CHAPTER III

MATERIALS AND METHODS

The present study was carried out to investigate the differential effects of Malathion, Deltamethrin and Carbofuran on different *Westiellopsis* species isolated from the rice field soils of Assam under controlled laboratory conditions.

3.1 Collection of soil samples:

The soil samples for the study were collected from different organically cultivated rice fields of Assam. Altogether, 20 sites from different parts of Assam were selected for isolation of the test organisms. The geographical coordinates of the collected sites are given in the Table. 1. The soil samples were collected from each site with spatula into the air tight pouches with proper labeling of the place from where it was collected. The samples were then brought to the Plant Ecology Laboratory of Department of Botany (Gauhati University) and then, air dried. The dried soil samples were ground with mortar and pestle, sieved through the sieve plates of 0.1 mm size and packed again in air tight pouches until further use. The prepared soil samples were then stored at a temperature of 20-30^0 C.

3.2 Sterilization of glass wares:

Sterilization is a process in which all the glass wares that were to be used for the experiment were washed with laboratory detergent and autoclaved at 150^o C for 1 hour and 30 minutes and then kept in the hot air oven at 110 ^{o} C for about three hours.

Table.1: Collection sites with their geo coordinates

Serial No.	Collection sites	Geo coordinates
1	Tinsukia	27°29′22″N & 95°21′36″E
2	Chabua	27°29'00"N & 95°11'00"E
3	Dibrugarh	27°42'30"N &95°29'08"E
4	Lakhimpur	27°13′48″N &94°06′00″E
5	Tezpur	26°39'04"N & 92°47'01"E
6	Sivasagar	26°59′04″N & 94°37′53″E
7	Demow	26°07'38"N & 94°44'23"E
8	Kohora	26°34'33"N & 93°10'01"E
9	Nagaon	26°21′00″N & 92°40′00″E
10	Karbi anglong	26°11′00″N & 93°34′00″E
11	Sonapur	26°07'12"N & 91°58'48"E
12	Panikhaity	26°12'12"N & 91°51'29"E
13	Basistha Chariali	26°06'00"N & 91°47'00"E
14	Lankeshwar	26°08'43"N &91°38'45"E
15	Rani	26°05'00"N &91°35'00"E
16	North Guwahati	26°10'48"N & 91°43'12"E
17	Nalbari	26°27′00″N & 91°26′24″E
18	Pathsala	26°30'42"N &91°10'51"E
19	Bongaigaon	26°28′00″N & 90°34′00″E
20	Goalpara	26°26′00″N & 90°22′00″E

3.3 Growth medium:

For enumeration of species belonging to genus *Westiellopsis*, which happens to be a nitrogen fixing cyanobacterial taxon the standard BG-11medium was prepared following Rippka *et al.* (1979) and used for their growth. As per methodology, four stock solutions containing different chemicals were prepared (Table.2) and all the four stocks were kept under aseptic conditions until further use. Taking the suggested volume from the four stocks along with Na_2CO_3 in 1000 ml of double distilled water, final BG-11 medium was prepared (Table.3). All the culture media containing flasks were then plugged with non absorbent cotton and autoclaved at 150^0 for 90 minutes and allowed to come to the room temperature before using for the experimental setup.

Table. 2: Stock Composition for BG-11 media according to Rippka *et al*. (1979)

Stocks	Chemicals	Distilled water(g/L)
STOCK 1	EDTA (disodium magnesium salt)	0.1
	Ferric ammonium citrate	0.6
	Citric acid	0.6
	Calcium chloride	3.6
STOCK 2	Magnesium sulphate	7.5
STOCK 3	Dipotassium hydrogen phosphate	3.05
STOCK 4	Sodium carbonates	0.02
STOCK 5	Boric acid	2.86
	Manganese chloride	1.81
	Zinc sulphate	0.22
	Copper sulphate	0.079
	Cobalt nitrate	0.050
	Sodium molybdate	0.01

Table. 3 Composition of BG-11 media

Stock solution	ml /L of distilled water
Stock 1	10
Stock 2	10
Stock 3	10
Stock 4	1
Sodium carbonates	0.02g

3.4 Preparation of Agar media:

The agar based solid medium was prepared for isolating the cyanobacterial colonies. 15 gm of agar was mixed with 250 ml of nitrogen free BG-11 media in an erlenmeyer flask. The solution was stirred with glass rod and heated on a hot plate to dissolve the mixture and then autoclaved.

3.5 Isolation and Maintenance:

About 10mg of soil sample was inoculated into a 250 ml erlenmeyer flask containing BG-11 medium (without nitrogen) and incubated at 27±1 °C under light intensity of 3600 lux alternating with light: dark photoperiod for 12:12 hour basis. The pH of the medium was adjusted between 7 to 7.3 for optimal growth. On observation of first cyanobacterial growth, the filaments were transferred to an agar based solid medium through the process of streaking from the mother culture. The process was repeated to make it pure.

3.6 Identification of the test organisms based on Morphological characters:

The morphology of each strain, from the late exponentially growing cultures were examined under a compound microscope (LM-52- 1601) in both 40X and 100X magnification having photographic attachment. The test organism *Westiellopsis* spp. was identified based upon the keys following standard monographs of Desikachary

(1959), Komarek and Anagnostidis (1989) ,Komarek (2013) and Komarek *et al*. (2014) and authenticated following Algaebase (www.algaebase.org).

The following keys were considered for morphological identification through microscopic examination as suggested by Whitton and Potts (2013) while consulting monographs.

(1). Size and shape of the filaments

(2). Size and shape of the heterocyst along with its frequency

(3). Size and shape of the akinete

(4). Branching pattern

(5). Presence or absence of sheath

3.7 Pesticide used

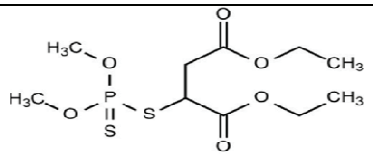
The commercial grade Malathion, Deltamethrin and Carbofuran (Manufactured by Biostadt India Ltd.) belonging to three different groups Organophosphate, Pyrethroid and Carbofuran respectively were taken into consideration for the present study. These pesticides are mostly used by the farmers in different rice grown areas of Assam to protect their crops from harmful insects and pests. All the three pesticides were obtained from an authorized dealer of Guwahati city of Assam (India).

3.7.1 Malathion

The Malathion (50%EC) is an Organophosphate and a broad spectrum insecticide. Due to its efficiency in killing pests, it has been widely used throughout the globe (Geed *et al*., 2016). It kills insects by inhibiting their acetylcholinesterase enzyme activity

resulting nervous and respiratory failure (Jebali *et al*., 2006). There are reports which revealed that malathion has adverse effects on soil microflora including a number of algal groups and cyanobacterial species (Anton *et al*., 1993; Tiwari *et al*., 2001). According to EPA, more than 30 million pounds of malathion are used annually (Donaldson and Kiely, 2002). In Assam, 3 lit/ha of malathion is being used to get rid of cricket, mole cricket, red ant and white ant as mentioned in the official document of Govt. of Assam: "Packages of practices for rabi crops of Assam (2009). The amount is much more in recent days.

Table 4: Physico-chemical properties of Malathion

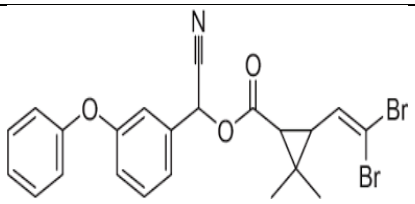
Name	Malathion
IUPAC name	Diethyl2- [(dimethoxyphosphorothioyl)sulfanyl]butanedioate
Chemical formula	$C_{10}H_{19}O_6PS_2$
Chemical group	Organophosphate
Chemical structure	
Common/local names	carbophos, maldison and mercaptothion
Physical state	Liquid
Density/Specific gravity	1.2076g/cm^3

3.7.2. Deltamethrin

The deltamethrin belongs to the pyrethroid group of insecticide. A wide range of insects are affected by its direct contact or sometimes affected due to ingestion of the

same (Worthing & Walker, 1987). Deltamethrin is considered as one of the most powerful toxic pyrethroid (Shrivastave *et al*., 2011). According to records, 2.5–15g ai/ha of Deltamethrin are being used in tropical rice fields (Mullie *et al*., 1991) per annum. The recommended dose of D-eltamethrin in Assam is 0.4 lit/ha It is mostly used to get rid of aphids and saw-fly (Package of practices for rabi crops of Assam 2009).Due to its high stability and recalcitrant nature, it contaminates the rice grown soils in Assam making it a matter of serious concern.

Table 5: **Physico-chemical properties of Deltamethrin**

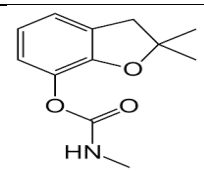
Name	Deltamethrin
IUPAC name	(S)-cyano-3-pehoxybenzyl(1R)cis-3-(2,2-dibromovinyl)-2,2 dimethylcyclopropane carboxylate
Chemical group	$C_{22}H_{19}Br_2NO_3$
Chemical structure	Pyrethroid
Chemical structure	
Common/local names	Decamethrin, Decis, K-othrin
Physical state	Liquid
Density/Specific gravity	1.5 g/cm^3

3.7.3. Carbofuran

The Carbofuran is a Carbamate pesticide which is used for killing of soil borne insects and nematodes. In India it is marketed as Furadan 3G in granular form. Carbofuran

residues are found to be very common in soil and water in any crop field having tremendous negative effect on the soil micro flora (Bhagabati *et al*., 2011). For which, it attracts world community to study its impact on living system (Noyfan *et al.,* 2017). It has been reported that about 22 -29 kg/ha of Carbofuran are in use in the rice fields of India which is much beyond the permissible limit. Though the Assam Agricultural University recommended about 3 g/sq. m of Carbofuran in the rice fields to repel or kill the root knot nematodes and stem borer (Package of practices for rabi crops of Assam 2009), the actual amount is much higher than that of the recommended doses as revealed during the preliminary survey.

Table 6: **Physico-chemical properties of Carbofuran**

Name	Carbofuran
IUPAC name	2,3-dihydro-2,2-dimethyl-7-benzofuranyl-N-methylcarbamate
Chemical formula	$C_{12}H_{15}NO_3$
Chemical group	Carbamate
Chemical structure	
Common/local names	Furadan, Curater, Furacarb
Physical state	Crystalline
Density/Specific gravity	1.18 g/cm^3

3.7.4. Pesticide treatment

The test organism from the exponential growth phase was inoculated to estimate the lethal concentration. LC_{50} of the pesticides was determined in terms of chlorophyll-a following Kumar *et al*. (2016). On the basis of that, treatments were set up for all the selected pesticides. Experiments were conducted in batch culture. The test organism was transferred to different test tubes containing 10 ml of BG-11 media with different pesticide concentrations along with the control (without pesticides) and incubated at the same culture condition as set for the axenic culture. At the end of each incubation period (4, 8, 12 and 16 days) as set up for the test, the cultures were washed thrice with double distilled water to remove the excess pesticides adhering to the samples and used for measuring the growth parameters. All the tests were conducted in triplicates and average calculated values were considered for further analysis.

3.8 Analytical methods (Growth estimation)

3.8.1 Biomass estimation (Gupta and Baruah, 2015)

For determination of dry biomass, the test organism was centrifuged and filtered through pre-dried Whatman No. 1 filter paper using sintered glass apparatus. The pellets obtained after centrifugation were washed thrice with double distilled water to remove the remaining pesticides and then dried in a hot air oven until constant weight was observed at 70^0 C. Weights were measured using an analytical balance readable to 0.01 mg.

3.8.2 Chlorophyll a estimation (Mackinney, 1941)

Reagents: 1). 95% Methanol

2). Double distilled water

Procedure:

Chlorophyll-a estimation was done following the protocol of Mackinney (1941). The pesticide treated test organism was centrifuged to obtain the pellet and each pellet was washed with double distilled water, homogenised with 95% methanol and kept in a water bath at 65^0 C for 30 min. The resultant suspension formed was centrifuged at 3000 rpm for 10 min and the supernatant was read at 650 nm and 665 nm against 95% methanol as blank in uv–visible spectrophotometer 119 (SYSTRONICS).

$$\text{Chlorophyll-a } (\mu g/ml) = A_{665} \times 13.42$$

Where, 13.42 = extinction co-coefficient at 665 nm

A = Absorbance at respective wavelength

3.8.3 Carotenoid estimation (Myers and Kratz ,1955)

Reagents :

1). 80% Acetone

2). Double distilled water

Procedure:

For estimation of carotenoid, the treated cyanobacterial pellet was extracted, washed with double distilled water, homogenised, suspended in 80% acetone and kept overnight at 4^0 C. Next day, the suspension was centrifuged at 3000 rpm for 10 min and supernatant was read at 480nm against 80% acetone as blank in uv–visible spectrophotometer. The value obtained was calculated following Myers and Kratz (1955).

3.8.4 Protein estimation (Lowry *et al.*, 1951).

Reagents:

1). BSA stock solution (1mg/ml)

2). Analytical reagents:

(a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.)

(b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Prepare analytical reagents by mixing 2 ml of (b) with 100 ml of (a)

3). Folin - Ciocalteau reagent solution (1N)- Dilute commercial reagent (2N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water)

Procedure:

Protein estimation was done following the method of Lowry *et al*. (1951). The test samples were centrifuged at 4000 rpm for 5 min. The pellet obtained after centrifugation was suspended in 1N NaOH and kept in a boiling water bath for 10 min. This was followed by addition of reagent A (prepared by adding 1 ml freshly prepared 1% Na–K tartarate solution containing 0.5% CuSO4 into 50 ml 2% Na2CO3 solution) and incubated at room temperature for 10 min. To it, 0.5 ml of reagent B (Folin ciocalteu's phenol reagent) was added, mixed thoroughly and incubated at room temperature for another 30 min. The absorbance of the supernatant was read at 650 nm against Folin reagent as blank. The protein concentration was determined from the calibration curve prepared using bovine serum albumin (BSA) as a standard.

3.8.5 Carbohydrate estimation (Spiro, 1966).

Reagents: 1). 100mg anthrone

2). 1gm thiourea

3). 75% sulphuric acid

Procedure:

The carbohydrate content was estimated according to Spiro (1966). 0.5ml of homogenised algal suspension was taken in a test tube and the volume was made to 1ml with double distilled water. It was followed by addition of 4ml anthrone reagent (prepared by addition of 100mg of anthrone and 1 gm thiourea to 100ml of 75% sulphuric acid). The tubes were then kept at boiling water bath for 10 min and then brought to the room temperature. The absorbance was read at 620 nm against anthrone reagent as blank. Carbohydrate content was determined from the curve calibrated with glucose as standard and expressed in µg/ml.

3.8.6 Nitrogen estimation (Das and De, 2018):

Reagents: (a). A digestion mixture (potassium sulphate and cupric sulphate was used in the ratio of 3:1)

(b). Mixed indicator (Bromocresol green (0.5g) and Methyl red indicator (0.1g) was dissolved in 100 ml of ethanol).

(c). Boric acid solution (Boric acid (40g) dissolved in 1000ml of distilled water along with methyl red and bromocel green as indicator (5ml).

(d). NaOH solution (NaOH solution(40%) was kept overnight to precipitate out the sodium carbonate and other impurities).

(e). Conc. H_2SO_4.

(f). 0.1 N Hydrochloric acid(standard)

Procedure:

To determine the N_2- fixation rate, "Microbial bioassay" method of Das and De (2018) was followed along with the method of Gafur and Parvin (2008) to estimate the nitrogen content of the organisms prior to estimating the nitrogen fixing rate. The microbial assay method was partially modified to fit in with N_2 fixing cyanobacterial populations. 1000 mg of soil was incubated in a BG - 11 medium (without nitrogen), to which 100mg of pure test organism was mixed and incubated under controlled laboratory conditions. From this mixed culture, 20 mg of soil was taken and treated with different concentrations of pesticides and incubated in the culture room maintaining the growth conditions. The samples were collected for estimating the nitrogen content during the first day i.e. 0^{th} day of treatment and then after 4 days of time interval up to 16^{th} day. The samples were dried, weighted and transferred to the 100ml kjeldahl flasks and to it 20 mg of digestion mixture and 10 ml of H_2SO_4 was added. The flasks were then heated for complete digestion. After cooling of the digested samples, 50 ml of distilled water was added to the flask. From it 5ml sample was added into distillation flasks with equal amount of NaOH solution and 1ml distilled water. The heater was then made on for distillation. The distillate was collected in 20ml of 1% boric acid solution with indicators. It was then kept in a conical flask (100ml) by immersing the tip of the condenser in it. 20 ml of distillate was collected and titrated against 0.1N hydrochloric acid till colour changes to light pink from blue. At last the amount of total nitrogen present was calculated and value obtained after calculation was used to estimate the rate of nitrogen fixation with the following equation.

$$\text{Rate of N2 fixation} = \frac{\text{Final nitrogen content} - \text{Initial day nitrogen content}}{\text{Incubation period} \times 20 \text{ mg of soil}}$$

3.9 Statistical analysis:

The experiment was carried out in triplicates and was expressed as mean ± standard deviation (N=3).The significant difference between the control and pesticide treated groups were analysed by doing One-way analysis of variance(ANOVA) with post hoc (Dunnett) test at significance level 0.05 using Software Graph pad prism version 5.