flat, deep, fertile, well drained, loamy to clayey, porous with good moisture holding capacity. The ideal range of soil pH is 6.2 to 6.8. The optimum pH required for mulberry is 6.5 to 6.8. Soil amendments may be used to correct the soil to get required pH.

A) Mulberry Cultivation in North West India under Rainfed Conditions:

I. Suitable Mulberry Varieties:

Kanva-2, S-13 and S-34 varieties were recommended for rainfed (rainfall: 500-800 mm) regions of North West India out of which K2 is still used for chawki rearing.

Kanva 2: Belongs to *Morusindica* - Diploid. Widely cultivated in North west India.

S-13: Belongs to *M.indica*. Selection from open pollinated hybrids of Kanva-2. Recommended for rainfed areas of North west during 1990..

S-34: Belongs to *M.indica* - Diploid. Selection from progeny of S30 x Berc 776. Recommended during 1990 for rainfed areas with black cotton soils of North west.

S146 : It is the most prominent variety of the region and it is under extensive use by farmers of Uttarakhand, Uttar Pradesh, Himachal Pradesh, Haryana and Punjab apart from rearers of these states.

II. Establishment of Mulberry :

i. Preparation of land : The land meant for mulberry cultivation is ploughed deep with heavy mould board plough up to a depth of 30-35 cm. Thereafter the land is repeatedly ploughed 2-3 times with a country plough to bring the soil to a fine tilth. The land should be properly levelled if it is sloppy. A basal dose of well decomposed Farm Yard Manure or compost is applied at the rate of 10 MT/ha and thoroughly incorporated into the soil.

ii. Spacing: The spacing commonly followed for a rainfed garden is 90 x 90cm Pits of 35 x 35cm. are prepared. About 1 kg farm yard manure/pit should be added.

iii. Preparation of cutting and planting: Branches of 8-10 months old and about 50 mm. in diameter should be used for preparation of cuttings of 22-25 cm. length with 5-6 healthy buds. Three cuttings are planted/pit in a triangular form with a spacing of 15 cm. leaving only one bud exposed above soil surface. If planting is done with saplings, then one sapling is sufficient/pit. Planting should be done during June /July after onset of monsoon.

iv. Inter cultivation: During first year of plantation, inter-cultivation should be done manually. Once the mulberry plants are established, bullock ploughing is carried out.

Application of fertilizer: 50N:25P:25K Kg/ha/yr in two doses.

First dose :Suphala (15:15:15) 167kg, after 2 months of planting

Second dose: Urea 55 kg orCAn (100kg) or Ammonium sulfate (125kg), end of September or early October before cessation of monsoon rains

v. Pruning and leaf harvest: First crop should be taken 6 months after plantation when mulberry gets well established. Then onwards, two more crops are taken during first year by leaf picking method. Mulberry should be pruned after the completion of one year at the onset of next monsoon. The pruning is carried out with a sharp sickle or a pruning saw at the height of 25-30 cm. from the ground.

vi. Greenmanuring and cover mulching: Green manure crops can be grown as inter-crop with mulberry. It should be done during monsoon only. The green manure crops (cow pea, horse gram, dhaincha) should be incorporated into soil by ploughing before the flowering starts and well before rains cease. After that the plots may be given cover mulching with any dry material or weeds in which seed is not a source of multiplication.

III. Maintenance of Mulberry Under Rainy Condition (Second year onwards):

Inputs required for rainfed garden (per ha. per year)

Recommended inputs for gardens maintained under rainfed condition.

Spacing: 90 cm. x 90 cm.

1. FYM/compost, 10 MT, single dose at the onset of monsoon
2. Azotobacterbiofertilizer, 4 kg/crop, two times/yr (during rainy season)
3. VAM inoculum, 1000 kg, once in life span of mulberry (inoculation through maize rootlets)
4. Suphala, 167 kg, I crop
5. Single Super Phosphate, 156 kg, I crop
6. Muriate of potash, 42 kg, I crop
7. Urea (55 kg) or Cam (100kg), III crop
8. Green manuring, 15 kg

Crops like horse gram, cow pea, sun hemp, dhaincha, etc., should be incorporated into soil by ploughing before flowering and cessation of monsoon.

Leaf harvest:

Individual leaf harvesting should be made. The yield for different varieties is as mentioned below:

K-2 : 10-12 MT/ha/yr

S-13 :14-15 MT/ha/yr

S-34 : 14-15 MT/ha/yr

S-146 : 15-17 MT/ha/yr

B) Mulberry Cultivation in North West under Irrigated Condition:

In North West India, mulberry is very scanty in irrigated conditions. However, V-1, Tr 10, S-635 and C-2035 are main varieties for irrigated conditions.

I. Suitable Mulberry Varieties:

Kanva-2, S-36, S-54, DD, MR2 and V-1 varieties are recommended for irrigated conditions.

Kanva 2: Belongs to *Morusindica* - Diploid. Widely cultivated in North west India after the same was recommended for cultivation in 1969 by CSR & TI, Mysore.

S-36: Belongs to *M. indica*. Developed at CSRTI, Mysore and recommended during 1984. Evolved from Berhampore local by chemical mutagenesis. Cultivated in North west India. Moderate rooting ability.

S-54: Belongs to *M. indica*. Developed at CSRTI, Mysore and recommended during 1984. Selected from Berhampore local by chemical mutagenesis (EMS). Recommended for assured irrigated conditions of North west.

DD: Selected from natural population of Dehradun variety and recommended by Karnataka State Sericultural Research and Development *Station*, Thalaghattapura. Recommended for North west India.

MR 2: Belongs to *M. sinensis* – Diploid. Selection from open pollinated hybrid population.Developed at CSR & TI, Mysore.Mainly cultivated in Tamil Nadu under both irrigated condition in plains and rainfed conditions in hilly regions.

V-1: Belongs to *M. indica* L. Recently developed from a cross of S-30 and Berc.776 at CSR&TI, Mysore. Recommended during 1996 for assured irrigated conditions.

II. Establishment of Mulberry

i. Selection of site: Mulberry flourishes well in soils which are flat, deep, fertile, well drained, loamy to clayey, porous with good moisture holding capacity. The ideal range of soil pH is 6.2 to 6.8. Mulberry can be grown in saline, alkaline and acidic soils after suitably amending the soils.

ii. Preparation of land: The land meant for mulberry cultivation is ploughed deep with heavy mould board plough up to a depth of 30 – 35 cm. Thereafter the land is repeatedly ploughed 2-3 times with a country plough to bring the soil to a fine tilth. The land should be properly leveled if it is sloppy. A basal dose of well decomposed farm yard manure or compost is applied at the rate of 20 MT/ha and thoroughly incorporated into the soil.

iii. Plantation can be raised by using both cuttings and saplings. The varieties ideally suited for irrigated conditions are Kanva-2, S-36 and V-1. Branches of 6-9 months old and about 15 ml..in diameter should be used for preparation of cuttings of 15-18 cm. length having 3-4 healthy buds for raising nursery or for planting directly in the field.

iv. Spacing: Plant spacing of 90 x 90 cm. is ideal for mulberry. While taking Plantation directly, two cuttings/pit required to be used. In case of using saplings, only one sapling/pit is sufficient. Presently a paired row plantation with the spacing of (90+150) cm x 60cm, is recommended.

v. Inter-cultivation: Two months after planting, weeding is done. A second weeding is done after another 2-3 months. Thereafter, inter-cultivation should be done after every shoot or leaf harvest.

vi. Irrigation: The plantation should be taken up during the onset of monsoon to take advantage of the rains. If the rain is not sufficient, the land should be irrigated at regular intervals of 8-14 days depending on type of soil. About one and a half to two acre inches of water is required/irrigation.

vii. Application of fertilizer: The total dose of fertilizer to be applied in first year is 100 N: 50 P: 50 K/kg/ha/yr. This is applied in two doses. The first dose is applied when the plantation is about 2 months old at the rate of 50 N: 50 P: 50 K/kg/ha. The second dose is applied after taking the leaf harvest at the rate of 50 kg N alone/ha.

viii. Pruning: After 6 months of plantation, mulberry attains a height of about 1.50 to 1.75 m. and is ready for harvest. The first harvest is taken by bottom pruning. The second leaf harvest is taken after 12 weeks of first leaf harvest and the third harvest is taken 12 weeks after the second harvest by shoot harvest. From second year onwards, harvest is made at an interval of 70 days by shoot harvest method.

Leaf harvest:

Leaf harvesting is done either through individual leaf harvest or shoot harvesting. Later one is more economical and useful for shoot method of silkworm rearing.

C) Mulberry Cultivation in Hilly Areas:

1. <u>Suitable Mulberry Varieties:</u>

S-1, S-7999, S-1635, S-146, Tr-10 and BC-259 varieties are recommended for hilly regions of north and north-eastern India.

III. Establishment of Mulberry :

i. Preparation of land: If the land is having gentle slope, it can be leveled with minor land shaping and providing suitable type of bunds across the slope. If the slope is more, contour budding terrace planting or contour line planting can be adopted. In more sloppy areas, platform for individual plants on contour lines is more suitable as the same involves less soil cutting.

ii. Spacing: Spacing for tree planting depends on soil topography, extent of land available for cultivation and training method. In case of gentle slope, 3`X 3`, 5'x 5' may be adopted. In case of more sloppy land 10' x 10' can be adopted.

Pits are to be prepared for plantation. In case of deep textured loose soils, 45 x 45cm. and in had shallow soils 60cm x 60cm x 60cm) pits are to be prepared. For each pit, 5kg (one iron pan) of FYM or compost must be applied.

iii. Planting: Saplings of 5 months ago with 5-6 feet is found suitable to plant during the regular onset of monsoon. One sapling/ pit should be planted. The saplings should be supported with stick to ensure straight growth.

iv. After care of plantation: After one month, all the buds except top 5-6 should be removed carefully without damaging the bark. Weeds around the plant should be removed and regular pot watering should be given. After 3 months of planting, second weeding should be given and 25g of suphala/plant should be applied in trench and should be covered with soil. 2nd dose of fertilizer (25g urea/plant) should be given before cessation of monsoon. Plants must be protected from grazing.

6. Leaf Chemical Composition :

Chemical composition of leaf varies with variety and maturity. However on the basis of the analysis carried out at RSRS, Dehradun, the chemical composition of the leaf is as follows:

7. Main Pests and Diseases of Mulberry

Maconellicoccushirsutus (mealy bug) – causing tukra in mulberry.

Diaphaniapulverulentalis – Leaf roller.

Spilarctiaobliqua – Bihar hairy caterpillar (Sporadic pest).

1. Review of literature

Mulberry is a member of the genus *Morus*of the family Moraceae and has many species and varieties. According to Hooker (1885), the wild taxa of the genus *Morus*in India are represented by four species *viz. M. alba*, *M. indica*, *M.serrata*and *M. levigata*which was later confirmed by Hooker (1888). But, Koidzumi (1917, 1923) classified the genus *Morus*into 35 species under two sections the Doluchostylac (long style) and Macromorus (Short style) under which he recognized the group Papillosae and Pubeseentae based on the nature of stigmatic hairs.There are about 68 species of the genus *Morus*.

The occurrence of mulberry is reported in different regions of India (Kanjilal *et al.,* 1940, Gamble and Fischer, 1957). In India the genus, *Morus*is represented by 4 species *viz. M. indica, M. alba, M. serrata*and *M. levigata*(Hooker,1885). (Dandin *et al.* 1993, 1995) reported the distribution of *M. indica, M. serrata* and *M. levigata*in forest flora of Kumaon region and North East India respectively. According to Ravindran *et al.* (1999) in India, *M. serrata*and *M. levigata*are the wild forms with the distribution of former confined to North-west India and the later distributed throughout India including Andaman and Nicobar Islands.

Mulberry plants are perennial. One generation of plants can survive for more than 10 years and at times for even 30-40 years. The features of mulberry vary according to the variety. Some varieties that suited well in particular regions while others do not. There are two important angles that are necessary to be carefully considered while selecting mulberry varieties. One of these is from the point of view of cultivating the plants. The other one is the suitability of the variety for the healthy growth of the silkworm larvae including the quality and quantity of the cocoons by them. In view of these facts, the selection of mulberry variety is made when these two conditions are satisfactory and moreover the variety suited for the region. Various workers have suggested average plant height, growth rate, number, of branches/plant, leaf size, number of leaves/branch etc., as the dependable characters in selection of genotypes for high leaf yield (Das and Krishnaswamy, 1969; Bari *etal*., 1989; Bindroo *et al.,* 1990; Sahu *et al.,* 1997; Vijayan *et al.,* 1997). Tojyo and Watanabe (1981) emphasized on average length of shoot, number of main and lateral branches per plant and weight of leaves as important parameter contributing to the leaf yield.

Leaf production of mulberry depends on various quantitative traits. Phenotypic observations of plants are mainly followed for selection of different traits for improvement of crops. Some workers reported about few traits *i.e.* branch number, single leaf weight, internodal distance, moisture percentage, etc. which have correlation with leaf yield and suggested to consider those traits for indirect selection (Sarkar *et al*, 1987; Susheelamma *et al.*, 1988; Bari *et al.* 1989) for crop improvement. Das and Krishnaswami, 1969 reported plant height and branch/plant have positive correlation and high heritability with leaf yield.

Susheelamma *et al.* (1992) evaluated 219 germplasm accessions for important yield components and found that higher number of primary and secondary branches and leaves per meter are the important traits associated with leaf yield. According to Bhat and Hittalmani (1992), shoot to root ratio by length and dry weight, number of roots per plant and volume of roots per plant are the best characters for selecting mulberry genotypes for further improvement. Rahman*etal.* (1994) reported that leaf yield was found to be positive and significantly associated with total stem weight, 100 leaf weight, total branch weight but insignificantly positively correlated with total branch number and 100 leaf area and length of the longest shoot.

Different mulberry varieties react to the climatic condition, soil quality differently. One of the primary criteria in selecting mulberry variety to a particular region is its degree of tolerance to different mulberry diseases. The resistance against various diseases differs among the varieties. Secondly, the suitability of mulberry to various climatic conditions particularly temperature vary considerably according to mulberry variety. Varieties that are strongly stable in winter conditions being able to withstand extreme cold. On the other hand, varieties which are suited to warm regions, even when practical features are excellent in a particular variety and if it can not withstand extreme cold conditions in winter in cold regions, it cannot be grown in the region. Although mulberry plants grow in almost all type of soil depending on varieties; however, some are suited for a particular quality of the soil. Particularly, the growth pattern of the root system differs to some extent in different varieties. Varieties in which the roots extend to a great depth would grow better in soil having relatively thick layer of surface soil. On the other hand, the varieties where the roots spread in shallow soil will grow well in shallow surface soil. This is an important aspect in relation to amount of rainfall in an area. Again there are varieties, which are highly resistant as compared to the common varieties in highly wet soil due to underground water (Aruga, 1994). Mulberry plant is harvested once in March-April in sub-tropical areas for rearing

purposes and then in September for autumn crop. Thus the trees are disturbed only twice in a year for sericulture purpose making them good trees under agro forestry. *(Dhar and Khan, 2005; Dzhafarov and Gadzhiev, 1972)*

India has rich resources of mulberry varieties, a couple of which are traditionally cultivated. In addition, a few exotic varieties (Kosen, Goshoerami, etc.) have also been introduced time to time. Research *station*s have released a few varieties such as Kanva-2 (or M-5), S-l, etc. The Kanva-2 and S-l variety is widely accepted by farmers of different parts of the country. It is an established fact that mulberry varieties recommended for field multiplication should be selected on the basis of leaf output/unit area, response to fertilizers and pruning (Gabriel and Rapusas, 1976) and also on the silkworm rearing results.

3. Materials and methods

3.1 Study Area:- Field work for the study of tropical mulberry varieties was conducted in Regional Sericulture Research Station, Sahaspur, Dehradun. The plants were maintained at germplasm bank of the research station.

3.2 Study Period:- April to May.

3.3 Usage Of Equipment:- Camera, Notebook, Pen, Weight machine, Scale, Calculator.

3.4 Methods :-

The plants were cultivated during the spring season. These plants were maintained at germplasm bank of the research station. Mulberry is propagated either by seeds, root- grafts or stem cuttings, the last one being most common. Cuttings, 22-23 cm long with 3-4 buds each and pencil thick, are obtained from mature stem. These are planted directly in the field or first in nurseries to be transplanted later. After the plants have grown, pruning is carried out routinely which serves two purposes, induction of growth and sprouting of new shoots. Harvesting of leaves for feeding larva is done in three ways: leaf picking, branch cutting and top shoot harvesting.

Several varities of mulberry plant are found in Regional Sericuture Research Station, Sahaspur. The different varities found here are listed below:

SERIES	VARIETY
LF Series (2)	LF 1, LF 2
K2 Series (2)	K 2 (M), K 2 (S)
Alkaline Series (2)	AR 12, AR 14
RFS Series (2)	RFS 135, RFS 175
CW Series (2)	China White, China White (M)
Mandaley Series (2)	Mandaley (S), Mandaley (M)

HP Series (3)	HP 02/8, HP 02/12, HP 02/13
Tr Series (4)	Tr 4, Tr 5, Tr 8, Tr 10
Exotic (5)	Rokokuyaso, Kokuso 27, Ichinose, Kairorosa, Goshoerami
L Series (6)	L1, L2, L3, L4, L5, L6 (Shivalik)
MS Series (8)	MS 1 to MS 9
C Series (10)	C4, C740, etc
Others (15)	V 1, Local lobed etc
S Series (19)	S 13, S30, etc.

The methods used for cultivating the mulberry plant include the following steps:

i) Firstly, the land meant for mulberry cultivation is ploughed deep with heavy mould board plough up to a depth of 30-35 cm. Thereafter the land is repeatedly ploughed 2-3 times with a country plough to bring the soil to a fine tilth. The land should be properly levelled if it is sloppy. A basal dose of well decomposed Farm Yard Manure or compost is applied at the rate of 10 MT/ha and thoroughly incorporated into the soil.

ii) The spacing commonly followed for a rain fed garden is 90 x 90cm Pits of 35 x 35cm. are prepared. About 1 kg farm yard manure/pit should be added.

iii) Branches of 8-10 months old and about 50 mm. in diameter should be used for preparation of cuttings of 22-25 cm. length with 5-6 healthy buds. Three cuttings are planted/pit in a triangular form with a spacing of 15 cm. leaving only one bud exposed above soil surface. If planting is done with saplings, then one sapling is sufficient/pit. Planting should be done during June /July after onset of monsoon.

iv) During first year of plantation, inter-cultivation should be done manually. Once the mulberry plants are established, bullock ploughing is carried out. Application of fertilizer should be 50N:25P:25K Kg/ha/yr in two doses as shown:

First dose: Suphala (15:15:15) 167kg, after 2 months of planting

Second dose: Urea 55 kg or CAn (100kg) or Ammonium sulfate (125kg), end of September or early October before cessation of monsoon rains

i) First crop should be taken 6 months after plantation when mulberry gets well established. Then onwards, two more crops are taken during first year by leaf picking method. Mulberry should be pruned after the completion of one year at the onset of next monsoon. The pruning

is carried out with a sharp sickle or a pruning saw at the height of 25-30 cm. from the ground.

ii) Green manure crops can be grown as inter-crop with mulberry. It should be done during monsoon only. The green manure crops (cow pea, horse gram, dhaincha) should be incorporated into soil by ploughing before the flowering starts and well before rains cease. After that the plots may be given cover mulching with any dry material or weeds in which seed is not a source of multiplication.

After the Mulberry cultivation, study of specific varieties is done by following methods:-

(a) Three plants of each genotype were studied. The quantitative characters observed are number of branches, shoot length, inter nodal distance, moisture percentage and leaves weight.

(b) For counting number of branches the short branches are excluded and all other branches are counted from each plant. Finally from all three plants average branch number is considered as branch number for the genotype.

(c) For Shoot Length the longest three branches of plant are selected and measured, the average of three branches is taken as the shoot length for that plant. Further the final shoot length for variety is taken as average of shoot lengths of the three plants of the genotype.

(d) For Inter nodal distance, distance between nodes is measured from three different branches of a plant, further final average of those three is considered as final nodal distance of plant. For the variety further average of all three plants is taken.

(e) Leaf yield was observed in the branches cut for shoot length. For this, leaf yield of one shoot of a plant is taken and multiplied with 3, which is taken as final leaf weight for that variety.

(f) The leaves as plucked for leaf yield were weighed immediately after plucking for fresh weight and were subjected to drying. The degree of drying was observed when the dry weight data showed consistency.

(g) The leaf moisture was calculated as follows:

Moisture Content (%): Fresh weight-Dry weight X 100

Fresh weight

4. Observation and Discussion:

Mulberry varities in germplasm bank of rsrs, sahaspur:

Several varities of mulberry plant are found in Regional Sericulture Research Station, Sahaspur. The different varieties found here are listed in the table below:

SERIES	VARIETY
LF Series (2)	LF 1, LF 2
K2 Series (2)	K 2 (M), K 2 (S)
Alkaline Series (2)	AR 12, AR 14
RFS Series (2)	RFS 135, RFS 175
CW Series (2)	China White, China White (M)
Mandaley Series (2)	Mandaley (S), Mandaley (M)
HP Series (3)	HP 02/8, HP 02/12, HP 02/13
Tr Series (4)	Tr 4, Tr 5, Tr 8, Tr 10
Exotic (5)	Rokokuyaso, Kokuso 27, Ichinose, Kairorosa, Goshoerami
L Series (6)	L1, L2, L3, L4, L5, L6 (Shivalik)
MS Series (8)	MS 1 to MS 9
C Series (10)	C4, C740, etc
Others (15)	V 1, Local lobed etc
S Series (19)	S 13, S30, etc.

Discussion:

The study was conducted on L Series found in the Germplasm Bank of Regional Sericuture Research Station, Sahaspur. Total 6 varitiesof L Series (L1, L2, L3, L4, L5, L6) were studied. All the varities of L series are of Indian origin and collected by RSRS, Sahaspur, Dehradun from Uttarakhand.The mulberry, a perennial woody plant having fast growth and short proliferation period, belongs to family Moraceae and genus *Morus* (Yang *et al*., 2010). In general, 10-16 species of genus Morus are found in subtropical, warm and temperate regions of Asia, Africa and North America (Pérez-Gregorio *et al*., 2011). About 68 species of genus Morus occur, with most of them occurring in Asia. In China about 1000 varities are under cultivation.

Nutritive value of mulberry (*Morus*spp.) leaf is a key factor besides environment and technology adoption for better silkworm cocoon crop (Yogananda Murthy *et al*., 2013). Higher moisture content of mulberry leaves has a direct effect on growth and development of silkworm by favouring the ingestion, digestion and assimilation of nutrients. Mulberry leaves containing more water, total sugar and soluble carbohydrate and less mineral are best relished by silkworms. Nutritive requirement of silkworm larvae vary with the maturity of leaves fed. The plant is a very good source of ascorbic acid of

which over 90% is present in a reduced form, and also contains carotene, vitamin b1, folic acid, folinic acid, isoquercetin, quercetin, tannins, flavonoids and saponins (Mahesh *et al*., 2017)

The study was done during spring rearing of the silkworm. The aim was to study the qualitative and quantitative characters of the six varieties and to observe which variety is best among them for silkworm rearing. Five different characters of the Mulberry plants of L Series were studied. These include:

i. Moisture Content
ii. Number of Branches
iii. Length of the shoot
iv. Number of buds
v. Weight of the leaf on each branches

Detailed characteristics of different varieties of all the L series is described below:

L1 -It is of Indian origin. The tree usually grows erect with straight branching nature. The shoot colour of young plant is green, and on maturation turns greenish brown. The leaf is oval in shape, unlobed and green in colour. The margin of the leaf is serrate. Fruit obtained is black in colour, and sour in taste. The plant is monoecious.

L2- The nature of growth is spreading. Branching nature is straight. The colour of young shoot is green, while that of mature is greenish brown. The leaf shape is ovate, shallow lobed, and green in colour. The fruit is black in colour and sour in taste.


L3- The growth nature is erect, with straight branching nature. The colour of young shoot and mature shoot is green and greenish brown respectively. The leaf is oval in shape, unlobed and light green in colour. The margin of the leaf is serrate. Fruit obtained is black in colour, and sweet in taste.


L4- The growth nature is erect, with straight branching nature. The shoot colour of young plant is green, and on maturation turns greenish brown. The leaf is oval in shape, unlobed and light green in colour. The margin of the leaf is serrate. Fruit obtained is black in colour, and sour in taste.


L5- The nature of growth is spreading. Branching nature is straight. The colour of young shoot is green, while that of mature is greenish brown. The

leaf is oval in shape, unlobed and green in colour. The margin of the leaf is serrate. Fruit obtained is black in colour, and sour in taste.


L6- Thegrowth nature is erect, with straight branching nature. The colour of young shoot is green, while that of mature is greenish brown. The leaf is oval in shape, unlobed and green in colour. The margin of the leaf is serrate. However, unlike other varities, no fruits are produced by this genotype.


Data analysis:

The different parameters of the Local Varieties of Mulberry plant located in RSRS, Sahaspur, which were observed include

i. Moisture Content
ii. Number of Branches

iii. Length of the shoot
iv. Number of buds
v. Weight of the leaf on each branches

The following table shows the data of the parameters which were observed:

Table No. 1

VARITIES	MOISTURE CONTENT	NUMBER OF BRANCHES	SHOOT LENGTH	INTERNODAL DISTANCE	LEAF WEIGHT(gm)
	FRESH DRY				
L1	28.72 8.04	20 18 17	54, 48, 49 46, 54, 41 52, 42, 45	3, 5, 4 4, 5, 4 7, 4, 5	175 125 175
L2	42.37 6.97	12 9	44, 45, 31 35, 25, 35	4, 3, 5 4, 3, 3	150 75
L3	23.83 7.85	21 22 29	50, 61, 50 41, 38, 48 49, 48, 41	4, 5, 6 4, 5, 5 3, 3, 3	200 325 350
L4	32.41 10.32	23 22 15	44, 42, 35 64, 51, 48 64, 62, 57	4, 5, 4 6, 4, 4 5, 5, 5	375 250 325
L5	44.30 12.68	16 21 16	84, 29, 56 91, 83, 87 71, 73, 60	6, 6, 5 6, 7, 8 6, 6, 7	550 575 350
L6	23.83 7.85	20	49, 97, 31	4, 4, 3	75

Calculation:

The average for the parameters mentioned above is calculated as follows:

Moisture Content (%): Fresh weight-Dry weight X 100

Fresh weight

Number of branches, Shoot length, Internodal distance, Leaf weight: Average of given data

Table no. 2: shows the calculated data of the above parameters.

Varities	**Moisture Content (%)**	**Number of Branches**	**Shoot Lenght**	**Internodal Distance**	**Leaf Weight(gm)**
L1	72.01	18.33	47.88	4.55	158.3
L2	83.54	10.50	35.83	3.66	112.5
L3	67.05	24.00	47.33	4.22	291.66
L4	68.15	20.00	51.88	4.66	316.66
L5	71.37	17.66	70.44	6.33	491.66
L6	67.05	20.00	39.00	3.66	75.00

Table No. 2

Explaination:

From the above table, following conclusions can be drawn:

1. **Moisture Content:** Lowest moisture content was recorded in two varieties i.e., L-3 and L-6 (67.05%), while the highest was recorded in L-2 (83.54%).
2. **Number of Branches:** The highest number of branches were found in L-3 (24), and the lowest in L-2 (10.5).
3. **Shoot Length:** The shoot length was found maximum in L-5 (70.44), while the minimum shoot length was that of L-2 (35.83).
4. **InternodalDistance:** The least intermodal distance was found in L-6 and L-2 (3.66), and the maximum was recorded in L-5 (6.33). The minimum the internodal distance, maximum is the number of leaves. Thus the plant with minimum internodal distance has more leaves compared to others.
5. **Leaf weight:** The maximum weight of leaves was recorded in L-5 (491.66 g), while it was found least in L-6 (75g).

5. Conclusion

The main objective of this dissertation entitled "STUDIES ON tropical mulberry varities (l series) of regionalsericulture researchstation, sahaspur, dehradun, uttarakhand" was to study the qualitative and quantitative aspects of the six mulberry varieties of L series. The studies on the characteristics was done during spring season during which the silkworm was being reared in the sericulture research station. The plants were grown in the germplasm bank maintained at the Regional Sericulture Research Station, Sahaspur.

Though the rain fed and irrigated mulberry varieties of North West India have been discussed in previous pages, this study was on Local collection (L) and it has been concluded on the basis of the above data that L-5 variety has outperformed among all 6 varieties. L-5 variety has maximum shoot length and maximum leaf weight. The agronomical response of L-5 was found to be most suitable for rearing.

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Chapter - 5

Molecular Monitoring of Insecticide Resistance in Vector Borne Diseases

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Abstract

Insect vectors play a crucial role in the transmission of many new and re-emerging diseases. The resistance to insecticides is a major problem in the control of the major vector-borne diseases. Insects have evolved two major mechanisms to overcome toxicants; (1) behavioral resistance; and (2) physiological resistance. Physiological resistance refers to the ability of an insect population to survive exposure to a toxicant dose that would normally kill, including mechanisms of target-site resistance and metabolic-based resistance. Given the tremendous difficulty and investment associated with the development of new, safe and cost-effective insecticides, there is a grave need to preserve the efficacy of current and future insecticides. Understanding the molecular bases of insecticide resistance will provide valuable information as a model system for designing insecticide resistance management and monitoring strategies.

Keywords: Insect, vector, resistance, insecticide, target-site, metabolic

1. Introduction

Arthropod vectors play a crucial role in the transmission of many new and reemerging diseases. Sand flies transmit leishmaniasis [1]; ticks are vectors for lyme disease, babesiosis, ehrlichiosis and tick paralysis [2], mosquitoes transmit chikungunya, dengue, Japanese encephalitis, malaria, West Nile, yellow fever, and Zika [3]; fleas and lice transmit bartonellosis and rickettsiosis [4]. Attempts to control vector-borne diseases and vectors have been hampered because of parasite and host resistances. For example, the increase drug resistance has resulted in specific point mutations of the parasite's dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes that correlate with resistance to sulfadoxine-pyrimethamine [5] or the increased

insecticide application in the form of higher amounts, frequencies, doses and different varieties, decreased yields, environmental damage and outbreaks of vector-borne diseases in animals and humans leads to the resistance allele increasing over time [6_8]. Insect resistance to insecticides is a major problem in the control of the major vector-borne diseases. As the natural history of these diseases became better understood, the implemented control measures and protocols were highly effective in preventing the spread of the disease.

Given the tremendous difficulty and the investment associated with the development of new, safe and cost-effective insecticides, there is a grave need to preserve the efficacy of current and future insecticides. For these reasons, it is important to understand the mechanisms by which insects acquire resistance so that we can intelligently design strategies to delay the onset of the pesticide resistance problem. Insecticide resistance is believed to develop largely as the outcome of the selection pressures imposed by changed environmental conditions that allow some initially rare naturally occurring pre-adapted insects with resistance genes, to alive and pass the acquired resistance traits to their offspring. The resistant insects may multiply very rapidly than the susceptible ones, and when the continuous selection pressure is constant, it directly indicates that the insecticide is no longer effective [9].

2. Mechanisms of insecticide resistance

Resistance to organophosphate, organochlorine, carbamate and synthetic pyrethroid insecticides is evolved by a limited number of mechanisms in all insects analysed to date. Insects have conferred two major mechanisms of resistance to overcome the main chemical classes (toxicants); (1) behavioral resistance; and (2) physiological resistance so that it is no longer susceptible to alone or in combination insecticide resistance mechanisms. Behavioral resistance is defined as the ability of an insect to prevent or minimize lethal insecticide exposure [10]. This type can be further subdivided into direct contact excitation (‘irritancy’) and non-contact spatial repellency (repellent effect) responses [11]. The term ‘repellent effect’ involves an insect moving away from the insecticide-treated area without making direct contact, whereas ‘contact irritancy’ is when an insect leaves an insecticide-treated area only after making physical (tarsal) contact with the chemical [11].

In contrast, physiological resistance refers to the ability of an insect population to survive exposure to a toxicant dose/concentration that would normally kill, including reduced cuticular penetration, increase in excretion of insecticide before metabolism, increase in metabolic detoxification or

sequestration through overexpression or amplification of certain genes, and decreased sensitivity of pesticide target sites (Fig 1). Behavioral and physiological resistance often coexist in insect pests [10] and both forms can be involved with insect resistance. Identifying the prevalence and frequency of mechanisms conferring resistance may help inform the potential use of insecticides as alternatives to synthetic chemical pesticides for public health and crop protection and to prevent/manage resistance. Understanding the molecular bases of insecticide resistance will provide valuable information as a model system for designing appropriate insecticide resistance management strategies. In this chapter, we review the data currently available regarding molecular insecticide resistance in vector-borne diseases.

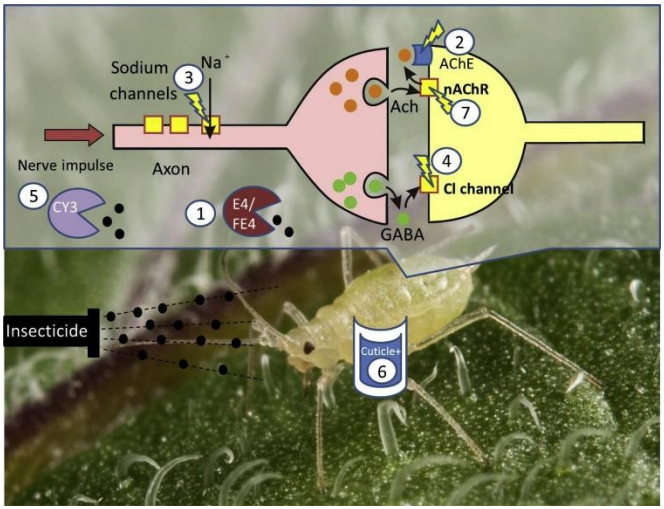

Figure 1. There are multiple distinct physiological resistance mechanisms in various insect species whose graphical representation is described in *Myzus persicae* [12]. These include (1) enhanced expression of E4/FE4 esterase confers resistance to carbamates, organophosphates and pyrethroids by metabolising and sequestering these insecticides before they reach the nervous system. (2) Mutation of the acetylcholinesterase (AChE) results in resistance to organophosphates and carbamates. (3) Mutation of the voltage-gated sodium channel confers knockdown resistance (kdr) to DDT and pyrethroids. (4) Mutation of the GABA receptor confers resistance to phenylpyrazoles and cyclodienes. (5) Enhanced P450 activity can be mediated resistance to organochlorine, organophosphate and pyrethroids. (6) Reduced penetration of insecticide through the insect cuticle confers resistance to neonicotinoids. (7) Altered the nicotinic acetylcholine receptor (nAChR), which confers resistance to neonicotinoids.

2.1. Target site resistance mechanisms

Target site resistance is caused as a result of a change in the affinity of the insecticides to their binding sites such as the voltage-gated sodium channel which causes knockdown resistance (kdr), mutations in the acetylcholinesterase (*ace*) gene, gamma-aminobutyric acid (GABA) receptor genes and ryanodine receptors (RyRs).

2.1.1. Voltage-Gated Sodium Channel (VGSC)

Mammalian VGSCs are composed of one pore-forming α subunit and one or two non-pore-forming β subunits, β1 and β2. The VGSC contains multiple α-subunits in mammals. The α-subunits consist of four homologous domains (I–IV), each of which contains six transmembrane segments (S1–S6), an intracellular N-terminal and C-terminal [13]. In contrast to conserved genes of the sodium channel α subunit in mammalian species, insect VGSCs transcript undergo both alternative splicing and RNA editing of only a single gene (para in *Drosophila melanogaster* and its orthologs) to generate pharmacologically and functionally distinct channel variants (Fig 2). Like the VGSC α-subunit genes, *DSC1* from the *D. melanogaster* sodium channel 1 gene and its orthologous, *Blatella germanica BSC1* gene, belong to the I–IV × S1–S6 transmembrane domain family. Despite *DSC1* and *BSC1* similarity to para gene, they originally encode calcium-selective channels [13, 14]. The sodium channel β subunit family of vertebrates were not identified in the genomes of insects and instead appear to use a novel family of glycoproteins, encoded by the *D. melanogaster TipE* gene and its orthologs, as an auxiliary subunit of the para sodium channel (Table 1) [13, 15].

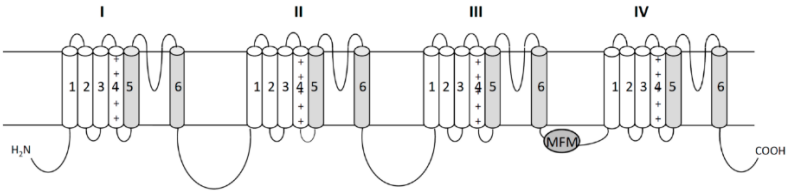

Fig 2. Voltage-gated sodium channel from mosquito. The α-subunit of the voltage-gated sodium channel has four homologous domains (I–IV), each domain is made of six α-helical transmembrane segments (S1–S6), an intracellular N-terminal and C-terminal. "+" represent positively charged residue in segment S4 [15].

The most well-characterized target site resistance for diseases vectors such as mosquitoes is *kdr* as it confers resistance against pyrethroids and DDT. The target of organochlorines (DDT and analogues) and synthetic pyrethroids are the sodium channels of the nerve sheath. Pyrethroid insecticides, including

type I (non-alpha-cyano group, such as permethrin) and type II (alpha-cyano group, such as alpha-cypermethrin and deltamethrin) formulations, are registered and used for the prevention and control of outbreaks of vector-borne diseases and personal protection at household level applications [16]. The type of their interaction holds the channels open for such long periods that the membrane potential ultimately becomes depolarized, to the point at which generation of the action potential is not possible [17]. The chronic depolarization of the nerve and muscle cell membrane resulting from the persistent inward sodium conductance induces repetitive nerve firing and hyperexcitability, leading to paralysis and eventual death of the insect.

The channel modulators are selective for insect sodium channels than for mammalian sodium channels, which coupled with differences in temperature-dependent activity, rates of detoxification and create them selective and potent insecticides [18]. Several *kdr* mutations have been shown associated with resistance to pyrethroids and DDT in *Aedes aegypti* from, Latin American, Caribbean and Asian countries [19, 20]. The *kdr* mutations at codon 1534 (F1534S/L/C) and I1532T are described in populations of *Ae. albopictus* in Italy [21], Singapore [22] and China [23].

Six novel mutations and three well-known substitutions (M815I, T917I, L920F) have been reported as a possible knockdown resistance-associated mutation in head and body lice, louse populations. The novel amino acid substitutions clustered in the IIS1-2 extracellular loop (H813P) and IIS5 (I927F, L928A, R929V, L930M, and L932M) of the α-subunit [24]. F815I, N818D and K794E mutations were also found for the first time not only in head lice but also in insect species. F815I, N818D mutations occurred in the linker IIS1-2 extracellular loop and K794E occurred in IIS1of the α-subunit [25]. Substitution of a Leu to His amino acid detected upstream of *VGSC* gene in position 29 of exon I the previously known *kdr* mutation site could be an indication for other insecticide resistance-related mutations in field populations of *Anopheles culicifacies* from Baluchistan of Iran [26]. A very low frequency of *kdr-his* resistance mutation (L1014H) was observed in *Musca domestica* collected from northwestern Iran [27]. The *kdr* mutation of L1014F in *B. germanica* which is resistant to pyrethroid was also found from various locations in the United States, Germany, Iran, India, China [28].

Different studies have attempted to reconstruct the evolution of *kdr* alleles in various *Ae*. *aegypti* populations. A number of mutations have been identified to be associated with *kdr* resistance to insecticides, most of which are located in the S6 segment of domain II and III (Table 1) [29]. These include G923V, L982W, I1,011M, V1,016G and V1005G/I mutations were

identified in 2003 [15; 19], V1,016I and I1,011V in 2007 [16], V1016G+D1763Y in 2009 [30], S989P+V1016G, F1534C and F1552C in 2010 [31, 32], V1016G+-F1534C and S989P+V1016G+F1534C in 2014 [33], T1520I+F1534C in 2015 [34], V410L+F1534C in 2017 [35], V410L, V410L+F1534C, V410L+V1016I and V410L+V1016I+F1534C in 2018 [36, 37], V1011M+V1016G in 2019 [38], Q1835R [39]. However, only a few mutations including V1016G/I, S989P, I1011M, F1534C, V1005G, D1763Y, T1520I, F1552C and V410L, alone or in combination, have been confirmed to be associated with resistant phenotypes or functionally corresponded well to reduce the sensitivity of *VGSC* gene to pyrethroids [31, 37, 40–42]. Additionally, the substitutions (e.g., F1534C/L/S and I1532T) in the *VGSC* have been shown to significantly reduce the channel's sensitivity to type me but not to type II pyrethroids [43].

A high frequency (71%) of the V1016I *kdr* mutation in *Ae. aegypti* populations have been revealed from Martinique [44]. The frequency of the F1534C resistant mutation ranges from 40 to 80% shown in *Ae. aegypti* and *Ae. albopictus* from Malaysia whereas the V1016G mutation is found at around 20 to 39% and a significant correlation was also established between 1534C genotypes and pyrethroid resistance, while no significant correlation was found for the V1016G mutation. However, an additive effect to pyrethroid resistance was observed when both 1534C and 1016G were present [45]. In some cases, mutation co-occurrence can have an additive or even synergistic effect resulting in very high levels of resistance [46]. A synergistic effect has been reported in the presence of the triple mutant V1016G+F1534C+S989P, which substantially reduced sensitivity to both permethrin and deltamethrin by 1100-fold and 90-fold, respectively [47].

There are multiple *kdr* alleles in *Ae. aegypti*; some with global distribution and others that are found in more limited areas of the world. For example, the alleles containing only the F1534C mutation were widely distributed in the populations from Asia and the Americas. Alleles containing the S989P+V1016G mutations were found only in Asia and alleles with the V1016I mutation were only found in the Americas [39]. In *Ae. aegypti* mosquitoes, the functional expression system of a sodium channel, AaNav1–1, was successfully established in *Xenopus* oocytes to examine whether any of the seven (S996P, I1018V/M, V1023G/I, F1565C and D1794Y) pyrethroid-resistance mutations found in *Ae. Aegypti*. Two mutations (L1021F/S) in pyrethroid-resistant *Anopheles* and *Culex* mosquitoes affect pyrethroid sensitivity of a sodium channel. The involvement of three *Ae. aegypti* mutations (I1018M, V1023G in IIS6, and F1565C in IIIS6) and also two *Anopheles*/*Culex* mutations (L1021F/S in IIS6) of the nine mutations reduce

sodium channel sensitivity to pyrethroids (Table 1) [48].

2.1.2. Acetylcholinesterase (AChE)

Acetylcholinesterase is a critical enzyme for hydrolysis of the neurotransmitter acetylcholine in cholinergic nerve synapses and is a target for organophosphate (e.g., malathion, fenitrothion, temephos) and carbamate insecticides (e.g., propoxur, pirimicarb, sevin) [49]. The anticholinesterase insecticides inhibit enzyme activity by forming a covalent bond with a serine residue at the active site of the enzyme and disruption of this mechanism leads to accumulation of AChE that causes overstimulation of target membrane receptors and eventual blockage of neurotransmission [50]. Most insects possess both AChE1 and AChE2 enzymes that encode by *ace1* and *ace2* genes, respectively. Drosophila melanogaster and other flies possess only one gene that is probably due to a secondary loss during the evolution of the Diptera [51]. Between the two AChEs, AChE1 has been proposed as a major catalytic enzyme based on its higher expression level and frequently observed point mutations associated with insecticide resistance [52].

Insensitive AChE has been reported from *Cx quinquefasciatus* [53, 54], *Cx. pipiens* [55], *Cx. tritaeniorhynchus* [56, 57], *Cx. vishnui* [56], *An. gambiae* [53, 58, 59], *Ae. aegypti* [50]. Across all insect species, there are two major classes of target site insensitivity mutations, conferring high carbamate and low OP resistance due to a Ser-Phe mutation in the vicinity of the acyl pocket of acetylcholinesterase, or high OP with either equivalent or low carbamate resistance [60]. All the mosquito AChE target site resistances to date fall into the former category. In *ace-2*^{R} of *Cx tritaeniorhynchus*, the F455W mutation was identified to confer insecticide insensitivity of acetylcholinesterase [57]. The mutation encoded by the *ace-1*^{R} gene induces substitutions G119S (glycine to serine) and F331W (Phenylalanine to valine) in resistant mosquitoes [57, 61].

The F331W substitution was selected only in resistant *Cx. tritaeniorhynchus* in East Asia [57]. In contrast, the G119S substitution was found independently in several mosquitoes' species including *An. sinensis* from China and China-Vietnam border [62, 63], *An. gambiae* from Mali and Cameroon [58, 59], *Cx. vishnui* from China [55] and *Cx. pipiens* from Morocco [64] in which it is largely spread worldwide. The Phe-to-Val substitution of residue 290 (F290V) was shown at *ace-1*^{R} gene in resistant *Cx. pipiens* in Cyprus and substituting phenylalanine to a smaller residue may prevent large substrate with a probable orientation of the valine side chain or inhibitor molecules to interact efficiently with the catalytic serine (Ser200) [55]. These

mutations constrict the neck of the active site gorge, limiting the accessibility of inhibitors to the catalytic serine located at the base of the gorge [61], as the acetylcholine turnover number of G119S *An. gambiae* is reduced to 3% of the wild-type (WT) enzyme [65]. The structure of G119S acetylcholinesterase (*ace1*) mutation of *An. gambiae* reveal unique properties within the active-site gorge distinct from human acetylcholinesterase, including a distinct open channel at the base of the gorge, and provide a means for improving selectivity of species in the design of effective insecticides for malaria vector control strategies (Fig 3) [66].

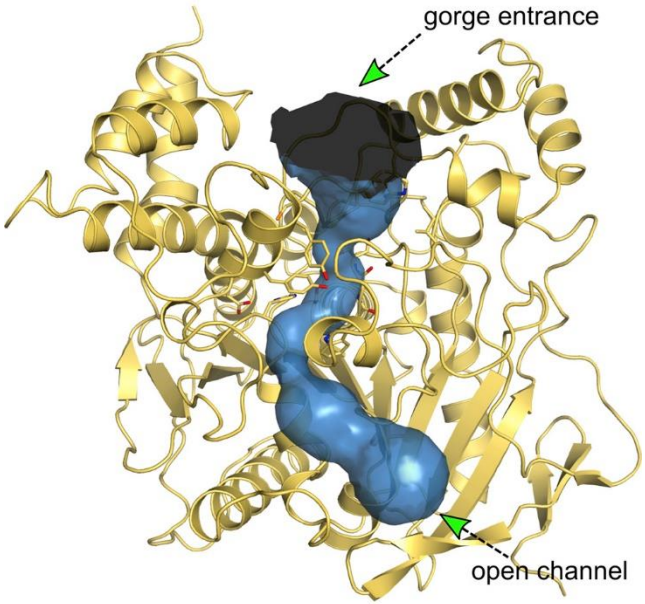

Figure 3. Crystal structure of the G119S mutant AChE of *Anopheles gambiae* reveals basis of insecticide resistance [66].

All resistant specimens by the G119S mutation in *An. gambiae* populations carried the resistant allele (R) in its heterozygous form [58]. Several studies have reported that homozygous resistant individuals are more likely to die during pupation than susceptible individuals [54, 61]. In *M. domestica*, several different point mutations in and around the catalytic site of the AChE gene (I162M, V260L, G342A, G342V, F407Y, G445A, V180L, G262A/V, F327Y, G365A) were described either alone or in combination to confer wide spectrum of resistance to various insecticides [67, 68]. In *D. melanogaster* and *M. domestica*, the F290Y substitution of AChE2 alone provides a low level of resistance to many distinct insecticides while G262V confers much stronger

levels of insecticide resistance to certain compounds (Table 1) [67, 69].

2.1.3. The gamma-aminobutyric acid (GABA) receptor

The neurotransmitter gamma-aminobutyric acid (GABA) is widely distributed in the mammalian and insect central nervous systems and the neuromuscular junction of insects. The type a receptor for the GABA neurotransmitter belongs to a superfamily of ligand-gated ion channels that contain an intrinsic anion; by binding to these receptors, endogenous GABA causes the opening of chloride ionophore channels resulting in hyperpolarization of the membrane. The target site of several classes of insecticides including cyclodienes (e.g., dieldrin and α-endosulfan), Phenyl pyrazoles (e.g., fipronil and ethiprole) and Hexachlocyclohexanes is $GABA_A$ receptors and these insecticides bind to a specific site (the picrotoxin site) on the chloride channel, thereby blocking its opening and thus antagonizing the inhibitory effects of GABA [70]. Type II pyrethroids (e.g., deltamethrin), but not type I compounds, also bind to and inhibit $GABA_A$-gated chloride channels, albeit at higher than usual concentrations that affect the inhibition of sodium channels [17].

GABA receptors comprise five subunits arranged around the central ion channel. Each subunit contains an extracellular N-terminal domain that encompasses the ligand-binding domain and four transmembrane segments (TM1–4), with a variable intracellular loop between TM3 and TM4. The TM2 segment lines the channel pore [71]. In insects, three genes encoding GABA receptors have been identified: resistance to dieldrin (*RDL*), GABA and glycine-like receptor of Drosophila (*GRD*), and ligand-gated chloride channel homologue 3 (*LCCH3*). The insect GABA receptor that has been studied most intensively is *RDL*, which was originally identified in *D. melanogaster* with the replacement of a single alanine (Ala301) at the start of the second transmembrane region with a serine or more rarely to a glycine [72]. This mutation is presumed to be the principal constituent of the ion-channel lining and appears to confer both insensitivity to the insecticide and a decreased rate of desensitization [65, 73].

Subsequently, additional resistance-associated target site mutations have been identified in *RDL* from the different species. For example, the A296S substitution of TM2 domain was associated with dieldrin resistance in mosquitoes such as *An. arabiensis* [74], *An. funestus* [75], *An. sinensis* [76, 77]) and *An. maculatus* [78]. The A296G mutation in *Ae. albopictus* and *Ae. aegypti* showed lack of responsible for insecticide resistance but it does decrease the fitness which significantly affects neuronal signaling [79]. Several other

mutations have been detected that associated with resistance including A296G and T345S substitution in *An. gambiae* [74], A302S in *Cx. p. quinquefasciatus* and *Ae. albopictus* [80], V327I, located in the intracellular loop between TM2 and TM3, in *An. funestus* [67], V327I, A296S, T345S in *An. sinensis* [76] and N530K and H539Q in *An. coluzzii* [81].

Other studies, measuring the prevalence of mutations at A302S/G to predict the extent of resistance in varying countries noted 11% of 154 *Anopheles* mosquitoes of various species (*An. vagus, An. barbirostris, An. nigerrimus* and *An. farauti*) from Indonesia [82], whereas 100% *An. sinensis* populations from China were found to have either 327I and 345S mutation in addition to 296S [76]. Insecticide resistance in *An. sinensis* may be mediated by other mutations R356G, 357L/N and Q408H on the binding site of GABA receptors. So, more functional studies are needed to confirm it (Table 1) [83].

2.2. Metabolic-based resistance

Metabolic-based resistance involves the insecticide bio-transformation by detoxifying enzymes and is now considered as one of the key mechanisms of insect resistance to the chemical insecticides [65]. This mechanism can be resulted from two distinct but additive genetic events, referred to a mutation in coding sequence of enzyme leading to a better metabolism of the insecticide and a mutation in a non-coding regulatory region leading to the amplification and overexpression of an enzyme capable of metabolizing the insecticide [44]. The metabolic resistance mechanisms involve three major enzyme families, the cytochrome P450 monooxygenases (P450s), carboxy/cholinesterases (CCEs) and glutathione S-transferases (GSTs) [72] and ATP-binding cassette (ABC) transporters are also involved in the metabolic detoxification and excretion [84].

2.2.1. The cytochrome P450 monooxygenases

Cytochromes P450 enzymes include a superfamily of hydrophobic and heme-containing monooxygenases that catalyze the oxidation and metabolism of a large number of endogenous (hormones, fatty acids and steroids) and bioactive exogenous (drugs, pesticides and plant toxins) compounds [85]. To date, insect P450 genes have been assigned to four major clades; the mitochondrial P450s (Mit P450s), *CYP2*, *CYP3* (including *CYP6* and *CYP9*) and one, *CYP4*, is shared with sequences from vertebrates [86, 87]. Genes in the *CYP2* clade are evolutionarily conserved and play a role in the sense of organs development in insects and ecdysone synthesis [88]. Genes from the *CYP3* clade, particularly those belonging to *CYP6* and *CYP9* families,

have been involved in xenobiotic metabolism and resistance to insecticides across a range of insect species [89].

P450 genes have different functions and are differentially expressed. For instance, some P450s are larvae-specific (e.g., *CYP6B2*, *Helicoverpa armigera*; ISOP450, *Cx. pipiens quinquefasciatus*) whereas others are adult-specific (e.g., *CYP6Z1*, *An. gambiae*) [90, 91]. Cytochrome P450 enzymes are encoded by microsomal and mitochondrial *CYP* genes and each P450 is assigned to a family (designated by a number), subfamily (designated by a letter) and individual (designated by a number) [92]. P450s can be identified in a broad range of insect tissues where they are expressed in response to environmental and physiological stimulators. The resistance P450 genes were often present in the brain, midgut and Malpighian tubules and other organs such as the central nervous system may critical function for detoxification and response to insecticide resistance [93].

Insects are evolving resistant to insecticides. So, it is an urgent need to identify more P450 genes involved to become an effective monitoring tool. The development of molecular-based studies was helped us to identify several P450s genes in insects associated with many insecticides' resistance, such as organochlorine (eg., DDT, Lindane), organophosphate (e.g., Chlopyriphos-methyl, Pirimiphos-methyl) and especially pyrethroids (e.g., Permethrin, Deltamethrin). These include *CYP6P9a/b* [94], *CYP6P9b* [95], *CYP6BB2*, *CYP6Z8*, *CYP9M5* and *CYP9M6* [96], *CYP6Z9* [97], *CYP6Z1* [98], *CYP6M2* [99]. Several studies have directly (e.g., in vitro and in vivo metabolism) or indirectly (reduction in resistance with the P450 inhibitors such as piperonyl butoxide (PBO)) implicated P450-mediated metabolism as a mechanism of resistance in mosquitoes. In *Ae. aegypti*, *CYP9M4*/*5*/*6*/*9*, *CYP6M6* and *CYP6M11* have been found over-transcribed in pyrethroids-resistant strains [44, 100]. *Anopheles gambiae* and *An. funestus* are the most important vectors of malaria in sub-Saharan Africa. In *An. gambiae*, *CYP6Z1*, *CYP6P3*, *CYP6Z6* have been frequently found constitutively over-transcribed in pyrethroids, carbaryl and DDT-resistant strains [101, 91]. In *An. funestus*, *CYP6P9*, *CYP6P4*, *CYP6Z1*, *CYP6Z3* and *CYP6M7* as being strongly associated with pyrethroid resistance [102, 103]. The results indicate that contrary to *Anopheles* mosquitoes, genes from the *CYP9* family play a more important role than those from the *CYP6* family in insecticide resistance in *Ae. aegypti* [45].

Molecular mechanisms of P450-mediated resistance to multiple insecticides can be attributed to changes in gene expression (e.g., regulatory

region), a coding sequence or post-translational protein modifications (e.g., regulatory protein and phosphorylation) and copy-number (e.g., gene amplification or gene duplication) [96]. For example, the CNV (copy number variation) affecting the P450s, *CYP9J21/22* from Thailand and French Guiana [104], *CYP6BB2* and *CYP6P12* from Laos [78], in *Ae. aegypti* are associated with pyrethroid and possibly DDT resistance, while the overexpression of *CYP9M6* in *Ae. aegypti* was partially due to gene amplification in pyrethroid-resistant strains from USA (Table 1) [100].

2.2.2. Glutathione S-transferases (GSTs)

The glutathione S-transferases (GSTs) are encoded by a large gene family and found almost in all eukaryotes and restricted to certain bacteria including proteobacteria, cyanobacteria and a few Gram-positive bacteria [105]. According to cellular locations in eukaryotes, GSTs are divided into at least three different families, namely cytosolic GSTs, mitochondrial GSTs and microsomal GSTs [106]. To date, only the cytosolic and microsomal groups have been discovered in insects [105]. Cytosolic GSTs are typically small proteins (200–250 amino acids) that are activated as either homo- or heterodimers. Based on sequence homology in the N-terminus, substrate specificity, immunoreactivity, and sensitivity to several inhibitors, cytosolic GSTs in insects, which constitute the largest GST family are divided into seven distinct classes (delta, epsilon, omega, sigma, theta, zeta and unclassified) [107]. Microsomal GSTs, with a completely different structure from the cytosolic enzymes, consist of approximately 150 amino acids which are membrane-associated that are active in ecosanoid and glutathione metabolism [108]. High expressions of GSTs were detected in the midgut and the fat body for detoxification of xenobiotic compounds [109].

GSTs are multifunctional enzymes involved in the detoxification of xenobiotics and endogenous compounds. High levels of GST activity have consistently been found in resistant insect strains and play a major role in resistance to various insecticides such as organophosphates, chlorinated hydrocarbons, and pyrethroids [95, 110]. GSTs-mediate resistance confers via the major known mechanisms including the glutathione (GSH) conjugation to insecticides that lead to the production of a less toxic soluble conjugate, the dehydrochlorination reaction by removing a hydrogen atom from the substrate leading to the elimination of chlorine (using GSH as a co-factor), peroxidase activity that reduces the toxic peroxides generated by oxidative stress caused by insecticide intake and finally, the confer resistance through the non-catalytic passive binding of the insecticide [111]. In addition to their catalytic properties, GSTs also function as non-enzymatic carrier proteins or ligandins

in intracellular transport [112].

The delta and epsilon GSTs are unique and exist only in insects [113] and these specific classes were shown to be involved in the neutralization of toxic compounds and to play a key role in conferring insecticide resistance [105]. The epsilon class is represented at least by eight members in the mosquitoes: *GSTe1-8*. Several reports revealed that *GSTe2* is the most over-expressed detoxification gene in DDT and pyrethroid-resistant mosquitoes [114]. Five of the eight epsilon classes have elevated expression levels of the GSTs in a DDT resistant strain of *An. gambiae* [115]. *AgGSTe2* is the only member of the five *GSTes* with confirmed DDT-dehydrochlorinase activity. *GSTe2* and *GSTe3* were characterized from *Spodoptera litura* and *SlGSTe2* with a high-affinity binding site for DDT has higher DDT-metabolizing activity than *SlGSTe3* and more likely plays a role in conferring insecticide detoxification [116]. In addition to its over-expression, a single amino acid mutation on position 119 on the gene (*L119F-GSTe2)* confers high levels of metabolic resistance to DDT and pyrethroids in *An. funestus* mosquitoes [114]. The over-expression of *GSTe2* and *GSTd3* coupled with metabolic resistance was found in *An. gambiae* populations [117, 118]. The sigma class also confers resistance to insecticides including DDT and fenitrothion in *Phlebotomus argentipes* and *Hyphantria cunea* species in response to oxidative stress (Table 1) [110, 119].

2.2.3. Carboxylesterases or Carboxy/Cholinesterases (CCEs)

The classification and nomenclatures of a large group of esterases enzymes are so difficult and complex. Many researchers proposed some classification system based on the interaction of esterases with OP molecules (Aldridge 1953), inhibition criterion, substrate and electrophoretic mobility criterion and phylogenetic/genomic criterion [120]. Based on the phylogenetic/genomic criterion, analysis of 425 insect genes segregated the carboxyl/cholinesterases (CCEs) into 14 clades (e.g., α-esterase, gliotactins, β-esterase (I, II), neuroligins) that are within three classes [121]. These classes were created based on the phylogenetic similarity of carboxylesterases genes and some different physiological characteristics. (i) The neuro/developmental class contains proteins that are catalytically inactive comprising the neuroligin, gliotactin and neurotactin lineages except for the catalytic active acetylcholinesterases. (ii) Hormone/semiochemical processing class includes secreted esterases (e.g., integument, beta, glutactin) that have specific substrates (hormones and pheromones). (iii) The dietary/detoxification class contains intracellular enzymes (e.g., mitochondrial, cytosolic and microsomal alpha estrases) with dietary and xenobiotic detoxification functions [120].

Insecticide resistance can be conferred by mutant enzymes in all three classes.

Many critical biological functions perform from detoxification of foreign chemicals (xenobiotics) to physiological regulation of endogenous compounds by CCEs in Arthropoda. The α/β and acetylcholine esterase groups are associated with resistance to insecticides such as organophosphates, carbamates and pyrethroids. Esterase-mediated resistance involves two main mechanisms in more insect species: target sites mutations or metabolic [121, 122]. The target sites mutations can alter the catalytic properties of acetylcholinesterases (AChEs) giving them less sensitivity to inhibition by insecticides, whereas metabolic resistance mainly occurs due to hydrolysis of insecticide with an increase in the expression or activity of α/β-carboxylesterases (CESs) enzymes [122]. According to Aldridge 1953 classification system, the esterases are also grouped into A-, B-, and C-esterases. Esterases A (Est-A) are capable of hydrolysing OP compounds, esterases B (Est-B) are inhibited by them and esterases C (Est-C) do not interact with OPs [120, 123].

Organophosphates induce toxicity largely by targeting serine enzymes of CESs and AChEs. The enzymes use the serine esterases, a catalytic triad: serine, glutamic or aspartic acid, and histidine, for hydrolysis. The catalytic residues act as the nucleophile in the triad. Inhibition of CES and AChE through organophosphates takes place by replacing the active-site serine residue of CES (S203) and AChE (S200) with a leaving group of organophosphates (Fig 4) [124]. The hydrolysis reaction of ester bonds catalyzed by the esterases to release an acid and an alcohol as metabolites with a two-step reaction. The first reaction begins with the nucleophilic attack by the catalytic serine hydroxyl on the carbonyl carbon atom of the substrate, forming an acyl-enzyme (acylated, carbamylated and phosphorylated when the substrate is an ester of carboxylic, carbamic and phosphoric, respectively) by liberating the alcohol metabolite. In the second step, a water molecule is needed for a nucleophilic attack on the acyl-enzyme bonds resulting in the liberation of the acid metabolite and the free active enzyme [120].

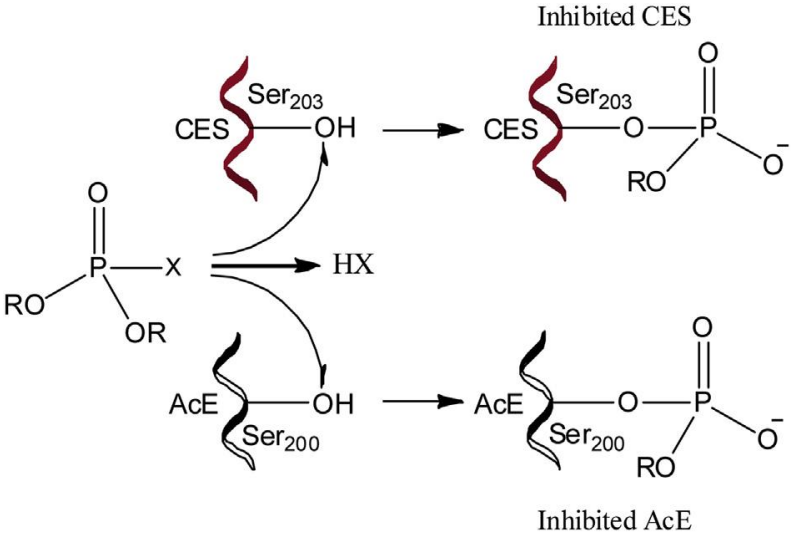

Fig 4. Inhibition of carboxylesterase (CES) and acetylcholinesterase (AChE) by organophosphates [120].

Overproduction of the non-specific carboxylesterases is a common resistance mechanism of mosquitoes to organophosphate insecticides [72]. For example, resistance alleles in five consecutive *CCE* genes on chromosome 2 induced by selection with malathion also confer cross-resistance to other organophosphates at larval and adult stages but not to the pyrethroid deltamethrin in *Ae. aegypti* populations in Laos [125]. Two gene loci, Est-2 (encoding esterase B) and Est-3 (encoding esterase A), encode amplification or up-regulation of non-specific esterases in the *Cx. pipiens* complex. To date, 13 Ester alleles conferring OP resistance have been identified at the Ester locus in the *Cx. pipiens* complex [126, 127]. Esterases A and B were also found to be significantly overexpressed (> 10-folds, $P < 0.05$) in very high pyrethroid resistance in *Cx. quinquefasciatus* populations from Cameroon [8].

According to dietary/detoxification class classifications, the alpha esterases of *CCEae3a* and *CCEae6a* were upregulated more than 60- to 29-fold respectively in conferring temephos resistance in some populations of *Ae. aegypti* larvae from Thailand. The worldwide temephos insecticide resistance is supported by the over-expression of the two genes through gene amplification in *Ae. albopictus* populations of Greece and Florida. All individuals from Greece and Florida with *CCEae3a* and *CCEae6a* co-amplicon share a common haplotype, indicating a single amplification event that spread between the two countries, highlighting the importance of

monitoring of disease vectors carrying resistance mechanisms, which is mainly facilitated by anthropogenic activities [128]. Two types of amplification were also found in *Ae. albopictus* populations: a *CCEae3a* gene amplification and a *CCEae3a- CCEae6a* co-amplification [16, 128]. Temephos resistance was particularly identified with *CCEae3a* overexpressed through gene amplification. Despite increasing reports of the incidence of temephos resistance in *Ae. aegypti* populations from Brazil [130] and Greece [12, 128], the overexpression of the *CCEae3a* gene was not observed in resistance to the OPs temephos and malathion in *Ae. aegypti* samples from Pakistan (Table 1) [129].

Table 1: The resistance mechanisms of insects of medical importance to chemical insecticides, including the target-site mutation **(**Voltage-Gated Sodium Channel (VGSC), Acetylcholinesterase (AChE), Gamma-Aminobutyric Acid (GABA)**)** and enhanced metabolism **(**cytochrome P450, Glutathione S-transferase (GSTs), Carboxy/Cholinesterase (CCE)**).**

Taxon	**Mutations identified**					
	Target site mutations			**Enhanced metabolism**		
	VGSC	AChE	GABA	CYP450	CCE	GST
Musca Domestica	D600N, M918T, T929I, L1014F/H, L932F [12, 27, 131, 132]	I162M, V260L, G342A, G342V, F407Y, G445A, P119S, V182L, G265A, F327Y, D342V, W233G/L, V180L, G262A/V, F327Y, G365A, F290Y, A201S, G227A [67, 68, 72, 132]	-	*CYP6A1*, *CYP6D1/3* [133, 134]	G137D, W251L, G139N (E3 esterase) [122]	-
Aedes albopictus	I1532T, F1534C/LS, V1016G [135,	-	A302S [80]	*CYP4G16*, CYP6Z6, *CYP6N3*, *CYP6AG6*,	*CCEae3a*, *CCEae6a* *[79]*	-

	136]			*CYP6P4/12* [45, 79, 88]		
Aedes aegypti	I1011M/V, V1016G/I, V1005G/I (IIS6), S989P, D1794Y (IIL5-6), T1520I, F1552C, F1565C, F1269C and F1534S/L/C (IIIS6), V410L (IS6), S982P, G923V (IIS5), I1018M/V, V1023G (IIS6) [19, 30, 32, 37, 74, 79, 137]	-	-	*CYP4H25/28*, *CYP6AH1*, *CYP4C50*, *CYP6BB2*, *CYP6CB1*, *CYP6F3*, *CYP6M6/10-11*, *CYP6N12/17*, *CYP6P2/12/9a/9b*, *CYP6Z6-8*, *CYP9J10/11/19/21-24/26-28/32*, *CYP9M4-6/9*, *CYP304C*1, *CYP12F6* [44, 78, 79, 100, 125, 138-140]	*CCEae3a/4a*, *CCEae6a* [16, 79, 139, 114, 140]	*GSTe2*, *GSTe7* [79, 139-141]
Anopheles spp.	L1014F/S/C/W, V1010L, N1013S, N1575Y (III-IVL) [48, 95, 117, 142, 143]	G119S	A296S/G, T345S/M, V327I, A302S/G, N530K, H539Q, N485I [75, 78, 80-83, 94]	*CYP6AA1*, *CYP314A1*, *CYP325A3*, *CYP6AG1*, *CYP325C2*, *CYP12F1*, *CYP4H24*, *CYP9K1*, *CYP4G16*, *CYP*6M2*/7*, *CYP6CM2*, *CYP6N1*/3 *CYP6P3/4/4a/4b/9/9a/9b*, *CYP6Z1-3/6* [91, 95, 101-103, 138, 144-146]	Overexpression of *Esterase A (α)* and *Esterase B (β)* [147]	Overexpression of *GSTe1-3*, *GSTd1-5* [95, 117, 141]
Culex spp.	L1021F/S, L1014F/S/C (IIS6), L982F (II6), A109S (LI1), W1573R	G119S, F331W, F290V, F455W [8, 55, 56, 58, 60]	A302S/G [80]	*CYP6AA7*, *CYP4H34*, *CYP9J34/40*, *CYP9J40*, *CYP9M10*, *CYP6Z10*	Overexpression of *Esterase A (α)* and *Esterase B (β);* Ester[1] (A1), Ester[2]	*GSTd* (CPIJ002678 gene) [150]

	(III-IVL), L932F, I936V (IIS5) [8, 48, 148, 149]			[8, 93]	(A2-B2), Ester^{4} (A4-B4), Ester^{5} (A5-B5), Ester^{8} (A8-B8), Ester^{9} (A9-B9), Ester^{11} (A11-B11), Ester^{B1} (B1), Ester^{B6} (B6), Ester^{B7} (B7), Ester^{B10} (B10), Ester^{B12} (B12) [79, 126]	

3. Conclusion

This article reviews recent studies on the mechanism of resistance to insecticides in most insect vectors of human pathogens, mainly focusing on the distribution of structural modifications of proteins (sodium channels, AChE and GABA receptors) and metabolic detoxification genes (cytochrome P450 monooxygenases, GSTs and CCEs) to contribute to control the pests and vector-borne diseases and to improve vector control services. These findings highlight the importance to monitor and quantify the level of insecticide resistance in insects. Taking action to limit the spread of resistance alleles into the non-infected areas would be helpful to prevent or delay the development of insecticide resistance.

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