EFFICACY ASSESSMENT OF BIO-PESTICIDES AGAINST RICE LEAF FOLDER, *CNAPHALOCROCIS MEDINALIS* (GUENEE) (LEPIDOPTERA: PYRALIDAE) USING DNA QUANTIFICATION METHOD

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ABSTRACT

The study was carried out to check the efficacy of different bio pesticides at equal concentration against *Cnaphalocrocis medinalis* (Guenee) (Lepidoptera: Pyralidae). In this study DNA quantification was used as pesticide screening and evaluating tool for plant genome health. Different botanicals crude extracts i.e. *Azadirachta indica, Melia azedarach, Eucalyptus globulus* and one microbial insecticide Bt was used in this experiment for control of rice leaf folder under Randomized Complete Block Design (RCBD) with 3 replications. The neem crude extract and microbial insecticides (Bt) gave excellent results with lowest post treated increase in leaf infestation (2.76, 3.07, 6.60% and 4.48, 6.06, 9.33 % after 3, 9 and 16 days of application, respectively) caused by *Cnaphalocrocis medinalis*. On the other hand, *Melia azedarach* and Eucalyptus also controlled the *Cnaphalocrocis medinals* but with least efficacy. Maximum leaf folder infestation was observed in control plots. Different bio pesticides were used instead of chemical pesticides that harmful to the plant (cause genotoxicity) and checked it may bond or not with plant genome. Rice samples treated with different bio pesticides along with control were collected and extracted the DNA by using CTAB method. All the samples were run on gel electrophoresis for further confirmation and quantification DNA using spectrophotometer. The bio pesticide treated rice samples showed DNA quantification value i.e. 34.45 µg/mL, 33.65 µg/mL, 31.05 µg/mL, 34.55 µg/mL, 33.88 for control, Bt, Eucalyptus, Darek and Neem, respectively. The main objective of the study was to check the genotoxicity effect of bio pesticide treated rice and concluded that no harmful effect of bio pesticides on rice plant structure and cause no genotoxicity of plant genome.

Keywords: *Bio-pesticide; Rice leaf folder; DNA; Treatments, Genotypes*

INTRODUCTION

Rice plays diverse role in Pakistan economy and is main export product (Sherawat *et al*., 2007). In Pakistan it is cultivated on 10% cultivable land. It accounts for 5.5% value added goods in agriculture and I.1 percent in GDP (Gross domestic product). Insect pests attack is a major yield limiting factor in rice. A large number of insect pest attack it, from nursery to harvesting. Different insect pest species (128) were found to attack on rice crop in Pakistan (Ahmed, 1981). From these near about 15-20 insect species are important which cause major loss in tropical Asia (Pathak and Dyck, 1973).

Outbreak of severe damage of *C. medinalis* has been recorded in numerous Asian countries containing Japan, china, Malaysia, India, Korea, Vietnam and Sri Lanka (Wada *et al*., 1980; Heong, 1997). During August-September 1989-90 this pest spread and multiplies severely in Pakistan with severe incidence. In Punjab, Pakistan leaf infestation due to rice leaf folder was recorded 25% and reduces the yield near about 30% and in few areas 50% leaf folder infestation was observed (Salim *et al*., 1991). Bautista *et al*. (1984) in his study have described loss in rice yield by *C. medinalis* directly related to the leaves damage percentage and observed that infested leaves 17.5% caused loss in yield about 16.5% and damage leaves 26.6% caused 21.3% yield loss in rice. It is observed that attack of pest have increased with change of pest complexities in last few decays (Ahmad *et al*., 2005).

In Pakistan mostly insect pests of rice are controlled by granular and sprayable synthetic insecticides formulations (Mustafa and Razzaq, 1991). Totally dependence on chemical pesticides is not a wise and sustainable insect pest control tactic as it may cause insect resistance problems, mortality of naturally occurring bio control agent and other fauna , outbreak of sporadic and secondary pest , severe environmental pollution and food product contamination problems (Heong, 2005).

Moreover chemical pesticide binds with DNA, as numerous pesticides possess electrophilic agent which is potentially reactive to different DNA site. Reaction of DNA bases with pesticide or their metabolite can change the

structure of nucleic acids and does not allow proper normal replication. Degradation of genomic DNA leads to poor development of roots, leaves or fruits and ultimately fewer yields of Agriculture commodities. Some bio pesticide caused genotoxicity and damage the plant DNA and plant genome and adduct formation occurred **(**University of Mass Dartmouth).

This effect was checked by DNA isolation and assaying of DNA quantification through experiments. Rice leaf folder management through synthetic chemicals has failed due to pest resurgence, Environment pollution and insecticide resistance (Dale, 1994). Synthetic insecticides are persistence in Environment and toxic for human (Linka *et al*., 2005), domestic animals (Covaci *et al*., 2004) and beneficial insects (Youn *et al*., 2003). There is renowned interest to use the Botanical insecticide and Microbial pesticide (Bt) because they are safe and ecologically acceptable.

Present study were carried out to check the efficacy of different bio pesticides and effectiveness interval of these bio pesticide for sustainable management of rice leaf folder (*Cnaphalocrocis medinalis*) with DNA quantification of plants samples treated with bio pesticides separately to check genotoxicity effect.

MATERIALS AND METHODS

Experiment was carried out in the Institute of Agricultural Sciences, University of the Punjab Lahore during 2016. By following all the agronomic practice raised bed is well prepared (Ashfaq *et al.*, 2011) and seed was sown at the rate of 1kg /m^2 by followed wet sowing method. Super basmati rice seeds were sown on 18 June 2016. 35 days old seedling were transplanted in plots outlined according to RCBD following three replicates according to planed research experiment design (Ashfaq *et al*., 2011). The Nurseries were transplanted in standing water by maintaining Plant to Plant and Row to row distance of 9 inch (Sagheer *et al*., 2008). Two Plants per hill were used by maintaining almost 80000 Hill/acre (160000 Plants /Acre). Recommended doses of Fertilizers and standard Agronomic practice were done.

Random sampling method was used and 21 hill from each sub plot is selected randomly and leaf infestation was calculated. Leaf folder infestation was started in last week of August. Folded leaves were seen and larvae of rice leaf folder were also observed feeding inside the folded leaves. Observation was done on daily basis. ETL for Rice leaf folder is 2 percent infested leaf (Arshad *et al*., 2012). When population was reached at than Bio pesticides (Botanical & Bt) were applied at the ETL stage.

Preparation of Crude Extracts

Fresh leaves of *Azadirachta indica*, *Euclayptus camaldulensis* and *Melia azedarach* were collected and identified from the department of botany, university of the Punjab Lahore. Leaves were washed efficiently and then dry enough to evaporate the water. Fresh leaves were used instead of dry leaves (Ashfaq *et al*., 2011) Cut these leaves into small pieces separately and soaked individually for 6 hour than boiled these chopped leaves in water at rate of 1:3 in water bath at 100 ^{0}C for two hours (Ali *et al*., 2016) and shake twice during boiling. At the end material was sieved through muslin cloth and preserve in bottle for used (Ashfaq *et al*., 2011).

Formulations and Field Application of Azadirachta indica, Eucalyptus camaldulensis and Melia azedarach crude Extracts

Fifteen percent (15%) dilution of each (*Azadirachta indica, Euclayptus camaldulensis and Melia azedarach)* crude extract with water was used for field application. Calibration of area was done and mixture was applied on rice crop to control the rice leaf folder.

Bacillus thuringensis var Kurstaki (Bio Pesticide)

As foliar application Bt (LIPEL TM) is mixed in water and spray on crops. 2 g/L of water for 18,0000 IU /mg WP formulation were used. Knapsack sprayer was used for spray application.

Calculation of Cnaphalocrocis medinalis Infestation

The following formula was used for calculation of leaf infestation caused by *Cnaphalocrocis medinalis*

$$\text{Leaves folded (\%)} = \frac{\text{Infested leaves number/hill}}{\text{Number of total leaves/hill}} x \frac{\text{Infested hill}}{\text{Number of total hill in sample area}} x100$$

DNA Quantification as a Pesticide Screening and Evaluating Tool

DNA Extraction

One gram plant sample was weighed and grinded into fine powder using liquid nitrogen in pestle and mortar grinded material was transferred to 50 ml falcon tubes and 15 mL of pre warmed CTAB buffer was added in it. 200µL of β-mercaptoethanol was added to 100 ml of CTAB buffer before using it. Falcon tubes were placed in a water bath or in an incubator at 65^{o}C. Equal volumes of chloroform and iso-amyl alcohol in a ratio of 24:1 were added to the falcon tubes after 30 min and were centrifuged at 5000 rpm for 15 minutes at temperature 15^{o}C. After centrifugation, three layers were appeared, upper aqueous layer representing DNA which was taken with the help of micropipette and was transferred to a new falcon tube. Other layers showed the presence of proteins and RNA. Cold iso-propanol was added to the falcon tubes in a 2/3 V of total liquid. There may be cloud formation or not. If cloud formation is occurred than looped out it and if no cloud formation then store falcons in refrigerator overnight at -4^{0}C. Next day falcon tubes were centrifuged again at 5000 rpm for 15 min at 4 ^{0}C. DNA pellet was appeared in falcon tubes after centrifugation and supernatant was discarded. 1ml of wash buffer was added to the falcon tubes to re-suspend DNA pellets and then transferred to eppendorfs. Eppendorfs were centrifuged for 10 minutes at 12000 rpm at 4^{o}C. Supernatant was discarded and DNA pellet was allowed to dry. DNA pellets were allowed to dissolve for a week by adding 1mL TE buffer into the eppendorf tubes containing DNA pellets. 1 µL RNAase per sample were also added to avoid the RNA's contamination.After DNA extraction rice samples treated with different bio pesticide were further analyzed on gel electrophoresis (Doyle and Doyle 1990).

Gel Electrophoresis and Spectrophotometer

To check the quality of DNA, 0.84% agarose gel was prepared and ethidium bromide was added (20mg/mL) for visualizing the DNA. Solidification of gel was carried on room temperature and comb was removed carefully by avoiding the rupture of wells 8µL DNA of rice leaf sample treated with each bio pesticide with 2µL 5x gel loading buffer was loaded in each wells and run the gel for 40 minutes at 700amp+80volts (Fig. V) and then quantified at Spectrophotometer (Maniatis *et al.,* 1982).

RESULTS

Analysis of variance regarding replication and treatments showed significant variation of rice leaf folder infestation before treatments. The result showed in the (Table 1). Present data was non-significant which means rice leaf folder population in all blocks is near to ETL level and it was best time to initiate the control measure. Means having similar letter significantly correlated with each other and in all these plots leaf folder infestation were found non-significant. Different bio pesticides were applied to check the leaf folder infestation percent rate (Table 2). High F values showed that data is highly significant. According to (Fig. 1) all the values showed significant variation among each other along with the application of different bio-pesticides to analyze the leaf folder infestation. This graph showed highest leaf folder infestation in control plot where no pesticide were applied and lowest post treated infestation in plot treated with *Azadirachta indica* (Neem), while Bt gave good control after Neem, Darek and Eucalyptus intermittent in efficacy. In (Fig. 2) after nine days data highlighted Eucalyptus showed leaf folder infestation in middle between Neem, Darek and Bt, control. Highest post treated infestation were observed in control plot and lowest in Neem treated plot but this infestation in all treated plots after 16 days were almost greater than double as compared to 1-9 days data which indicated that these bio pesticide were effective for 9 days and after nine days other spray should be applied (Fig. 3). Yield indicating the bio pesticides treated effect for controlling the leaf folder infestation on rice crop. Highest productions were recorded on Neem treated plot and lowest yield in control plot (Fig. 4).

Analysis of variance regarding replication and treatments showed significant variation of rice leaf folder infestation with different treatments after 3, 9, 16 days The result showed in the Table 3 to 5. All the treatments showed significant variation due to high F value in different time interval.

Analysis of variance regarding replication and treatments showed significant variation of ifferent yield plots treated with various bio pesticides. The result showed in the (Table 6).

This graph showed highest leaf folder infestation in control plot where no pesticide were applied and lowest post treated infestation in plot treated with *Azadirachta indica* (Neem), while Bt gave good control after Neem, Darek and Eucalyptus intermittent in efficacy. After nine days data highlighted Eucalyptus showed leaf folder infestation in middle between Neem, Darek and Bt, control.

Table 1. Analysis of variance for data of rice leaf folder Infestation before treatment at ETL.

Source of Variation	D.F	S.S	M.S	F. Value
Replication	2	0.47956*	0.23978*	
Treatments	4	3.51580*	0.87895*	1.39 $^{N.S}$
Error	8	5.05244	0.63156	
Total	14	9.04780		

Table 2. Pretreatment Data Mean Infestation Caused by *Cnaphalocrocis medinalis.*

Block	Percent mean infestation± S.E.
B1	2.1000 ± 0.48a
B2	3.0667 ± 0.53a
B3	2.0767 ± 0.21a
B4	1.5967 ± 0.56a
B5	2.0100 ± 0.21a

Table 3. Analysis of variance for data of rice leaf folder after three days of bio pesticide application on rice.

Source of Variation	D.F	S.S	M.S	F. Value
Replication	2	0.2378**	0.1189**	
Treatments	4	82.2396*	20.5599*	32.21
Error	8	5.1073	0.6384	
Total	14	87.5847		

Table 4. Analysis of variance for data of rice leaf folder after 9 days of bio pesticide application on rice.

Source of Variation	D.F	S.S	M.S	F. Value
Replication	2	1.102**	0.5511**	
Treatments	4	349.994*	87.4985*	154.92
Error	8	4.518	0.5648	
Total	14	355.615		

Table 5. Analysis of variance for data of rice leaf folder after 16 days of bio pesticide application on rice.

Source of Variation	D.F	S.S	M.S	F. Value
Replication	2	4.518**	2.259**	
Treatments	4	517.955*	129.489*	203.53
Error	8	5.090	0.636	
Total	14	527.563		

Table 6. Analysis of variance for data of different plots yield treated with various bio pesticides.

Source of Variation	D.F	S.S	M.S	F. Value
Replication	2	360.5**	180.27**	
Treatments	4	29434.9*	7358.73*	50.69
Error	8	1161.5	145.18	
Total	14	30956		

Table 7. DNA quantification of five samples treated with bio-pesticide and their absorbance value without dilution.

Absorbance value at A_{260}		DNA Quantification	
Sample Name	A_{260}	Rice Sample Treated With Bio Pesticide	DNA Quantification µg/ml
Neem	0.676	Neem	33.88
Darek	0.691	Darek	34.55
Eucalyptus	0.621	Eucalyptus	31.05
Bt	0.673	Bt	33.65
Control	0.689	Control	34.45

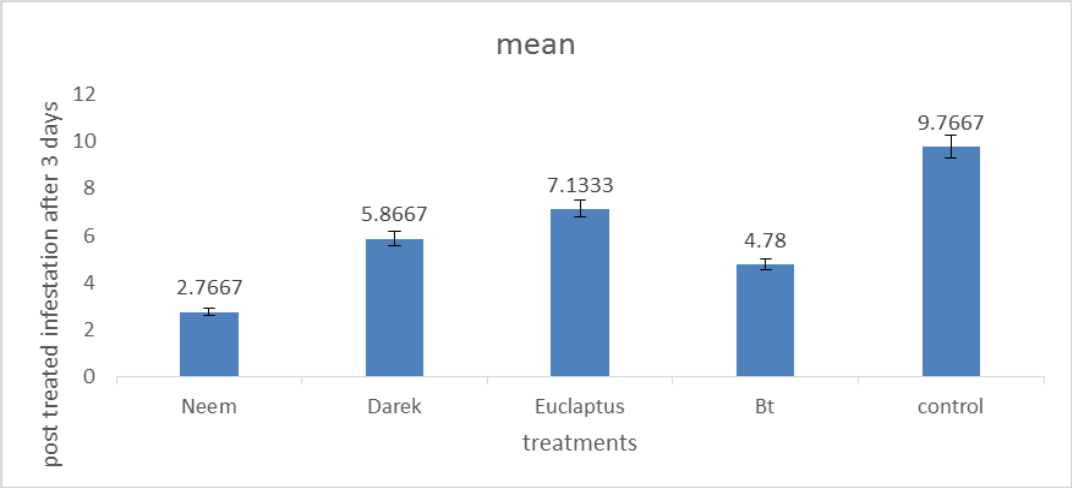

Fig. 1. Graphical representation of post treated mean infestation data after 3 days

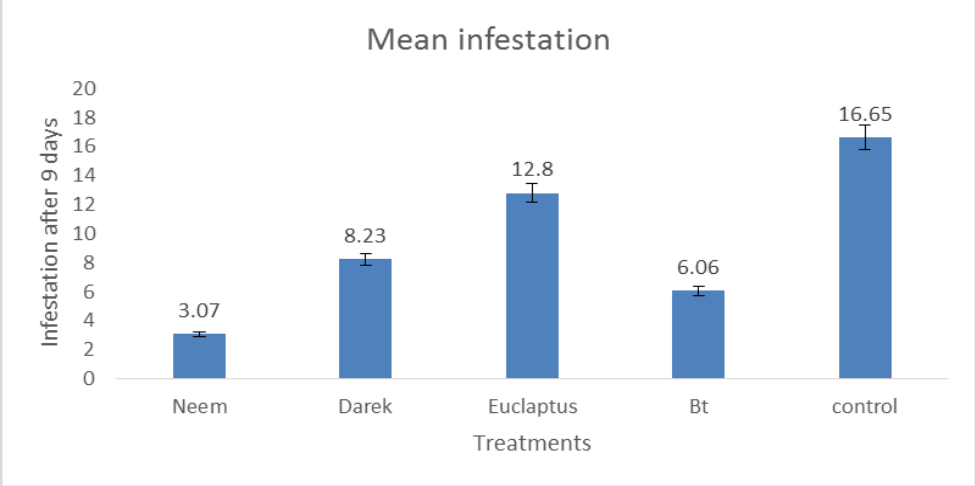

Fig. 2. Graphical representation of post treated mean infestation data after 9 days.

Highest post treated infestation were observed in control plot and lowest in Neem treated plot but this infestation in all treated plots after 16 days were almost greater than double as compared to 1-9 days data which indicated that these bio pesticide were effective for 9 days and after nine days other spray should be applied.
Yield indicating the bio pesticides treated effect for controlling the leaf folder infestation on rice crop. Highest productions were recorded on Neem treated plot and lowest yield in control plot.

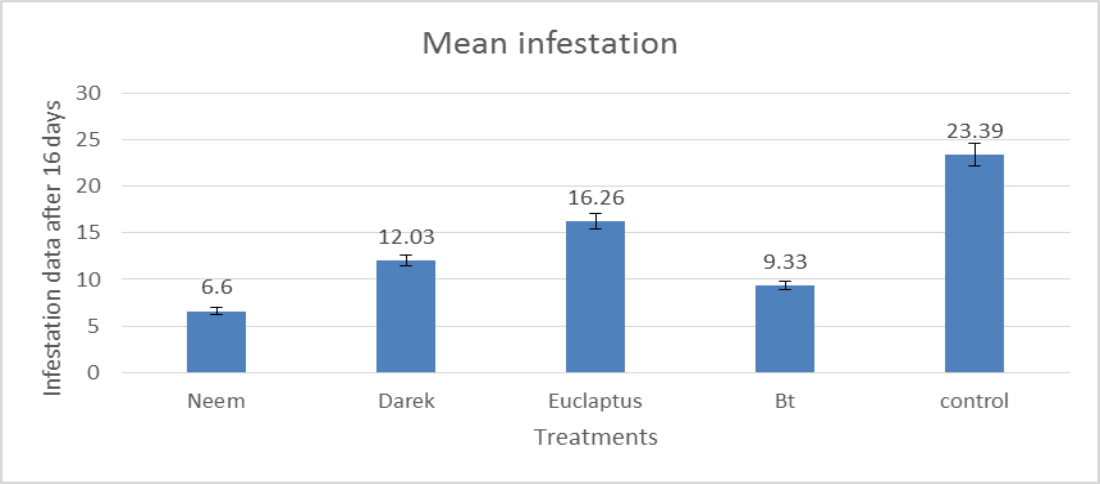

Fig. 3. Graphical representation of post treated mean infestation data after 16 days .

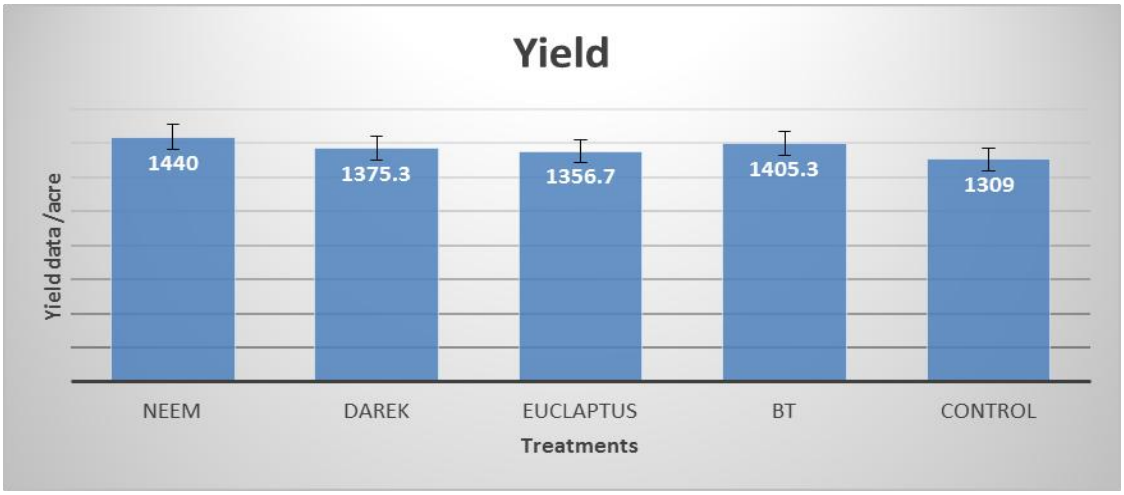

Fig. 4. Graphical representation of mean yield data of different bio pesticide treated plots.

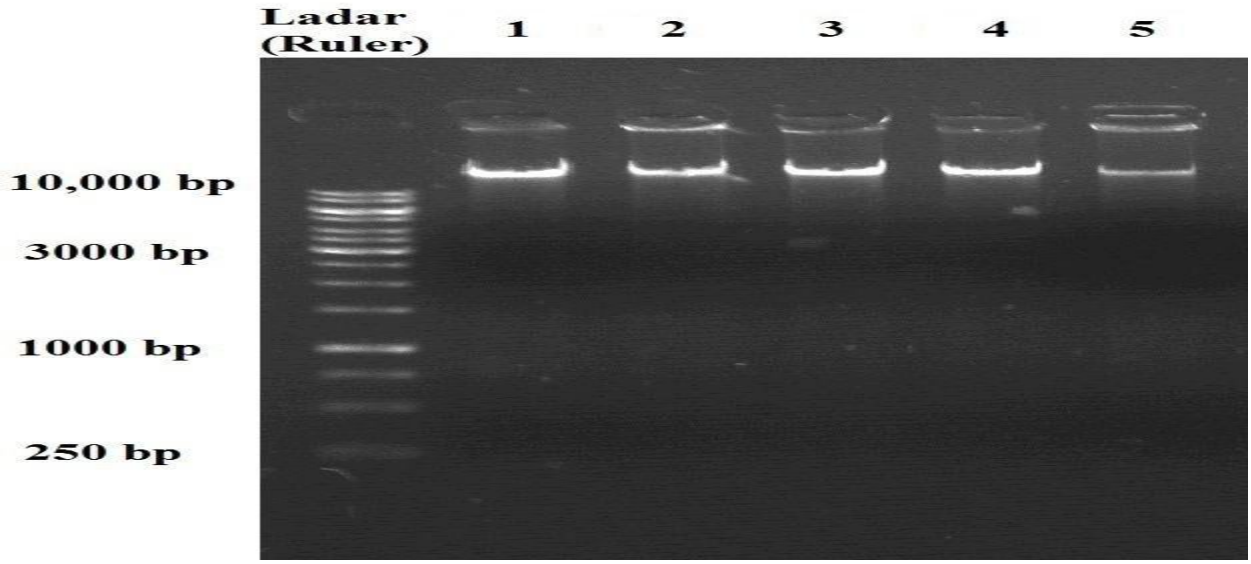

Fig. 5. DNA bands on gel electrophoresis.

DNA Bands on Gel Electrophoresis

In (Fig .5) five bands indicating quality of DNA of five Rice sample treated with different bio pesticide (Neem, Darek, *Eucalyptus*, Bt and control, respectively.

DNA Quantification

Holo BD 20 spectrophotometer was used to quantify the DNA. Absorbance was taken at 260 nm.

$$\text{DNA in } \mu g/mL = 50 \times A_{260} \times DF \times 1$$

Absorbance of Pure DNA (Without making dilutions) five sample reading were noted on spectrophotometer at absorbance wavelength 260 values indicated five samples of DNA have negligible difference of absorbance wavelength.

Quantification Formula

$$= \text{DNA in } \mu g/mL = 50 \times OD260 \times DF \times 1$$

It was found negligible difference of rice bio pesticide treated sample DNA Quantification values with control. This negligible difference may be due to device calibration and concluded that bio pesticide are safe for rice plant genome health and vigor and it did not make bond with DNA bases (Table 7)

DISCUSSION

Bio Insecticides

On the basis of results indicated that Neem crude extract gave excellent result with lowest post treated increase in leaf infestation (2.76, 3.07 and 6.60% after 3, 9 and 16 days of application, respectively) caused by *Cnaphalocrocis medinalis*. Bt showed good results with least increase in post treated infestation of 4.48 %, 6.06 and 9.33 after 3, 9 and 16 days, respectively of application after neem, *Melia azedarach* and Eucalyptus also control the *Cnaphalocrocis medinalis* but with least efficacy. It was observed that these bio pesticides showed good results up to 9 days, after this efficacy in control was decreased and infestations were increased more as compared to previous days. The similar work done by Nathan (2006) who described rice plant treated with *Melia azedarach* suppressed the leaf folding behavior of *C. medinalis* and it showed high activity at all doses when *Bacillus thuringiensis*, *vitex negundo* and neem seed kernel extract were used in laboratory experiment against *Cnaphalocrocis medinalis.* Leaf folder behavior suppressed and even larvae treated with very low dose of bio pesticide stopped spinning activity and feeding were reduced larvae become sick and regurgitate semi solid substance and die

Melia azedarach extract have different effect on insect. It affects the behavior, Fecundity of insect, reduced growth, retardation and morphological defect (Ascher *et al.*, 2002). Anti-feeding effect was observed against many insect of *Melia azedarach* (Sexana *et al*., 1984). *Azadirachta indica* seed kernel extract act as growth regulator and anti-feedant against numerous insect (Schmuttere, 1990; Mordue and Blackwell, 1993; Adnan and Rubae, 2009). Kandibane *et al.* (2010) conducted two field trials to evaluate *Bacillus thuringiensis* bio efficacy and concluded that high doses 2.5kg/ha gave better results than low doses 1kg /ha (Akhtar *et al*., 2008). On the other hand, the efficacy of Bt decrease with high temperature. All above results that supports our results with respect to the application of different bio-pesticides at various time intervals.

DNA quantification as pesticides evaluating tool for plant genome health

Different chemicals pesticides were applied on variety of crops to control the insect pest in which some pesticide are very harmful for plants. Chemicals pesticides make bond with DNA bases and cause genotoxicity (Zhang *et al*., 2013). Crops are mostly susceptible to genotoxicity **(**Boerth, 2005). Genotoxicity or bond formation of chemical pesticide with DNA bases leads to abnormal natural transcription and translation process which may affect the plant molecular process to produce a particular defense protein against particular insect and plant natural defense system become weak resulted more attack of insect pest as outbreak (Xi *et al*., 2003; Ascenso *et al*., 2013; Santos *et al*., 2015).

Our results showed DNA quantification value 34.45 µg/mL, 33.65 µg/mL, 31.05 µg/mL, 34.55 µg/mL, 33.88 for control, Bt, Eucalyptus, Darek and Neem respectively. These differences were negligible and may be due to device calibration or observation error and found no effect on plant health after spraying the different bio-pesticides. Hence bio pesticides are safe and cause no genotoxicity of plant genome (Katti, 2013).

CONCLUSION

From the study it was concluded that the *Azadirachta indica* (Neem) crude may be very useful to control *Cnaphalocrocis medinalis* and also increasing the yield potential of the crop. This bio pesticide is environment friendly and harmless for human. DNA quantification as pesticide evaluating tool for plant genome health is best one to check genotoxicity effect of pesticide which were used in this experiment for bio pesticide evaluation. This test is not only for bio pesticide but it is more necessary for chemical pesticide to check genotoxicity effect.

On the other hand, such types of studies could be helpful both for entomologists, scientific community and farmers for further improvement and enhancement of the yield potential of rice crop by controlling rice leaf folder to strengthen the economy of the country.

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