Isolation, Culture and Characterization of Insect Cell Line for *In- Vitro* Screening of Insecticides

Research Synopsis for Ph. D.

Submitted to

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Introduction:

Pests and diseases, the prime factors causing low agricultural productivity, are mostly controlled by chemical means. Despite the advantages of applying chemical pesticides in improving food quantity and quality, negative effects on human health and serious environmental problems challenge these benefits (Konradsen, 2007; Dhaliwal and Singh, 2000). Misuse and overuse of insecticides cause resistance and increase the survival rate of insect pests.

The problems associated with these general toxicants, including insect resistance and environmental concerns, have encouraged the development of more insect-specific insecticide screening procedures. For screening purposes, there is increasing interest in the development of *in vitro* methods to replace conventional animal toxicity tests, because mass rearing of these entire pests is difficult and *in vivo* bioassay are more tedious to perform. The ultimate goal is to achieve an alternative system that allows rapid testing of candidate compounds, formulations and finished products which enables the accurate prediction of toxic efficacy at the whole animal level. Thus, Insect cell culture is an alternative to insect mass rearing and its bioassay for entomopathogens and their toxins, growth regulators or various chemicals (Smagghe *et al.,* 2009). They are preferred as it reduces the time and is cost effective (Watts *et al.,* 2003; Decombel *et al.,* 2004). Apart from this it has wide application in the studies on morphogenesis, pest control, virology, pathology, biochemistry, genetics and other fields of biology and medicine. Such research in insect science through cell culture systems can be part of the modern front line of agricultural science necessary for safer production of healthier foods to meet the demands of a rapidly increasing human population.

Cell lines provide unlimited material resources because the cells can be readily maintained at relatively low costs with minimum labour input. Cell lines also provide a population of cells to generate consistent samples and reproducible results. Most insect cell lines are usually derived from embryos, ovaries, hemocytes, testis, newly hatched larvae, pupae, fat body, silk glands, epidermis and midgut cells (Sudeep *et al.,* 2002a, 2002b; Shao *et al.,* 2008; Goodman, 2012; Grasela *et al.,* 2012; Imanishi *et al.,* 2012; Zhang *et al.,* 2006, 2013; Ding *et al.,* 2013; Zheng *et al.,* 2013, 2010; Hu *et al.,* 2014). The alimentary canal particularly the midgut, is one of the most important tissues playing a crucial role in the insect physiology, as

it is the site of digestion, detoxification, transport, and semiochemical production (Hall *et al.* 2002a, 2002b; Nardi *et al.* 2002). Histologically, the midgut epithelium of Insects is a complex and dynamic tissue composed of a monolayer of columnar and goblet cells with stem cells lying along the base of the epithelial cells. The midgut epithelium is responsible for the digestion, absorption, and transport of nutrients and inorganic ions. In addition, the midgut epithelium is also the target site for the initiation of most viral infections as well as for insecticidal toxins (Liu *et al.,* 2011). Studies on the development and physiology of the midgut epithelium and on the mode of action of pesticides are difficult to carry out *in vivo.* However, significant progress has been made in the preparation of primary cultures of differentiated midgut insect cells *in vitro* (Garcia *et al.,* 2001; Li *et al.,* 2015; Young and Black 2004; Li and Xie 2005). More than 500 cell lines from over 100 different insect species have been described worldwide form the order Lepidoptera, Diptera, Coleoptera, Blattaria, Hymenoptera, Orthoptera, Hemiptera, etc. Among them, cell lines from Diptera and Lepidoptera has the largest number (Zheng *et al.,* 2013).

As far as the India is concerned, in 1967, the first attempt was made to establish two cell lines at the National Institute of Virology (NIV), Pune from the larval tissues of *Aedes aegypti* and *Aedes albopictus*. Further, the attempts were made to establish a new cell line from the embryonic tissues of *culex tritaeniorhynchus* (Athawale, 2002) and of *Helicoverpa armigera* (Sudeep *et. al.,* 2002a). Later, Khurab and his team in 2006 developed a cell line from ovarian tissue of a commercial variety of the silkworm and proved that cell line was highly susceptible to BmNPV infection. A number of well characterized, dipteran and lepidopteran cell lines are available with NIV but the potentials of these cell lines are not yet fully exploited. In Gujarat no organization till now has dealt with study of such establishment of insect cell line. The renewed interest in developing new insect cell lines is due to their potential application in biotechnology, virology, pathology, biochemistry, genetics and other fields of biology and medicine. (Li and Bonning, 2007; Soin *et al.,* 2009; Monti *et al.,* 2014 and Mandrioli *et al.,* 2015).

Keeping in view the above mentioned facts the present study is an attempt to isolate, culture and characterization of insect cell line in order to provide tool for physiological studies as well as in vitro bioassays and insecticide screening studies.

Objectives

Objective 1: Rearing of stored grain pests.

Objective 2: Isolation of cells from the insect organs.

2(a): Establishment of cell line from insect organs.

Objective 3: Characterization of insect cell cultures.

3(a): Morphological characterization.

3(b): Molecular characterization of insect cell line

3(c): To check the epithelial and mesodermal markers of cells.

Objective 4: Toxicity studies on insect cells exposed to insecticide.

4(a): Cell viability assay.

4(b): To check cell death using Annexin/PI staining.

4(c): To check the oxidative status using ROS parameters.

Material And Methods:

Animal model: *Sitophilus oryzae*

Rice infected with the pest were collected and brought to the laboratory. The pests were identified and then were separated from the infected lot, and fresh rice grains were provided. They were then reared in the laboratory for several generations. The Colonies were maintained in the dark in a climatic chamber set at 25±2 ºC and 60±5% r.h. Adult *S. oryzae*, 2–4 weeks old, were then used for the experiments.

Objective 1:

Experimental regime:

Stock insect culture preparation:

The collection and maintenance of the pest was done in which they were monitored till the third generation so as to obtain the fresh pest population. *S. oryzae* were collected from the stored grain warehouse of Vadodara, Gujarat. The collected pest species were first acclimatized to the laboratory conditions, the dead and live were separated and were then

identified using proper taxonomical keys. Twenty five pairs of adult *S. oryzae* were introduced into 200 g grains of rice in 1 Kg capacity plastic jars covered with mesh lids and were allowed to feed, mate, and oviposit. The insect cultures were kept under the maintained conditions i.e. 25–28°C temperature, 60–70% relative humidity, and 12-hour photo phase. Culture arenas were observed daily until new progenies emerged; the whole set up was replicated for four times. The pure breeds from the fifth generation were removed and sexed using morphological characters described by Halstead *et al.*, 1969. This stock culture was used as source of *S. oryzae* throughout the period of the study.

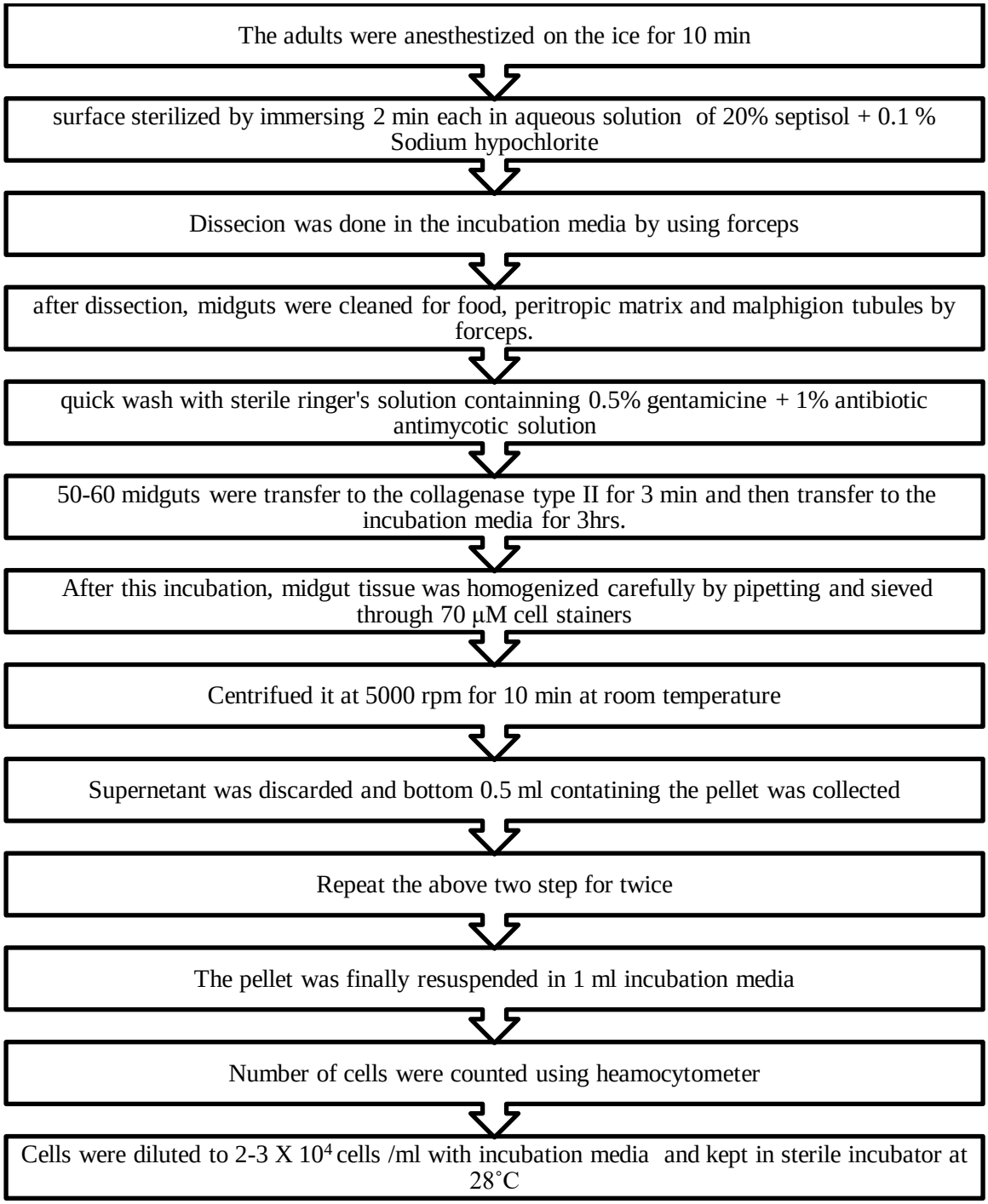
Insect development and morphometry analysis:

From the stock the pests were then monitored for their incubation time, Male: female ratio and total body length. Along with observing their developmental time their fecundity and longevity was also observed. In addition an attempt was made to note down the total grain loss. From the stock pest, ten pairs of one-week-old male and female *S. oryzae* were introduced into 200 g grains of maize (*Zea mays*), rice (*Oryza sativa*), chickpea (*Cicer arietinum*) and millet (*Pennisetum glaucum*) were weighed and kept in 2 kg capacity plastic jars covered with mesh lids. These grain jars with adult *S. oryzae* were kept under ambient temperature of 25–28°C, 60–70% relative humidity, and 12-hour photo phase. Weevils were allowed to feed, mate, and oviposit for 7 days. The grains were observed for infestation, which was identified by the plug formation on the grains. Once the infestation was observed, the live adult weevils were removed and the culture was monitored till the adult emergence. Overall, the whole experiments were maintained for 60 days and the observation of newly emerged *S. oryzae* were recorded.

To determine the fecundity and longevity, one adult pair of the pest were introduced into 20 g of maize (*Zea mays*), rice (*Oryza sativa*), chickpea (*Cicer arietinum*) and millet (*Pennisetum glaucum*) were weighed and kept in plastic jars covered with mesh lids. They were kept under ambient temperature of 25–28°C, 60–70% relative humidity, and 12-hour photo phase. After the exposure for 7 days these pairs of *S. oryzae* were shifted to fresh batch of heat sterilized grains and again kept for 7 days. They were observed for a total period of 27 weeks, during which the fresh change was given on every 7th day till the end of the experiment. The infested grains were kept in the separate jar and were also observed till new pest emerged. The total number of the new adults emerged were recorded for their fecundity and longevity. For loss of grains, the weight of the grain was taken before the exposure of the pest and after

the removal of the pest from the plastic jars. All the experiments were performed in triplicates.

Objective 2: Newly emerged one week old adult *S. oryzae* were separated from the culture and were starved for 2 days before starting the experiments. All dissections and transfers were done in the sterile environment of the biosafety cabinet. The isolation of the midgut cells were carried out by modified protocol of Hakim *et al.,* 2009.



Incubation media contains:

Grace's insect media (Himedia IML001A)	25% FBS (Himedia IML1112)
3X Antibiotic antimycotic solution (HimediaA002)	Vitamin mixture (3mg /ml riboflavin, 1.2 mg/ml vitamin B12 +1.5 mg/ml of folic acid)
1X gentamicine (HimediaA005)	
0.1 % amphotericine B (HimediaA011)	Ecdysone (Sigma E9004)

Objective 3: Morphological characterization was made under the microscope. Further the molecular characterization was done using sanger sequencing of COI and 16srRNA gene. The epithelial and mesodermal markers were screened using the conserved markers for proliferation and differentiation. Primers for the markers will be selected from NCBI and the gene expression studies were done using real time PCR.

Objective 3 (a): Morphological characterization:

Optimization of insect cell growth media: Two insect growth media (Grace's Insect media and TNM-FH medium) were used to conduct this study. Six-well plates containing 3 ml of insect growth media, 2 X 10^4 cells/ ml were seeded. Cells were counted in triplicate every 24hrs for each medium with neubauer hemocytometer. Cell density and viable cell percentage were observed up to 96 hrs to find the optimum growth media for *S.oryzae* midgut cell line (SoMG). Cell viability was calculated by the following formula:

$$\% \text{ Cell viability} = \frac{\text{Total viable cells (Unstain)}}{\text{Total no of cells (stain + unstain)}}$$

Objective 3 (b): PCR Reaction Mixture & Condition:

PCR Reaction Mixture	
Master mix	**5 µl**
Forwad Primer	**1.0 µl (10pmol)**
Reverse Primer	**1.0 µl (10pmol)**
DNA Templete	**100 ng**
Nuclease free D/W	**Make up to 10 µl**

2X Master mix Composition	
2mM	**$MgCl_2$**
50 mM	**KCL**
10mM	**Tris HCL**
0.2 mM	**Each dNTPs**
0.5 mM	**Taq polymerase**

PCR Conditions

For COI gene

Stage 1 (1 cycle)	Stage 1 (5 cycle)	Stage 2 (35 cycle)	Stage 3 (1cycle)
94° C 1.30 min	94°C 1.00 min	94°C 1:00 min	72°C 10:00 min
	45°C 1:30 min	50°C 1:30 min	4.0°C stop
	72°C 1:30 min	72°C 1:00 min	

For 16s gene

Stage 1 (1 cycle)	Stage 2 (35 cycle)	Stage 3 (1cycle)
94° C 3.00 min	98°C 0:10 min	72°C 08:00 min
	50°C 0:30 min	4.0°C stop
	72°C 1:30 min	

Primers details:

Name of DNA marker and primer	Primer sequence (5' to 3')	Reference
Cytochrome c oxidase subunit 1		
LCO-1490	5'-GGTCAACAAATCATAAAGATATTGG -3'	Folmer *et al.,* 1994
HCO-2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer *et al.,* 1994
16S Gene		
16Sar	5'-CCGGTCTGAACTCAGATCACGT-3'	Simon *et al.,* 1994
16Sbr	5'-GCTCAGAACGAACGCTATC-3'	Simon *et al.,* 1994

Objective 3 (d): RT-PCR for Epidermal and mesodermal markers of Cells

qPCR Reaction Mixture	
Master mix	5 µl
Forwad Primer	1.0 µl (10pmol)
Reverse Primer	1.0 µl (10pmol)
cDNATemplete	500 ng
Nuclease free D/W	Make up to 10 µl

qPCR conditions:

Step 1 (1 cycle)	**Step 2 (1 cycle)**	**Step 3 (40 cycle)**
50°C- 02:00 min	**95°C- 02:00 min**	**95°C- 00:15 min**
		55°C- 60°C 00:15 min
		72°C- 01:00 min

Primer details

Gene ID	Gene Name	Sequence	Tm	Amplicon size (bp)
XM_022975529.1	β –actin F	5'-GTATCGTGCTGGACTCCGGT-3'	61.0	154
	β–actin R	5'-GTGAAGGAGTAGCCACGCT-3'	59.0	
EU526836.1	Cup 1 F	5'-GGCAAGAAGGGTTCCGAAGT-3'	59.0	134
	Cup 1 R	5'-TTCTCGTTGGCGACGTACC-3'	59.0	
EU526837.1	Cup 4 F	5'-GAGGGTGGATACCAGTTCGC-3'	61.0	118
	Cup 4 R	5'-CGCGCACAACCACTACTTTG-3'	59.0	
DQ847154.1	hmg-176 F	5'-AAGTTTCCTTGTCATCGCCC-3'	57.0	105
	hmg-176 R	5'-GTCTCCGAACTCTCGGGTA-3'	59.0	

Objective 4: Toxicity studies on insect cells exposed to insecticide

Insecticide was selected according to their usage in agricultural field. An inhibition concentration of insecticide was performed and analyzed using probit analysis. After obtaining the Inhibition concentration (IC_{50}), sub lethal (1/5, $1/10^{th}$ and $1/20^{th}$ does of IC_{50}) concentrations were selected for further studies. The acute study were carried out in which, cell viability assay were performed for 24, 48, 72 and 96hrs using MTT. Further, the annexin-/Pi staining were performed to test whether the cells are undergoing apoptotic or necrotic death. Simultaneously, Oxidative status for ROS parameters were carried using standard protocol.

4(a): Cell viability assay

MTT assay:

SoMG cell suspensions (1 x 10^5 cells/ml) were seeded onto a 96-well culture plates (100 µL per well). After 24 hrs of incubation, varying concentration of fenitothion were added at final concentration of 25, 50, 100,125,150, 175, 200, 225 and 250 µg/ml for 24, 48, 72 and 96 hrs. Complete insect culture medium was used as control. Four hours prior to the assay, 10 µl of MTT solution (8mg/ml in incubation media) was added in to each well. To dissolve formazon crystals, the media in each well was replaced with 100 µL of DMSO. The optical density was measured using a microplate reader at 570 nm with a reference wavelength at 630 nm. Cell viability was obtained using the following formula.

$$\% \text{ Cell Viability} = \frac{\text{Average OD of test x 100}}{\text{Average OD of control}}$$

Calculation of IC_{50}: After treating the cells with the test chemicals, their mortality rate was determined from the formula given above. The results were plotted and the dose response curve and the IC_{50} value were obtained using the prism6 software.

Toxicity studies using the sub-lethal concentrations: Cell membrane blebbing, ROS generation, DNA damage, cell death, cell fragmentation etc was observed by time and dose dependent manner. Effects of the test chemical on the insect cell culture were studied using the sublethal concentrations –$1/5^{th}$ (HD), $1/10^{th}$(MD),$1/20^{th}$ (LD).

Results:

Objective 1:

Table 1 and figure 1 to 4 summarizes the incubation period, number of adults emerged, grain loss and variation in male and female body length. The incubation period and progeny size showed a significant ($p<0.001$) difference in the development period where, the least development period (from egg to emergence of adult) was observed with maize (32.5 days) followed by chick pea, rice and millet by 35, 39 and 42 days respectively. Along with the developmental time the progeny size of *S. oryzae* was also found to be significantly different. The mean emergence of F_1 progeny was found to be highest ($p< 0.001$) in maize (226 adults) and was least (126 adults) in millet. The average grain loss was found to be highest in maize (29.88%), followed by chick pea (26.54%), and was 22.02% in rice followed by least loss in millet (16.10%).

The body lengths of females were observed to be significantly bigger as compared to male in all four cultures, and were largest in maize and smallest in millet. The consumption of the grains by individual pest was also observed and was found to maximum in rice and least in millet. Table II and Figs (5) to (7) summarizes the fecundity, longevity and gender ratio. The highest fecundity and longevity was found to be in maize (453 adults), followed by chickpea (299 adults), rice (214 adults) and millet (142 adults) respectively. In addition, the gender ratio was also observed, among the four grains the highest male: female ratio was found to be in maize followed by chickpea, rice and the least in millet.

Type of Grain	Incubation period	No of adult emerge (Range)	Grain Weight Loss(in gram)(Range)	Body Length (in mm)	
				Male (Range)	Female (Range)
Maize	**32.5**	**226±5.77**	**59.77±3.02**	**4.28±0.76**	**4.6±0.41**
		(202-241)	**(49-65)**	**(4.17-4.31)**	**(4.51-4.87)**
Rice	**39**	**139±3.98**	**44.04±2.53**	**3.18±0.26**	**3.43±0.17**
		(122-158)	**(41-49)**	**(3.02-3.28)**	**(3.37-3.49)**
Chick pea	**35**	**176±4.43**	**53.08±2.94**	**4.02±0.54**	**4.14±0.24**
		(163-186)	**(51-68)**	**(3.82-4.07)**	**(4.10-4.26)**
Millet	**42**	**126±3.07**	**32.21±1.83**	**2.67±0.06**	**2.84±0.03**
		(116-132)	**(27-39)**	**(2.64-2.69)**	**(2.79-2.91)**

Table 1: Incubation period, Number of adult emerged, Grain weight loss and Body length of *S. oryzae* in different host grains

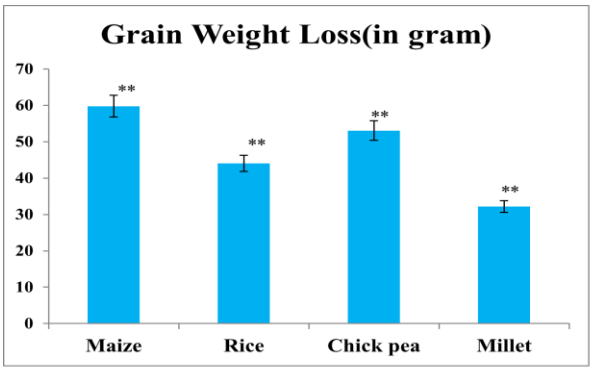


Fig 1: Amount of grain weight loss by *S. oryzae*

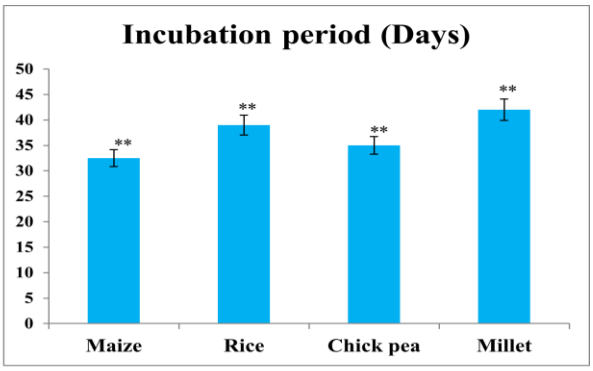


Fig 2: Incubation time taken by *S. oryzae* in different grains

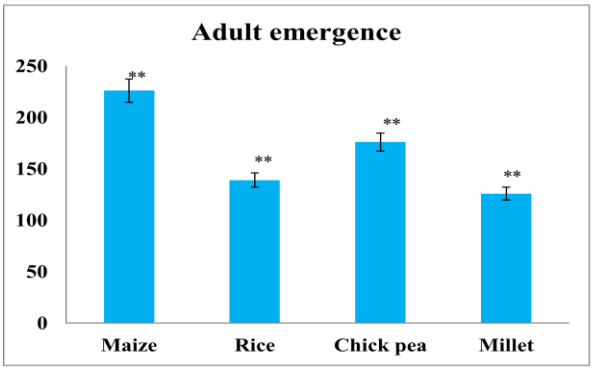


Fig 3: No. of Adult *S. oryzae* emerge in different grains

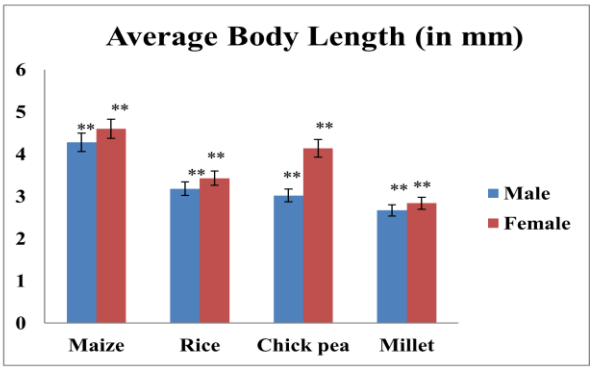


Fig 4: Body length of male and female in different grains

Significant level indicated by * (p<0.05); **(p<0.01);*(p<0.001)**

Type of Grain	Fecundity	Female: Male	Adult Longevity
Maize	453±2.37	2.36:1	184±1.52
	(422-494)		(179-198)
Rice	214±1.52	2.35:1	175±2.01
	(201-236)		(167-183)
Chick pea	299±1.75	1.98:1	180±2.57
	(271-315)		(169-192)
Millet	142±1.13	2.21:1	157±2.01
	(135-152)		(145-169)

Table 2: Fecundity, Female: Male and longevity periods (±SE) of *S. oryzae* on stored grains (24–30°C; 60 ± 10% RH; 12 h photophase).

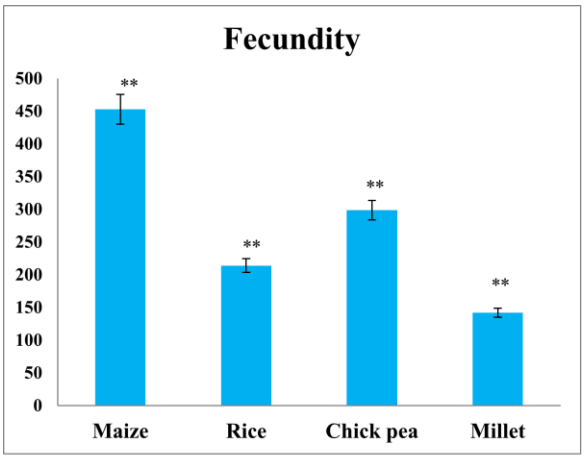


Fig 5: Effect of different grains on fecundity rate of *S. oryzae*

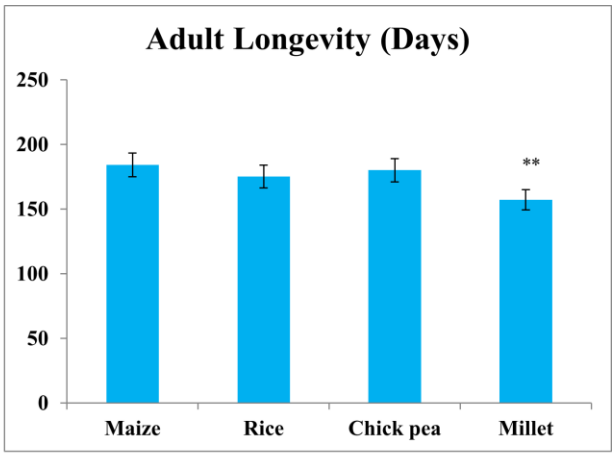


Fig 6: Effect of different grains on longevity of *S. oryzae*

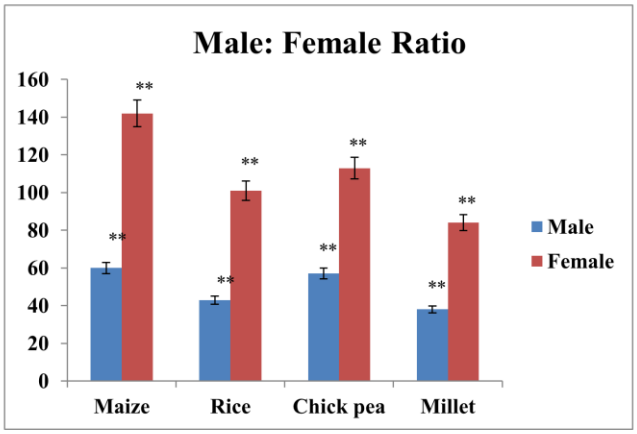


Fig 7: Male : Female ratio in newly emerge *S. oryzae*in different grains

Significant level indicated by * (p<0.05); **(p<0.01);*(p<0.001)**

Objective 2:

A cell culture was successfully initiated using midgut tissues in two different insect growth media i.e Grace's insect media and TNM-FH Medium with 10 % FBS in both media. A significant increase in the cell growth was observed at 72 hrs in Grace's insect media as well as TNM-FH media which reached to its maximum at 96 hrs (Fig 8). Further the additives were added to the media to enhance the proliferation rate of the cells (Table 1). The additives were added in different concentration following the protocol of Hakim *et al.*, (2009) with a slight modification. All modified media were assessed to find the optimum insect cell growth for SoMG cells, and the maximum cell density of SoMG was observed in the media with 25% FBS and 10pM Ecdysone with 93% of cell viability (Fig: 9-12). The initial Seeding cell density which was 2 $x10^4$ increased significantly to (4.1 x 10^4) at 72hrs and (5.1$x10^4$) at 96hrs for Grace's media . Whereas, a non-significant increase in the cell density in TNM-FH media was observed (Fig 9 -12). The first successful subculture was carried out on 6^{th} day after the

cells were seeded. The primary cell culture thus obtained was then sub-cultured for 17 passages with Grace's media and with TNM-FH media for 10 passages.

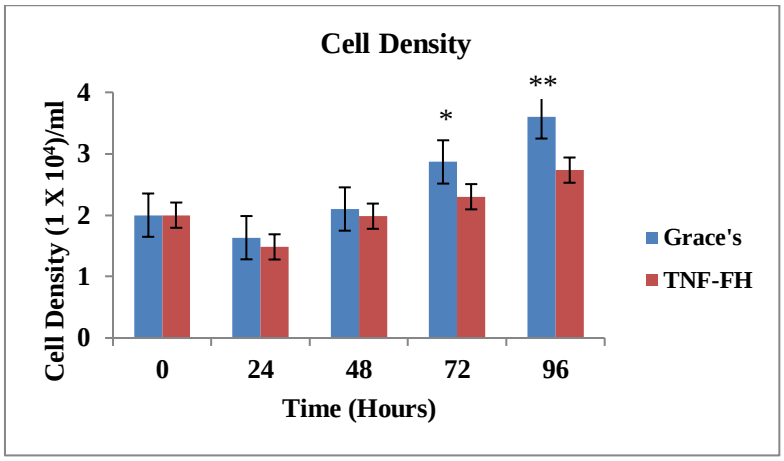


Fig 8: Cell growth density with different media.
Significant level indicated by * (p<0.05); **(p<0.01);*(p<0.001)**

Table 3: Different concentration of FBS and Ecdysone in Growth media

1	15% FBS +5pmol Ecdysone
2	15% FBS +10 pmol Ecdysone
3	15% FBS +15pmol Ecdysone
4	15% FBS +20pmol Ecdysone
5	20% FBS +5pmol Ecdysone
6	20% FBS +10pmol Ecdysone
7	20% FBS +15pmol Ecdysone
8	20% FBS +20pmol Ecdysone

9	25% FBS +5pmol Ecdysone
10	25% FBS +10pmol Ecdysone
11	25% FBS +15pmol Ecdysone
12	25% FBS +20pmol Ecdysone
13	30% FBS +5pmol Ecdysone
14	30% FBS +10pmol Ecdysone
15	30% FBS +15pmol Ecdysone
16	30% FBS +20pmol Ecdysone

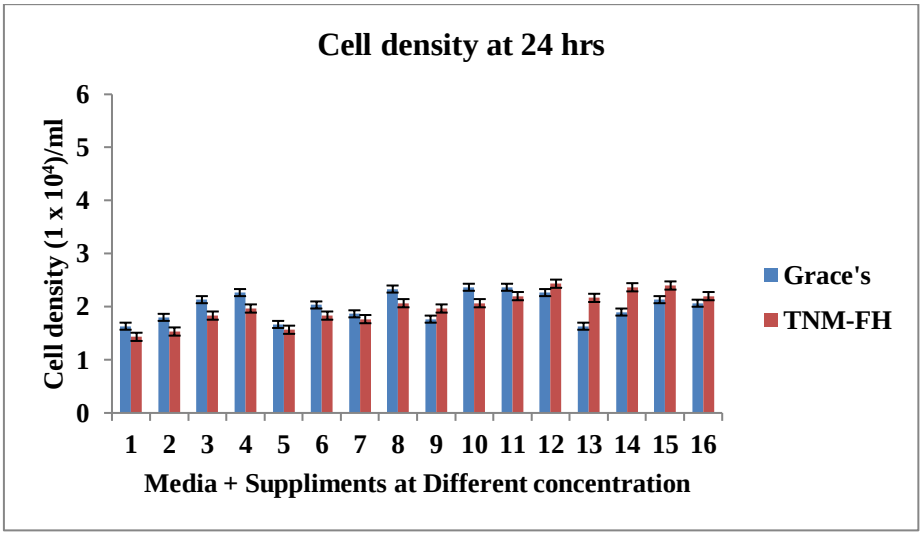


Fig 9: Cell density in different incubation media at 24 hrs
Significant level indicated by * (p<0.05); **(p<0.01);*(p<0.001)**

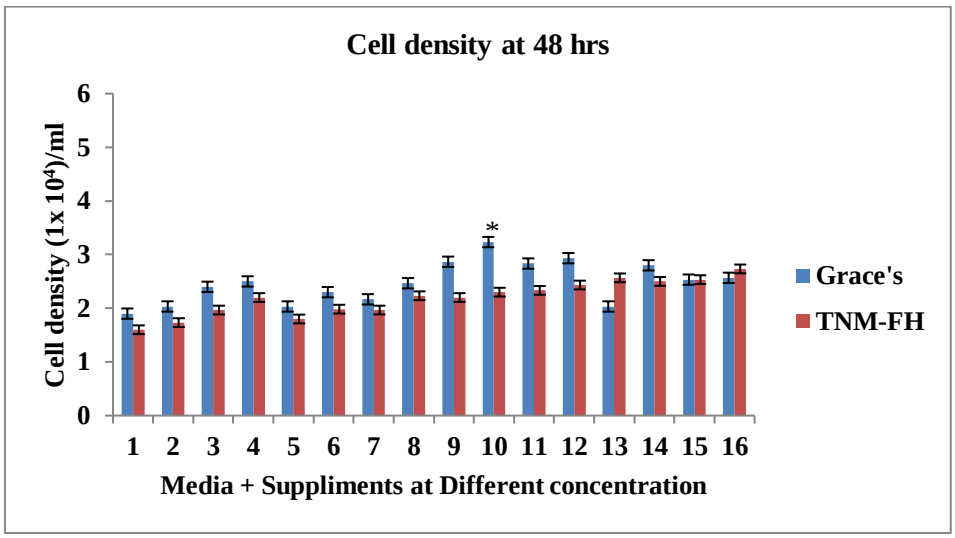


Fig 10: Cell density in different incubation media at 48 hrs

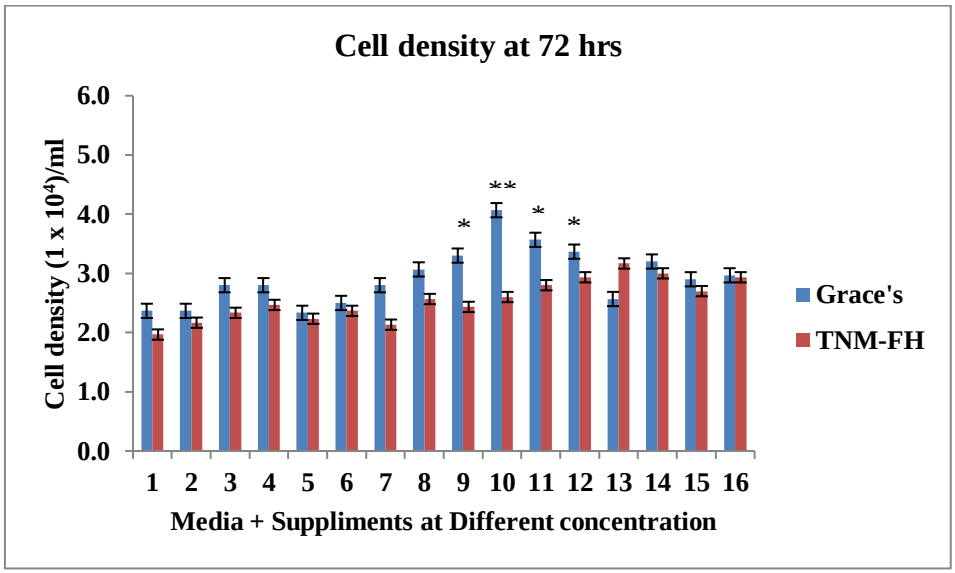


Fig 11: Cell density in different incubation media at 72 hrs

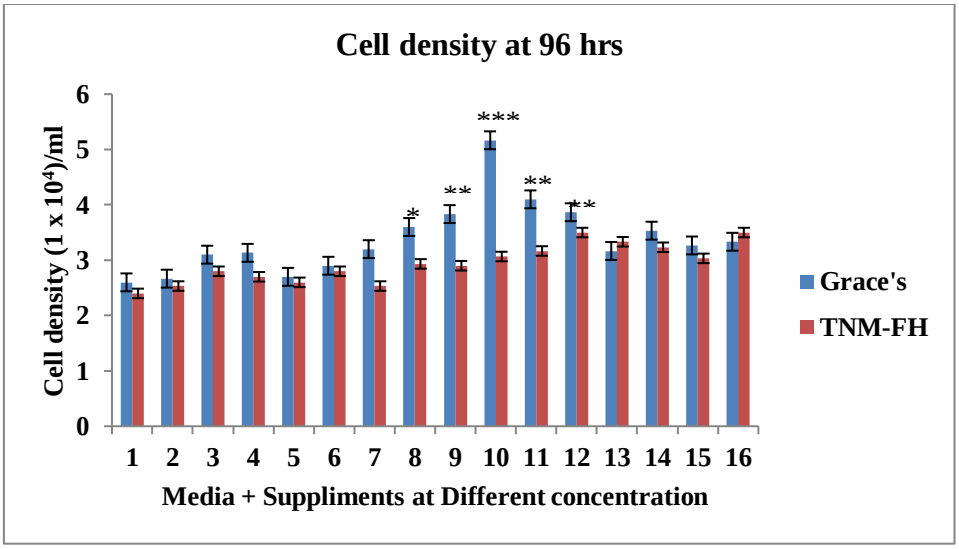


Fig 12: Cell density in different incubation media at 96 hrs

Significant level indicated by * (p<0.05); **(p<0.01);*(p<0.001)**

Objective 3:

Cell growth curve and cell doubling time: Primary cells culture were sub cultured with a 1: 1 split ratio when the cells reached to the 70% confluence in to 1:1 ratio in 12.5 cm^2 flask with an average duration of 6 days, from third passage it was transferred to the 25cm^2 tissue culture flask with average of 8 day intervals. Thereafter, the split ratio was increased gradually to 1:2 to 1:3 ratios on every 7^{th} to 8^{th} days. Growth curve was measured at the 9^{th} passage using the Hayflick formula. The curve demonstrates that the logarithmic growth phase occurred on 3^{rd} day and continued till 13^{th} day after the sub culturing. After which cells aged and died. Saturated cells formed approximately 9.00 x 10^4 cells in 25cm^2 flask, which denoted a 4.5 –fold increase over the initial population (2.0 x 10^4 cell/flask) on the 13^{th} day. Population doubling time during logarimatic growth was calculated and was found to occur at 72.3hrs using the Hayflick formula (Fig: 12).

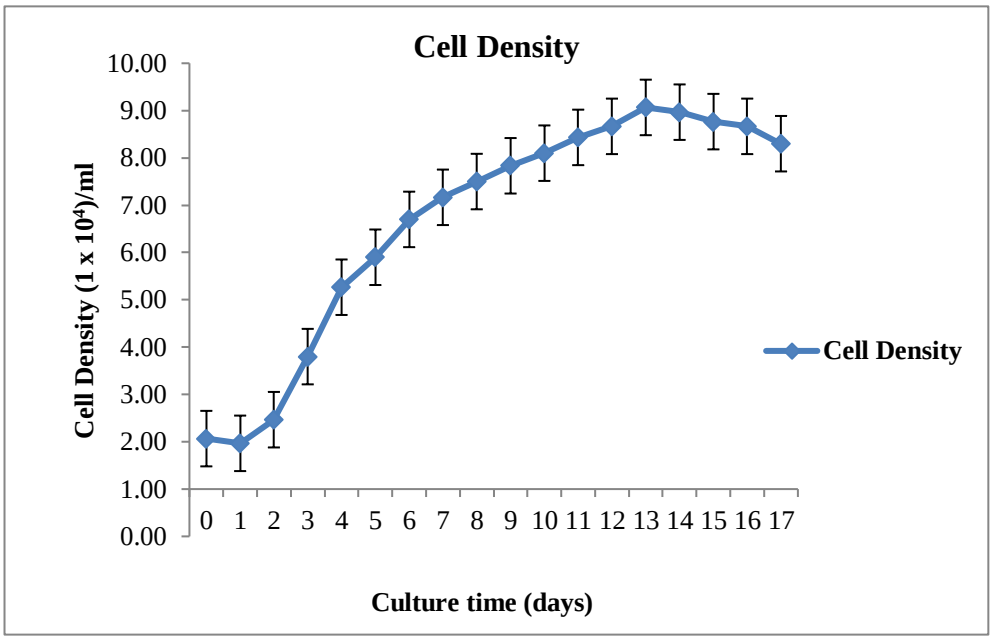


Figure 13: A Growth curve of SoMG cell line in Grace's medium containing 25% fetal bovine serum + 10pmol Ecdysone+ vitamin mixture

Cell morphology

At the beginning small tissue pieces of midgut gradually got attached to the bottom of the flask and many cells migrated from the tissue pieces and slowly got spread out. Cell growth was slow with round and sparse spindle shaped morphology. However, after few days the cell groups formed colonies which got loosely attached to the surface of the flask. The round cells grew faster than any other cells in the flask and the density of the cells was found to be increasing. Many cells aggregated to form multiple cell masses. Once the colony started

forming the spindle shaped cells were not seen. The size of the SoMG cells was measured and found to be of 16.4±2.1 μm in diameter using cellometer software (Fig: 14). The cell line was named as SoMG after it had been successfully sub cultured for over 17 generations of subculture.

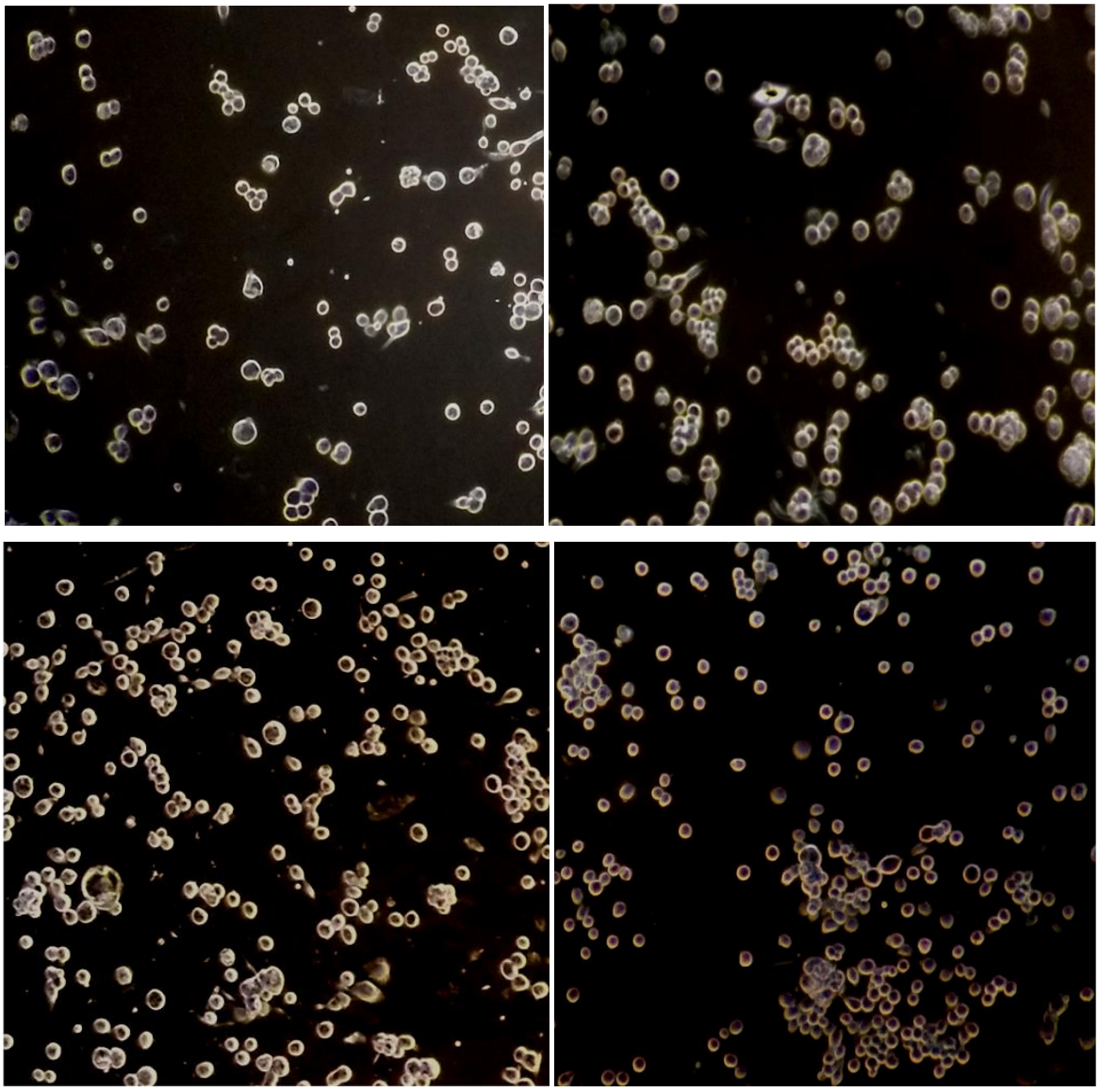


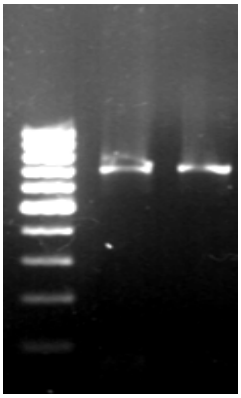
Figure 14: Cell growth after the seeding. A) at 24 hrs B) at 48 hrs C) at 72 hrs D) at 96 hrs (100X magnification)

Molecular characterization:

The molecular characterization was done by sanger sequencing of COI and 16s rRNA Gene. DNA sequencing of *Cytochrome oxidase I* (COI) and 16s rRNA was carried out to prove that the cell line belongs to *S. oryzae*. Sequence alignment and homology search was performed using MEGA 7 software. The results confirmed that there was 99.96 % for COI and 96% for 16s rRNA homology of this cell line with *S. oryzae.* COI and 16s rRNA sequence of *S.*

oryzae present on NCBI indicating the cell line belongs to this species. Furthermore, to identify the nature of cell line, expression pattern of candidate genes of cuticle (*cup1* and *cup4*), *hmg176* were analyzed. The results revealed that the highest expression was of *hmg176* followed by *cup4* and *cup1* proving that the SoMG cell line was epidermal in origin (Fig. 17).

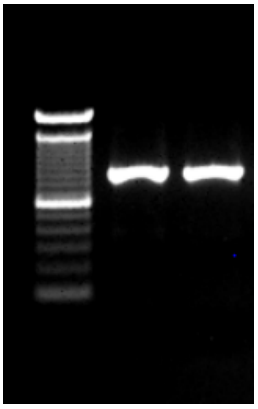
COI



CCCAATCTTTATGATTTGTTGACAGGTTAACTTACAGGGCTGACCAAAAATATCACCAATA
TCTTTATGATTTGTTGACAAGGTAAAATTTCAGGTTGCCAAAAAATGCCCCATAATTTTGA
GGATTGGTGGACAAATTCTTACTGCTCGTATAAAAAAGGTTATTCGTTCATATAATATTCC
TGAGGGTCGTATATTATAGGCTGTTGTAATAAAATTAATAGCTCCTAGAATAGATGAAATT
CCTGCTATATGTAAACTGAAAATGGCCAGATCAACAGAAGCTCCTTCATGGGCAATATTGG
ATGAGAGCGGGGGGTAGACGGTTCATCCTGTTCCTGCTCCCTTTTCAATAAATCTTCTTAT
TAGTAAAAGAGTTAAGGAGGGTGGAAGTAATCAAAATCTTATATTATTTAAACGGGGGAAT
GCTATATCTGGGGCTCCTAATATTAATGGGATTAATCAGTTTCCAAATCCTCCAATTATAA
TTGGTATTACTATAAAGAAAATTATAATGAATGCATGTGCTGTGACAATAGTATTATAAAT
TTGGTCATTTCCAATTAGTGATCCAGGATTTCCTAGTTCTGCCCGAATTAGCAAACTTAAG
GATGTACCTACTATTCCTGATCATGTTCCAAAAATAAAGTATAATGTTCCAATATCTTTATG
ATTTGTTGACCAAC

Figure 15: Gel image of COI lane 1 DNA ladder, lane 2 tissue, Lane 3. SoMG cell line

16s rRNA



CTTTTTGATTATAATTTAAAGTCTAACCTGCCCAATGATAAAATTTAAATGGCTGCGGTAT
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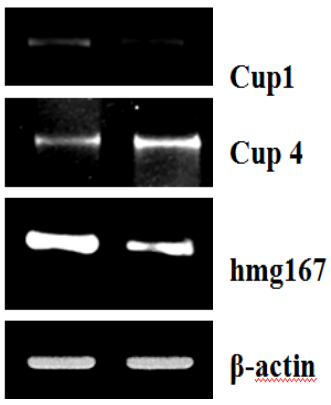
TGCATGAAAAAATGACTGTCTCTATTTAAATTTAATTGAATTTTATTTTTAAGTAAAAAAC
GCTTAAATTTTTCTAAAAGACGAGAAGACCCTATAGAGTTTTATAAATTTATTATAAGGA
ATTTTTAGGATTTAAAATAATTTATATTTAATTTATTTAATTGGGGTGATTGAAAAATTTG
TTAAACTTTTTTATTTATATTACATTAATTTATGAGTTTTTGATCCTTACTTTAAGATTAAA
AGATAAAATTACCTTAGGGATAACAGCGTTATGCGCATTGAGAGTTCAAATCGAAAAAAA
AGATTGCGACCTCGATGTTGGATTAAAATAAATTTTTGGTGTAGAAGCTAAATTTTTGAT
TATAATTTAAAGTCTAACCTGCCCAATGATAAAATTTAAATGGCTGCGGTATTTTGACCG
TACAAAGGTAGCATAATCATTAGTTTTTTAATTGAAAGCTGGAATGAAAGGTTGCATGAA
TGCATGACTGTCTCTATTTAAATTTAATTGAATTTTATTTTTAAGTAAAAAA

Figure 16: Gel image of 16s rRNA. lane 1 DNA ladder, lane 2 tissue, Lane 3. SoMG cell line

A. **B.**



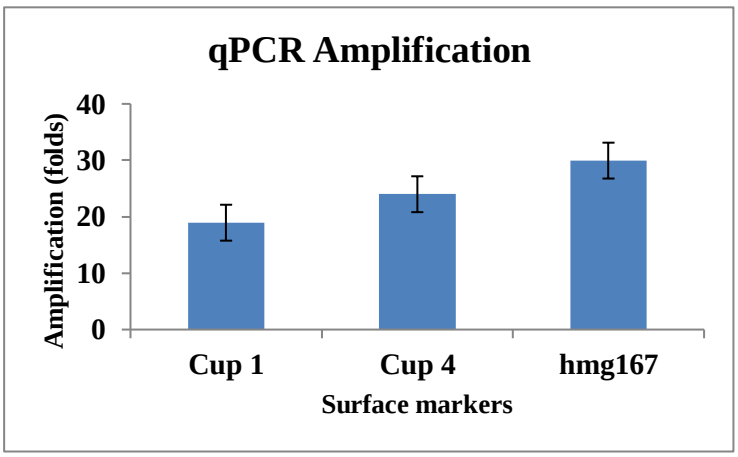


Figure 17: A. Semi-quantitative RT-PCR to compare gene expression patterns in SoMG cell line and tissues. B. Quantitative qPCR Amplification for 3 surface markers.

Objective 4: Toxicity studies

Cell viability assay

MTT Assay: For toxicity studies fenitrothion was used and cell viability was checked by MTT assay. The viability of SoMG cell line treated with increasing concentration of fenitrothion for 24, 48, 72 and 96 hrs was assessed by MTT assay. The result showed that fenitrothion inhibits cell viability in a dose and time dependent manner at concentration ranging from 10 to 250 µg/ml (Fig. 18). The IC_{50} value for fenitrothion treatments at 48 hrs as determined from the cell growth curve and was found to be 99.96 µg/ml based on the data retrieved by probit regression analysis (Fig: 18).

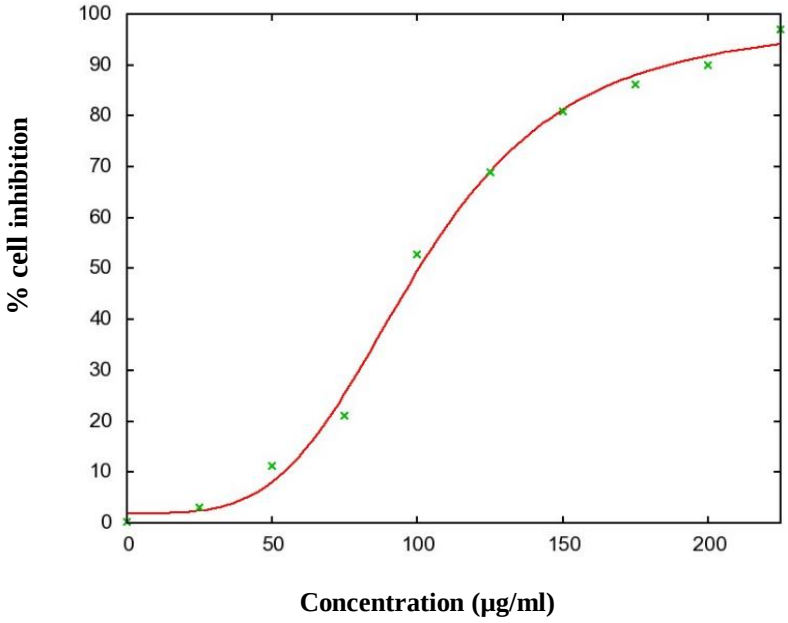


Fig 18: SoMG cell mortality against different concentration of fenitrothion

Discussion:

The host grain preferences of *S. oryzae* under laboratory condition were determined using maize, rice, chickpea and millet. With respect to preference of host grain by *S. oryzae* a significant decrease in grain weight with an increase in percentage grain damage and number of F_1 progeny were noted. Among the grains used, percentage loss in grain by *S. oryzae* were found to be highest in maize, followed by chickpea, rice and millet by 26.54%, 22.02% and 16.10% respectively. The variation in grain loss by *S. oryzae* indicates its preference for a particular grain on which they can lay large amount of eggs for healthy upcoming progeny.

Various studies reported an annual loss of stored grain by these pests from 15% to 57% with preference of different grains (Shivakoti and Manandhar, 2000; Upadhyay *et al.*, 2001;

Bhandari *et al.,* 2015). The present study also revealed that, there was a major loss in the grain content by *S. oryzae* larvae reared on stored grains. The highest average losses of grain on daily basis were also calculated and they were found to be 1.12 gm in maize, whereas, with rice, chick pea and millet the loss grains per day was found to be 0.83, 1.0 and 0.6 grams respectively. Our results are in agreement with the work of Ansari *et al.,* (2003) on varietal screening of rice weevil on wheat and maize. The grain weight loss has also been correlated with the susceptibility and resistance which is more in susceptible inbreeds and less in resistant ones (Masasa *et al.,* 2013; Derera *et al.,* 2014; Garcia-Lara and Bergvinson, 2014; and Zunjare *et al.,* 2016), probably in our study the maize is more susceptible compared to millet, chickpea and rice. Further, endosperm of susceptible inbreed promotes growth of the larvae much faster and in turn results into more grain weight loss as compared to resistant genotypes (Castro-Álvarez *et al.,* 2015).

Despite of the difficulty to visualize the eggs and larvae of *S. oryzae* in the different grains, the number of larvae transformed into adults that emerged out from the respective grain culture was observed and was taken as a total incubation time. The lowest time taken by *S. oryzae* to develop from an egg to adult was 32.5 days with maize, followed by chickpea, rice and millet (35, 39 and 43 days respectively). This difference in the total time of life cycle from egg to adult may be because of the germ layer of the grain, size of the grain and its nutrients content. The less time taken in transformation to adult with maize may attribute to the free availability of high nutrient. On other hand the highest time taken for maturation with millets culture suggests the less selectivity of this grain as this larva prefers to grow inside the grains. As the grain size of millets is smaller compared to larval body, it was observed to be least preferred source of food by *S. oryzae* in our study. Our work is in accordance with the previous studies which suggested that the variation exists in the development period of *S. oryzae* with different grains, which is depended on grain size, germ layer and hardness of the grain (Danho *et al*, 2002; Subedi *et al.,* 2009 and Ojo and Omoloye, 2016).

Fecundity is dependant on size of the insects. Large female insects usually have high potential fecundity. Thus, females of different size will have different amount of body reserves, size dependent allocation trade-offs between the mother's condition as far as egg production is expected (Berger *et al.,* 2008). In present study the size of maize was largest and the highest fecundity of *S. oryzae* is not a surprise. Further, as reported by Campbell (2002) seed size and competition among larvae also can impact offspring survival and

fecundity, to maximize fitness, females need to make decisions about which seeds to use, how many eggs to lay, and whether to lay eggs in hosts already parasitized. This fecundity rate also affected the male and female ratio, although the female ratio in all four stored grains were found to be higher than male ratio, but in maize the female ratio were found to be increased by 1.36 fold than male. This increase in the fecundity as well as the female ratio with maize may be attributed to the preference of grains by *S. oryzae* (Thakare, 2009 and Ojo and Omoloye, 2016). Parallel changes in longevity were also observed with respect to the fecundity rate of *S. oryzae* reared on different stored grains. With high fecundity rate the longevity were also found to be higher on maize. This alteration in fecundity and longevity in reared *S. oryzae* in lab condition would be owing to the nutrient and environmental condition which might have played a significant role in rice weevil (Barbhuiya, 2002).

In an attempt to develop the cell line form midgut, in the present study two different media were checked and the Grace's media was found to be more suitable. The further studies were then continued in Grace's media with additives (FBS and Ecdysone) with different concentrations and vitamin mixture at constant concentration so as to get maximum cell density. 25% FBS with 10 pmol of Ecdysone gave maximum cell density. Our results are in agreement with the earlier reported primary cell culture studies carried out by Aljabr *et al.,* (2013), who have established midgut cell culture from *R. ferrugineus* beetle and Li *et al.,* 2015, who has develop cell line from *H. armigera*. Over the past few years, most of the established coleopteran cell lines available are derived from embryonic tissues like hemocytes by Charpentier *et al.,* 2002 from *Leptinotarsa decemlineata*, larval fat body by Sarmento *et al.*, 2004 in *Eriopis connexa*, by Hoshino *et al.,* 2009 in *Plagionotus christophi* and whole pupae of *Leptinotarsa decemlineata* by Long *et al.,* 2002. Although numerous insect cell lines have been developed over the past few years, no attempt has been from stored grain pest *S. oryzae*, hence our study describes for the first time the cell line SoMG from the midgut of adult *S. oryzae.*

The time spent in cell growth and proliferation from midgut tissues of *S. oryzae* was relatively short, similar to that reported by Segura *et al.,* 2012 and Cruz and Bello 2013. Probably, the additives that were used in the Grace's media promoted necessary condition as well as nutrition which promoted the growth and spreading of cells on the surface of the culture (Zhang *et al.,* 2011). The continuity of the cell subcultures registered from this species was achieved only in the Grace's insect media because it included in its components two necessary nutrients for physiological processes of insects, trehalose and yeastolate, which

probably promoted the adhesion, growth, and spreading of the cells on the surface of culture flasks (Zhang *et al.,* 2011).

Mostly round cells were noted in SoMG cell line which has been characterized as stem cells by many researchers and has been confirmed as stem cells (O'Brien *et al.,* 2011; Scopelliti *et al.,* 2014; Castagnola and Jurat-Fuentes*.,* 2016). Similarly, round stem cells have been isolated from the midgut of several insect orders including Coleoptera (Hakim *et al.,* 2007; Aljabr *et al.,* 2013) and Lepidoptera (Hakim *et al.,* 2009), both from embryonic and adult tissues (Corley and Lavine, 2006), suggestive of their totipotency nature (Jiang *et al.,* 2017) No other morphologically well defined cells were observed probably because the cells were taken from adult tissue as reported by Segura *et al.,* 2012 that embryonic tissues usually proliferate with varied cell morphology.

After the successful survival of the cells, DNA barcoding was carried out using COI as a marker gene using bioinformatics tools of MEGA 7 and NCBI. The homology search was performed which suggested that there was 100% similarity between SoMG cells and *S.oryzae*, indicating that the cell belongs to *S. oryzae* species. Moreover, we characterized the cells using the marker genes of cuticle *cup1*, *cup4* and *hmg167* as reported by Shao *et al.,* 2008. They have analyzed these genes and had proven their differential expression in midgut, haemocytes, epidermis and fat tissue in lepidopteran larvae *H. armigera*. We found that all the three genes *cup1, cup4, hmg167* were expressed in midgut cells supplied with Grace's insect media with 25% FBS +10pmol Ecdysone suggesting the presence of epidermal cells of ectodermal origin in the midgut, which is also reported by Shao *et al.,* 2008. Thus, out of the all the cells found in midgut, we had successfully isolated and established epidermal cells till 17 passages. Moreover, it is known that the candidate genes are also expressed in fat and other body tissue, however, possible contamination was excluded as it was totally removed upon fine dissection, during initial preparation of the mid gut cells.

Hence, it is proved that midgut cell line expresses ectodermal genes, however, the exact function of these genes in the cells are yet to be discovered. Therefore, further studies are needed to carry out the mechanistic action and their role in these cells. Furthermore, to test the *in-vitro* applicability, the cell line was used to calculate the IC_{50} value of fenitrothion pesticide which is a commonly used pesticide for store grain pest. In the present study, the calculated IC_{50} value was found to be 99.96 mg/ml. Similar studies have been carried by several researcher across the globe where they had checked the effect of pesticides on

different insect cell lines for instance Zhong *et al.* (2008) had observed the cytotoxicity and cell growth inhibition of about 20 chemical insecticides, botanical insecticides, and microbial pesticides on *S. frugiperda* (Sf9) cells and *S. litura* Sl-1 cells.; Huang *et al*. (2011) inferred the inhibition of Sf9 cell proliferation with avermectin treatment and Decombel *et al*. (2004) reported that the exposure of pyridaben caused the highest cell growth inhibitory effect on *S. exigua* cell lines among other 13 insecticides. However, there is scarcity of area with respect to fenitrothion on other cells. Hence, this is the first study that accounts for the *in vitro* application of fenitrothion on SoMG cells.

Work to be carried out:

After establishing the IC_{50} using MTT assay of pesticide fenitrothion, the alteration in antioxidant status and cell death (objective 4) is under evaluation and the outcome will be incorporated in thesis.

Conclusion:

From the study it was seen that maize were found to be most effective grain for mass rearing of *S. oryzae* as the insect progeny in maize was highest in number as well as in body length. Further, we can conclude that the newly established adult *S. oryzae* mid gut cell line has its ectodermal roots and the cell line responded well in Grace's insect media with 25% FBS +10pmol Ecdysone. This opens up new avenue presenting a new cell line model of store grain pest for investigating the hormonal and other signaling transduction pathways in Coleoptera. However, further studies are needed for making the cell line immortal by transfecting it with bacculovirus.

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