CHAPTER III

MATERIALS AND METHODS

The present study was undertaken at the Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu Rice Research Institute (TRRI), Aduthurai and Tapioca and Castor Research Station (TCRS), Yethapur during 2013 – 2017.

The methodology and techniques adopted for conducting experiments based on the objectives set forth in the studies are presented hereunder.

3.1 Distribution and diversity pattern of mite species in different agro climatic zones of Tamil Nadu

3.1.1 Field Survey

Mite species (both herbivorous and predaceous) of various hosts were collected through direct survey from various locations in Tamil Nadu during 2013 - 2017. The locations of different climatic regimes and respective crops which were surveyed are given below.

Table 1. The locations of different agro climatic zones of Tamil Nadu and respective crops

S.No	Zone	Districts	Crops
1.	North zone	Salem and Namakkal	Tapioca, rice, castor, papaya, cotton and mango
2.	Western zone	Erode and Coimbatore	Jasmine, rose, chilli, brinjal, bhendi, papaya, grapes, rice, tapioca and cotton
3.	Cauvery delta zone	Thiruchirapalli, Thanjavur and Aduthurai	Rice, banana, black gram and cowpea

4.	Southern zone	Madurai, Tirunelveli and Periyakulam	Rice, maize, sorghum, brinjal, bhendi, chilli, jasmine and grapes
5.	Hilly zone	Ooty, Kothagiri and Valparai	Tea

3.1.2 Sampling and collection of mites

Intensive collections of mites were made from major crops at five climatic zones of Tamil Nadu. Mite infested leaf samples were collected separately from each locality in polythene covers, which were subsequently sealed. The covers were labeled properly giving details of crop, locality, date of collection *etc*., and brought to the laboratory for further examination. In order to prevent water accumulation in the covers, precautions were taken not to expose them directly to the sun.

Each sample collected was assigned with an accession number, and the details such as place and date of collection, host plant, age, stage of the plant, season of collection and mites details *etc*. In most of the cases, the samples were processed immediately. When they could not be processed on the same day, they were stored in a refrigerator at 4^{o}C and such samples were processed within four days.

In the laboratory, the leaves were observed under stereo binocular microscope with a magnification ranging from 10 – 35X. The mites were picked with fine needles moistened with media and mounted on glass slides.

3.1.3 Processing and preparation of permanent slides

Permanent slides of the mites collected during the survey were prepared for further taxonomic study. Hoyer's medium was used for mounting mites and the ingredients of the medium are given below.

Distilled water - 50 ml

Gum Arabic - 30 g

Chloral hydrate	-	200 g
Glycerol	-	20 ml

The mite was mounted on a drop of Hoyer's medium on a clean dry glass slide. The specimen was pushed down the media to rest on the surface of the glass slide and then oriented in such a way that the gnathosoma was facing towards the observer and the legs and gnathosoma of the specimen were stretched properly. A zero number glass cover slip of 12 mm diameter was placed gently on the media in such a way that no air bubble appeared between the glass slide and the cover slip. A single specimen was mounted on each slide and up to 20 such slides were prepared for each sample. The slides of the prey mites were also prepared wherever available.

The slides were labeled on the right side with the locality label furnishing the following details *viz*., place of collection, date of collection, habitat, host plant, name of the collector and the accession number. Identity label was pasted on the left side of the slide. The slides were serially numbered under each accession number.

The slides so prepared were kept in a hot air oven at 40^{o}C temperature for drying. After drying the mounted slides were ringed, i.e., the cover slips were sealed with a clear nail polish/glycerin using fine brush to avoid shrinkage of the medium. All mounted slides were stored in slide storage boxes in a cool place. The prepared slides were identified based on literature and some species were sent to the experts for correct identification and confirmation.

3.1.4 Illustrations

The photographs of the identified mite specimens depicting their morphological characters were made using Leica DM 500 phase contrast microscope with image analyzer software. The photographs on morphological characters in females and males were prepared.

3.1.5 Diversity Indices

The following alpha diversity indices were used to measure the diversity of species within a community or habitat. Species richness and diversity II (Pisces

Conservation Ltd., www.irchouse.demon.co.uk) (Henderson, 2003) programmes were used to assess and compare the diversity of mite in different locality.

3.1.5.1 Richness Index

The following richness index was worked out.

Margalef Index (Margalef, 1958)

$$D = \frac{S - 1}{\ln N}$$

where, S = Number of species

N = Total number of individuals

D = Richness index

3.1.5.2 Shannon – Weiner index

It is an information statistic index based on the proportional abundance of species. Variance in diversity was calculated using the equation of Whittaker (1972) and Pielou (1975).

$$\text{Var } H = \frac{\sum Pi\,(\ln Pi)^2 - (\sum Pi \ln Pi)^2}{N} - \frac{S - 1}{2N^2}$$

Where, Pi = Proportional abundance of i^{th} species

S = Number of species,

N = Total number of individuals

3.1.5.3 Simpson – Yule Index

Simpson (1949) gave the probability of any two individuals drawn at random from an infinitely large community belonging to different species, as:

$$D = \sum Pi^2$$

Where, Pi = Proportion of individuals in the i^{th} species

3.1.5.4 Species evenness or Equitability

Equitability J or evenness is the pattern of distribution of the individuals between the species. It is an important part of the description of a community and has important applications in ecological monitoring because highly stressed environments often show low levels of equitability as the system becomes dominated by disturbance or pollution tolerant species (Henderson, 2003).

$J = H/\log(S)$

Where, H = Observer Shannon Weiner Index

S = Total number of species in the habitat

3.1.5.5 Similarity Index

The similarity values were used for cluster analysis. Sequential agglomerative hierarchical non overlapping (SAHN) clustering were done using unweighted pair group method with arithmetic averages (UPGMA). Data analysis was done using NTSYSpc version 2.02 (Rolff, 1998).

3.2 Seasonal abundance of rice and tapioca mites

The rice leaf mite population was continuously monitored and recorded from March to September 2017 at Tamil Nadu Rice Research Institute (TRRI), Aduthurai (Ten hills were selected at random/plot. Population of mites at top, middle and bottom, 3 leaves/ hill/10 cm area/leaf was counted and recorded as no. per leaf length). The occurrence and the population of tapioca mites were continuously monitored in unprotected condition from February to November 2014 at Tapioca and Castor Research Station (TCRS), Yethapur (Ten plants were selected at random/plot. Population of mites at top, middle and bottom, 3 leaves/plant/cm^2 area/leaf was counted and recorded as number per cm^2). The population was observed at weekly intervals and the weather parameters *viz.,* maximum and minimum temperature, relative humidity, rain fall, wind velocity and sun shine hours *etc*., were recorded on daily basis to make correlation and regression studies, to ascertain whether the abiotic factors have any significant effect on the population dynamics of leaf mite, in rice and two spotted spider mite, in tapioca ecosystem.

3.3 Screening of certain varieties of rice to rice leaf mite and tapioca to two spotted spider mite

3.3.1 Mass culturing of mites

To carry out the pot culture screening experiments mite species were mass cultured on rice (ADT 43) and tapioca (Yethapur 1) in polycarbonate house, respectively. The mites were reared on plants raised in earthern pots (60 cm dia.) (Plate 1, 2). The initial mite population for inoculation were collected from rice field of TRRI and tapioca field of TCRS.

3.3.2 Collection of entries

A total of 31 rice entries (Table 2) from TRRI, Aduthurai and Paddy breeding station (PBS), Coimbatore and 30 tapioca entries (Table 3) from TCRS, Yethapur were collected for screening against rice leaf mite and tapioca mite, respectively.

3.3.3 Screening of rice varieties against rice leaf mite

3.3.3.1 Pot culture screening of rice under glass house condition

Thirty-one entries of rice varieties which showed resistance/ susceptible characters to rice leaf mite were selected and grown in pots at Department of Entomology, TRRI, Aduthurai during Nov'2016 (Plate 1). The potted plants were replicated thrice and arranged in completely randomized design (CRD). Ten numbers of mites (Nymph and adult stage) were released on each hill on 15 DAT to cause damage and population assessment was made on 30, 40, 50, 60 and 70 DAT.

3.3.3.2 Field screening of rice

Based on pot culture screening, field screening was conducted on thirty-one rice varieties raised by the Department of Entomology at TRRI, Aduthurai during Dec'2016 to Feb'2017.

The experiment was carried out in a randomized block design with two replications (Plate 2). The entries were grown in 5 m long row with spacing of 20 x 20 cm. The pest susceptible check TN1 was grown as infestor row for every 20 rows and along the borders. In the field screening, natural population build up of mites was allowed and population assessment was made on 30, 40, 50, 60 and 70 DAT. No acaricide application was given to ensure the maximum multiplication of mites under

natural condition. Utmost care was taken to remove other pests by application of mechanical methods.

3.3.3.3 Method of assessment

The mobile stage of mites was recorded from three leaves (1 x 10 cm) selected randomly from five hills at 10 days interval starting from 30 days after transplanting (DAT) up to 70 DAT with the help of 10 x lens and expressed as numbers/1 x 10 cm leaf length.

3.3.3.4 Leaf damage rating

Leaf damage rating was also made when the plants exhibited drying symptoms. Grading for damage was done based on the rating scale adopted by Archer (1987), as mentioned below.

Leaf area damage (%)	Grade
1-10	1
11-20	2
21-30	3
31-40	4
41-50	5
51-60	6
61-70	7
71-80	8
81-90	9
91-100	10

3.3.3.5 Resistance rating

The level of resistance was graded as per the procedure followed by Radhakrishnan and Ramaraju (2009).

Leaf damage (%)	Mean leaf damage rating	Level of resistance
0-40	1-4	Resistant (R)
41-60	> 4-6	Moderately Resistant (MR)
61-80	> 6-8	Moderately Susceptible (MS)
> 80	> 8	Susceptible (S)

3.3.3.6 Identification of the resistance sources in rice

Based on the mean mite population per 1 x 10 cm leaf length and leaf damage rating recorded from the glass house and field screening four entries *viz*., Co (R) 50, PTB 33, Co (R) 51 and ADT 50 as representative entries were selected to study the biology and biophysical as well as biochemical analysis. For each entry, 10 uninfested plants and 10 infested plants were maintained in experimental pots to carry out the tests.

3.3.4 Screening of tapioca varieties against two spotted spider mite

3.3.4.1 Screening of tapioca varieties under glasshouse condition

Thirty test varieties of tapioca were selected and grown in raised bed under the glasshouse of Tapioca and Castor Research Station (TCRS), Yethapur during Aug'2014 (Plate 3). The potted plants were replicated thrice and arranged in Completely Randomized Design (CRD). The entries were artificially infested with (TSSM) from a screen house raised entry by attaching two infested leaves, which had at least twenty adult mites each, onto each of the two plants per entry in every replication (Habekub *et al*., 2000). The petiole of each detached infested leaf was lightly tied with a string to the petiole of the attached first and second fully expanded leaf from the top of each of the two plants per entry. The infested and uninfested leaves were placed with their abaxial surfaces in contact with each other. The main lobes were lightly held together with a plastic coated paper clip leaving the other leaf lobes freely open. The infester leaves and paper clips were removed after 3 days. No acaricide, fertilizer and herbicide were applied, but the trial was kept weed free by frequent hand weeding.

The two spotted spider mite population density (PD) was estimated as suggested by Hahn *et al*. (1989), by counting adult mites on the third fully expanded leaf from the top on each plant. Leaf damage caused by Two Spotted Spider Mite (TSSM LD) was also assessed by estimating the proportion of leaf area (cm^2) covered by chlorotic spots on the same leaf.

3.3.4.2 Field screening of tapioca

Field screening was carried out at TCRS, Yethapur. Each entry was planted in 90 x 75 cm. After every ten rows of test entries two rows of the susceptible check Yethapur 1 were raised around the border to ensure mite infestation (Plate 4). The screening experiment was carried out in Randomized Block Design with three replications. The cultivation practices were followed as per the Horticulture Crop Production Guide by TNAU. Infestation was done by placing two lobes of a mite-infested tapioca leaf (50 to 100 mites) on the upper leaves of each test plant. Mites from the field were regularly reintroduced into the colony. Damage evaluations started from the second week after infestation and were made each week thereafter for four consecutive weeks. Second and third inoculations are made if the initial one was not successful. No acaricide or insecticide application was given to ensure the maximum multiplication of mites under natural conditions.

3.3.4.3 Damage scales used to evaluate mite resistance in tapioca entries

The level of resistance was evaluated with the damage scale followed by Bellotti *et al*. (1987) and Bellotti and Arias (2001).

0	:	No mites or symptoms
1	:	Mites on bud leaves, some yellow to white speckling on leaves
2	:	Many mites on leaves, moderate speckling of bud leaves and adjacent leaves
3	:	Heavy speckling of terminal leaves, slight deformation of bud leaves
4	:	Severe deformation of bud leaves, reduction of buds, mites on nearly all leaves, leaves have whitish appearance, some defoliation
5	:	Buds greatly reduced or dead, defoliation of upper leaves

3.3.4.4 Identification of the resistance sources

Based on the mean mite population and damage score recorded from screening four tapioca entries *viz*., Thailand, Co (DB) 4, Yethapur 1 and Sri Reka from 30 varieties were selected to study the biology and developmental duration and biophysical and biochemical analysis. For each entry, ten infested plants and ten uninfested plants were maintained in experimental pots to carry out the tests.

3.4 Mechanism of resistance

The following investigations were carried out in the selected four entries each in rice and tapioca.

3.4.1 Antibiosis

The antibiosis effect of the selected four test entries each in rice and tapioca on the mite was assessed by studying the fecundity, rate of development. For studying the biology of mite, detached leaf (leaf bit for rice and leaf disc for tapioca) method was used. The leaves of the same age from different test entries were used for this study.

3.4.1.1 Biology and developmental duration of rice leaf mite

The biology of the rice leaf mite was studied at room temperature (28 ± 2^{o}C) on selected resistant and susceptible rice entries at the Department of Entomology, TRRI, Aduthurai. Twenty five day old plants were selected and three uninfested leaf bits of 4cm length were placed over filter paper kept on moist cotton in a Petri Plate (50 mm diameter). Five gravid females were released in each Petri Plate for egg laying and after 24 hours all the females were removed. Freshly laid single unhatched eggs were transferred to each leaf bit using a fine camel hair brush and five replications were maintained for each entry. Observations were made every 6 hours until all mites reached adult stage. Data were collected on the incubation period, larval period, protonymphal and deutonymphal period, quiescent period and adult longevity (Plate - 5). The midpoint between two stages of nymph was considered as the time of moulting, whenever a change to the next instar was observed. Based on this duration, the nymphal stage was determined. Data were subjected to one-way analysis of variance and LSD was employed to separate the treatmental means.

3.4.1.2 Biology and developmental duration of tapioca mite

Tetranychus urticae was collected from tapioca leaves and then transferred and reared on fresh and clean tapioca leaves for several generations to provide a laboratory colony at TCRS, Yethapur (Plate - 6). The life history studies were performed at four different tapioca entries (Thailand, Co (DB) 4, Yethapur 1 and Sri Reka) under room temperature. Five adult female mites were picked randomly from stock colony and were placed on each of tapioca leaflet (2 x 2 cm) which was rested on a moistened cotton pad in the plastic boxes five replications were maintain for each entry. All mites and excess eggs were removed from each leaflet after 24 hours and only 1 egg was left on each leaflet. Observations were made every 6 hours until all mites reached adulthood. The number of stages and the duration for each developmental stage are recorded. Data were subjected to a one-way analysis of variance and LSD was employed to separate the treatment means (Suthida Sakunwarin *et al*., 2003).

3.4.1.2. (a) Longevity and fecundity

The reproductive potential of fertilized females was investigated on rice (4 cm)/ tapioca leaf bits (2 x 2 cm). One female teliochrysalis and 3 adult males were placed together on each of leaf bits. Observations were made every 12 hours until the first egg was laid and continued until the death of each mite. The data for female adult longevity, preoviposition, oviposition and post-oviposition periods and number of eggs for each female were recorded at 12 hour intervals. All eggs were kept until adult eclosion in order to determine the sex ratio of the offspring (Suthida Sakunwarin *et al*., 2003).

3.4.2 Physiological and biochemical bases of resistance

Physiological and biochemical analyses were done with the leaf samples of 45 DAT in rice and 90 days old tapioca entries.

3.4.2.1 Moisture content

The moisture content of the healthy and infested leaves of the entries was determined. Five samples of 100g leaves from upper, middle and lower portion were collected from each pot for estimation of moisture percentage in leaves. All the leaves were cleaned with muslin cloth, weighed, classified and kept in a drying oven, running at 70 ± 5^{o}C for 12 hours. Before keeping in oven the leaves were dried in shade for two days. The dry matter of leaves was weighed and put back into the oven, at the same temperature for another six hours. When the weight of dry material became constant, the

moisture content was calculated with the help of following formula and expressed in percentage.

$$\text{Moisture content (\%)} = \frac{\text{Wt. of fresh leaves} - \text{Wt. of dry leaves}}{\text{Wt. of fresh leaves}} \times 100$$

3.4.2.2 Chlorophyll

Chlorophyll content as chlorophyll 'a', chlorophyll 'b' and total chlorophyll was estimated following the method suggested by Yoshida *et al*. (1971). One gram samples of leaf tissue was ground to a fine pulp with addition of 20 ml of 80 per cent acetone. The pulp was centrifuged at 5,000 rpm for 5 minutes and the supernatant was transferred to 100 ml volumetric flask. The residue was again ground with 20 ml of 80 per cent acetone, centrifuged, the supernatant was transferred to the same volumetric flask, and the process was repeated until the residue became colourless. Then the volume was made up to 100 ml with 80 per cent acetone and the absorbance of the solution was read at 645 and 663 nm against solvent (80 % acetone) as blank.

The amount of chlorophyll present in the extract was expressed as mg chlorophyll per gram leaf tissue using the following equations.

$$\text{mg chlorophyll a/g of tissue} = 12.7\,(A_{663}) - 2.69\,(A_{645}) \times \frac{V}{1000 \times N}$$

$$\text{mg chlorophyll b/g of tissue} = 22.9\,(A_{645}) - 4.68\,(A_{663}) \times \frac{V}{1000 \times N}$$

$$\text{mg total chlorophyll /g of tissue} = 20.2\,(A_{645}) - 8.02\,(A_{663}) \times \frac{V}{1000 \times N}$$

A – Absorbance at specific wavelengths

V – Final volume of chlorophyll extract in 80 per cent acetone

W – Fresh weight of tissue extracted

3.4.2.3 Total carbohydrate

Total carbohydrate content was determined by Anthrone method (Hedge and Hofre, 1962). One hundred milligram of leaf sample (rice/ tapioca) was weighed and kept in a boiling tube. The sample was hydrolyzed by keeping it in a boiling water bath for 3 h with 5 ml of 2.5 N HCL and cooled to room temperature. The hydrolyses was neutralized with solid sodium carbonate until the effervescence ceased, after which the volume was made up to 100 ml with distilled water and centrifuged at 10, 000 rpm for 10 minutes. To 0.5 ml of the supernatant, 0.5 ml of distilled water and 4 ml of anthrone reagent were added. The reaction mixture was heated for 8 minutes in a boiling water bath and cooled rapidly. The solution turned green to dark green, which was read at 630 nm using spectrophotometer. D glucose was used as standard. Total carbohydrate content was calculated and expressed in terms of milligram of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.4.2.4 Reducing sugar

Reducing sugar was determined following dinitrosalicylic acid method (DNS) (Miller, 1972).

Hundred milligram of the leaf sample (rice/ tapioca) was weighed and the sugar was extracted using 10 ml of hot 80 per cent ethanol twice. The supernatant was collected and evaporated by keeping on a water bath at 80^{o}C. Ten milliliter of water was added to dissolve the sugars. Aliquot of 0.5 to 3.0 ml was pipetted out into a separate test tube and the volume was made up to 3.0 ml with distilled water. To this 3.0 ml of DNS reagent was added to each test tube. The tubes were then placed in boiling water bath for 5 minutes and cooled. The intensity of dark red colour was measured at 510 nm. A blank was run without the sample following the other steps. Glucose solution was used as working standard. From the standard graph, the amount of reducing sugar present in the sample was determined and expressed in percentage.

3.4.2.5 Protein

The protein content was estimated by the method suggested by Lowry *et al*. (1951). Leaf sample of 500 mg was ground well in a pestle and mortar with 5 to 10 ml of phosphate buffer solution and centrifuged for 10 min at 15, 000 rpm. The protein stock solution was prepared by dissolving 50 mg of bovine serum albumin in 50 ml of distilled water, which served as stock standard. Ten milliliter of the stock solution was made up to

50 ml with distilled water, which served as working standard. Series of 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard were pipetted out into test tubes. Sample extracts of 0.1 and 0.2 ml were pipetted out into two other test tubes. The volume was made up to 1.0 ml in all the test tubes with distilled water. A tube with 1.0 ml of water served as blank. Alkaline copper solution 5.0 ml (prepared by addition of 2 % sodium carbonate in 0.1 N sodium hydroxide plus 0.5 % copper sulphate in 1 % potassium sodium tartrate in 50:1 ratio) was taken, added to each test tube including blank, mixed well and allowed to stand for 10 min. To this Folin Ciocalteau reagent 0.5 ml was added to each test tube, mixed well and all the test tubes were incubated in dark at the room temperature for 30 min. On development of blue colour, readings at 660 nm were taken. The standard graph was drawn and the amount of protein in each sample was calculated and expressed in per cent.

3.4.2.6 Total free amino acid

Five hundred milligram of the leaf sample was ground with small quantity of acid washed sand, 5 ml of 80 per cent ethanol was added to this homogenate and centrifuged, the supernatant was saved and the extraction was repeated twice with the residue and the supernatants were pooled. The volume was reduced by evaporation and the extract was used for quantitative estimation of total free amino acid. To 0.1 ml of the extract, 1.0 ml of ninhydrin solution was added and the volume was made up to 2.0 ml with distilled water. The test tube was heated in boiling water bath for 20 min to this 5.0 ml of the diluents mixture was added and the contents were mixed. After 15 min, the intensity of the purple colour was read against a reagent blank in a colorimeter at 570 nm (Moore and Stein, 1948).

3.4.2.7 Phenols

Method described by Malik and Singh (1980) was followed for the estimation of phenol. Five hundred milligram of the leaf samples was ground with a pestle and mortar in 80 per cent ethanol and centrifuged at 10, 000 rpm for 20 min, the supernatant saved, the residue was re extracted five times with 80 per cent ethanol and the supernatants were pooled and evaporated to dryness. The residue was dissolved in 5.0 ml of distilled water. The aliquot (0.2 ml) was pipetted out into test tube and the volume was made up to 5.0 ml with distilled water, to this 0.5 ml of Folin Ciocalteau reagent was added after 3 min. 2.0 ml of 20 per cent Na_2CO_3 solution was added to the test tube and mixed

thoroughly. The tubes were placed in boiling water for exactly one minute, cooled and the absorbance were measured at 650 nm against a reagent blank. Standard curve was prepared using different concentrations of catechol. From the standard curve, the concentration of phenols in the leaf samples was determined and expressed mg phenols per 100 g leaf sample.

3.4.2.8 Tannin

Tannin content in the leaf samples were estimated following the Folin-Dennis method described by Oberbacher and Vines (1963).

3.4.2.8. (a) Standard tannic acid solution

100 mg of tannic acid was dissolved in 100 ml of distilled water, it served as stock solution. The working standard solution was prepared by diluting 5.0 ml of stock solution to 100 ml with distilled water.

3.4.2.8. (b) Extraction and quantification of tannin

250mg of the powdered leaf material was weighed and transferred to a 50 ml conical flask and 10 ml of water was added. The flasks were heated gently and boiled for 30 min. The flasks were then cooled and the contents were centrifuged at 5, 000 rpm for 20 min, the supernatant was collected and the volume was made up to 10 ml. The sample extract of 0.5 ml was transferred into test tube and the volume was made up to 7.5 ml with distilled water. To this 0.5 ml of Folin-Dennis reagent and 1.0 ml of sodium carbonate solution was added, shaken well and the absorbance was read at 700 nm after 30 min. Blank was run with water instead of sample. The tannin content was calculated as tannic acid equivalents as per the absorbance obtained for the samples from the standard graph.

3.4.3 Estimation of nutrients

3.4.3.1 Determination of total nitrogen (N) (Kjeldahl Method)

Total nitrogen was estimated using the Foss – Analyser Digestion. One gram of the leaf sample (rice/tapioca) was weighed and dropped into numbered Kjeldahl tube. Then, 0.89g of $CuSo_4$ + 7.0g K_2SO_4 and 12 ml of concentrated H_2SO_4 was added to each tube. If foaming was a problem 3 ml of 35 per cent hydrogen peroxide was added to reduce foaming. A blank was also run. The tubes were shaken gently to wet the sample. The exhaust was positioned and the scrubber was turned on. The samples were digested

for 60 min. The rack was then removed with exhaust still in place and allowed to cool for 20 min. After sufficient cooling, the digest was diluted with 80 ml of water.

Forty per cent of NaOH was placed in alkali tank of distillation unit and volume dispenser was adjusted to dispense 50 ml of the alkali into the cooled Kjeldahl tubes. Distillation was carried out for 5 min and distillate was titrated against 0.1000 NHCl with boric acid (4%) as indicator. The end point of the titration was disappearance of pink colour (Humphries, 1956).

The percentage of N present in the samples was calculated by

$$\text{Nitrogen (\%)} = \frac{V_s - V_b \times N \times 14.01}{W \times 10}$$

Where,

V_s - ml of standardized acid used to titrate sample

V_b - ml of standardized acid used to titrate reagent blank

N - Normality of standard HCl

4.01 - Atomic weight of nitrogen

W - Weight of sample in grams

10 - Factors to convert mg/ gram to per cent

3.4.3.2 Estimation of phosphorus (P)

One gram of the sample was digested with triacid extract to bring the mineral constituents into solution. The amount of phosphorus present in the samples was estimated based on the intensity of yellow colour developed with vanodomolybdate in the nitric acid medium.

Five ml of the triacid extract of the sample was pipette out into 25 ml volumetric flask, to this 5 ml of Barton's reagent was added and the volume was made up with

distilled water and allowed to stand for 30 min for the development of yellow colour and the intensity of yellow colour was measured at 410 nm in a spectrophotometer. From the standard curve, the concentration of phosphorous in the samples was calculated (Piper, 1966).

3.4.3.3 Potassium

The digested leaf samples were tested for estimation of potassium (K) using flame photometer. Depending upon the concentrations of K in the digested samples, a digest were used either directly or after dilution for flame photometric determinations. For standard, 5 ml of triacid extract were pipetted out into 25 ml volumetric flask, neutralized with ammonium hydroxide and the volume was made up with distilled water. The solution was mixed well to make a homogeneous solution. The concentration of potassium in the solution was measured using flame photometer. Concentration of K in the leaf samples was calculated using standard curve (Piper, 1966).

3.4.3.4 Estimation of micronutrients

3.4.3.4.1 Calcium (Ca)

Five ml of stock solution of leaf digest (0.5g leaf material in 50 ml aliquot/ solution) were taken in 100 ml China dish and diluted in to 10 ml with distilled water. Ten drops of 2% NaCN and hydroxylamine hydrochloride solutions were added. Pink to reddish colour were formed by adding 2.5 ml of 4N NaOH and 10-15 drops of calcon indicator. Blank solution was prepared exactly in the same way with distilled water instead of test solution. Add 2-3 drops of EDTA if blue colour not appears. Titration was done with 0.01N EDTA and calculation was done and expressed the results in per cent (Piper, 1966).

3.4.3.4.2 Magnesium (Mg)

Five ml of stock solution of leaf digest (0.5g leaf material in 50 ml aliquot/ solution) were taken in 100 ml China dish and diluted in to 10 ml with distilled water. Ten drops of 2% NaCN and hydroxylamine hydrochloride solutions were added. Purple colour were formed by adding 10 ml of NH_4OH - NH_4Cl buffer and 10 drops of Erichrome Black-T indicator. Blank solution was prepared exactly in the same way with distilled water instead of test solution. Add 2-3 drops of EDTA if blue colour not appears. Titration was done with 0.01N EDTA and calculation was done and expressed the results in per cent (Piper, 1966).

3.4.3.4.3 Iron (Fe)

The atomic absorption spectrophotometer (AAS) was calibrated with working satandard. The aliquots (leaf digest containing 0.5g leaf material in 50ml volume) were feed directly and calculation was done by the reading observed on AAS and expressed as ppm. Different concentrations of standards were prepared by Fe (Piper, 1966).

3.4.4 Assay of enzymes

The selected entries with respect to resistance were used for the assay of studies. The leaf samples were drawn from each entry for assaying the biochemical constituents.

3.4.4.1 Peroxidase (PO)

Using pre-chilled pestle and mortar, 1g of leaf sample was homogenized with 5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and to which a pinch of polyvinyl pyrollidone (PVP) was added. The homogenate was centrifuged at 10, 000 rpm for 10 min at $4^{o}C$ and the supernatant was used as the enzyme extract for the assay of peroxidase activity.

The reaction mixture consisting of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of H_2O_2 (1%) was incubated at 28 ± $1^{o}C$. At the start of the enzyme reaction, the absorbance of the mixture was set to zero at 420 nm in spectrophotometer and changes in the absorbance were recorded at 30 sec. intervals for 3 min.

The peroxidase activity was expressed as change in the absorbance of the reaction mixture per minute per gram on fresh weight basis (Hammerschmidt *et al*., 1982).

3.4.4.2 Polyphenol oxidase (PPO)

Enzyme extract was prepared as per the procedure adopted for estimation of peroxidase. The polyphenol oxidase activity was assayed using the modified method of Mayer *et al*. (1965). Standard reaction mixture contained 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5), 0.5 ml of the enzyme preparation and 0.5 ml of 0.1 M catechol.

The reaction mixture was incubated at 28 $\pm$ $1^{o}C$ and absorbance was set to zero at 495 nm in spectrophotometer. The changes in the optical density (OD) were followed at 30 sec. intervals for 3 min. and the PPO activity was expressed as change in the OD of the reaction mixture per minute per gram on fresh weight basis.

3.4.4.3 Phenylalanine ammonialyase (PAL)

For preparation of the enzyme extract, 1 g of sample was homogenized with 5 ml of 0.1 M sodium borate buffer (pH 8.8) containing a pinch of polyvinyl pyrollidone using chilled pestle and mortar. The homogenate was centrifuged at 10, 000 rpm for 20 min. The supernatant was used for the assay of PAL activity.

The PAL activity was determined at 30^{o}C by direct spectrophotometric measurement of the conversion of L-phenylalanine to trans cinnamic acid at 290 nm (Dickerson *et al*., 1984). The reaction mixture contained 3.1 ml of 0.1 M sodium borate buffer (pH 8.8), 0.2 ml of the enzyme extract and 0.1 ml of 12 mM L-phenylalanine prepared in the same buffer.

The blank was run by taking 3.1 ml of 0.1 M sodium borate buffer (pH 8.8) and 0.2 ml of the enzyme extract. The reaction mixture and the blank were incubated at 40^{o}C for 30 min. and the reaction was stopped by adding 0.2 ml of 3 N HCl. The absorbance was read at 290 nm in a spectrophotometer. The PAL activity was expressed in moles of cinnammic acid produced per minute per gram on fresh weight basis.

3.5 Field experiments

3.5.1 Field experiment I – Management of rice leaf mite, *O. oryzae* on rice

Two trials were conducted at TRRI, Aduthurai, during Mar' 2017 – Jun' 2017 (First season) and Aug'2017 to Nov'2017 (Second season). The field experiment was conducted with following treatments in a randomized block design replicated three times using ADT 43 (Plate – 7 and 8).

T1 : Neemazal 0.15 EC @ 2 mL/L

T2 : Fenazaquine 10 EC @ 1. mL/L

T3 : Fenpyroximate 5 SC @ 1.5 mL/L

T4 : Mineral oil 2% @ 20 mL/L

T5 : *Beauveria bassiana* CFU 1x10^{8} /mL

T6 : *Hirsutella thompsonii* CFU 1x10^{8} /mL

T7 : Propargite 57 EC @ 2.5 mL/L

T8 : Untreated control

3.5.2 Field experiment II – Management of two spotted spider mite, *T.urticae* on tapioca

This experiment was conducted at TCRS, Yethapur, during Feb'2014 – Nov'2014 (First season) and Jan'2016 – Oct'2016 (Second season). The field experiment was conducted in a randomized block design replicated three times with treatment mentioned in 3.5.1 using Sri Vijaya variety (Plate – 9 and 10).

Two rounds of foliar applications were given at 15 days interval for two season. First spray was given after sufficient population build up of mites. Besides pretreatment count, the post treatment counts were recorded on 1, 3, 7 and14 days after each spraying for all trials.

3.5.3 Assessment of mite population

The mite population which occurred in each treatment was assessed by adapting the following methodology. Ten hills were selected at random from each replication. Three leaves were selected from each hill (top, middle and bottom) which were examined for the presence of mites for rice. Ten plants were selected at random from each replication. Three leaves were selected from each plant (one each from top, middle and bottom) which was examined for the presence of mite for tapioca. On each selected leaf, the mites were observed on the lower and upper surface. Assessment of the mite population in the sample was made at various stages of the infested leaves. The number of mites (Adults & nymphs) was observed in an area of 10 cm length for rice and 1cm^2 for tapioca (using a square card). 10x hand lens was used in field condition for observation of mites. The mean population was worked out, before subjecting the data for transformation the data were subjected to square root transformation to compare the means and to find out the most effective treatment by DMRT as suggested by Gomez and Gomez (1984).

3.5.4 Assessment of yield

The yield was recorded from each treatment replication wise and computed in terms of kg/ha. The increase in yield in treated plots over the untreated plot was worked out.

3.6 Statistical analysis

The method suggested by Goulden (1972) was adapted for statistical analysis of the data obtained from field experiments. Prior to analysis, the data on mite counts were subjected to square root transformation. The mean values of treatments were then compared using Duncan's multiple range test (DMRT) (Gomez and Gomez, 1984) to identify the most effective treatments.

Corrélation and régression analyses were applied to find out the relationship between the abundance of mite population and the possible influence of weather parameters. The weekly observation of mite and weekly mean of weather data such as rainfall, maximum and minimum temperature, relative humidity, sun shine hours and wind velocity were related for correlation coefficient adapting the formula suggested by Regupathy and Dhamu (2001).

Table 2. List of rice entries screened for resistance against rice leaf mite, *O.oryzae*

Entry No.	Entry	Entry No.	Entry
1.	ADT 36	17.	White ponni
2.	ADT 37	18.	ASD 18
3.	ADT 38	19.	TKM 9
4.	ADT 39	20.	TKM 13
5.	ADT 40	21.	Co 48
6.	ADT 41	22.	Co 49
7.	ADT 42	23.	Co 41
8.	ADT 43	24.	Co (R) 50
9.	ADT 44	25.	Co (R) 51
10.	ADT 45	26.	BPT 5209
11.	ADT 46	27.	Paiyur 1
12.	ADT 47	28.	TN 1
13.	ADT 48	29.	Co 43
14.	ADT 49	30.	PTB 33
15.	ADT 50	31.	Co RH 4
16.	CR 1009		

Table 3. List of tapioca entries screened for resistance against tapioca mite, *T.urticae*

Entry No.	Entry	Entry No.	Entry
1.	Co 2	16.	Sri Visagam
2.	Co 3	17.	Sri Prakash
3.	Co (DB) 4	18.	Sri Harza
4.	Co (DB) 5	19.	Sri Jeya
5.	Yethapur 1	20.	Sri Vijaya
6.	Mulluvadi 1	21.	Sri Reka
7.	Rasi 20	22.	Sri Prabha
8.	Burma	23.	Sri Bathmanaba
9.	Kunguma rose	24.	Sri Athulya
10.	Thailand	25.	Sri Apoorva
11.	H 226	26.	Sri Pavithra
12.	Kerala rose	27.	Sri Swarna
13.	H 97	28.	M (4)
14.	H 165	29.	Vellagani
15.	Sri Sahiya	30.	Harswa