General Consideration

The separation of cells from animal tissues and their subsequent and successful growth in artificial culture situations is referred to as cell culture (Phelan and May 2016; Reid *et al.,* 2016). Cells for cell culture might be removed directly from the tissue and separated by mechanical or enzymatic means, or they might be collected from a previously established cell line or cell strain (Jedrzejczak-Silicka, 2017). Cells thus extracted from tissues of a variety of living organisms may be preserved in culture vessels under appropriate conditions (Annathur *et al.,* 2003; Harrison and Jarvis, 2006; Rieffel *et al.,* 2014) including appropriate culture medium, incubation temperature, atmospheric CO_2 concentration and humidity (Landauer, 2014; Spasojevic, 2016).In addition, the culture medium consists of various amino acids, salts, vitamins, serum or other proteins and antibiotics. (Abitorabi and Wilcox, 2011). Primary cell cultures are those derived from cells removed from the tissue without any cell multiplication. Any subsequent cell multiplication cultures are referred to as secondary cultures (Fischer *et al.,* 2010; Sullivan, 2010). Many cell lines are anchor-dependent and cultivated when attached to the substrate and are known as monolayer cultures or adherent cell cultures (Danielson *et al.,* 2010), while others that can be grown suspended in the culture medium are called suspension cultures (Rowghani *et al.,* 2004; Willems and Jorissen, 2004).

Day and Grace (1962) outlined the history of insect cell culture as of that date, divided into three phases. The first phase involved work primarily on gametogenesis with hemolymph-cultivated tissues and simple salt solutions. (Glaser, 1917). Mitosis was rarely noted in these studies and cultures usually did not survive beyond several weeks. The second phase emphasized medium culture production and processing, while researchers primarily looked at the composition for vertebrate tissues for their study. These cell cultures did not

survive beyond 3 months; nonetheless, these short periods allowed progress toward the propagation of viruses in cultured cells (Trager, 1953). The third phase was considered a milestone due to its reliance on developing media based on insect tissue chemistry. This phase led directly to the development of an insect cell line from the pupal ovary tissues (Grace, 1962). A similar breakthrough in insect cell culture occurred where Gao and colleagues (1989) used a variety of silk moth tissues and showed the cultivation of viruses in these cultures (Gaw, 1958; Liu *et al.*, 1959; Vlak, 2007). Gao's and Grace's successes were followed by the establishment of numerous cell lines from Lepidopteran, Dipteran, Orthopteran, and Coleopteran insects (Li and Bonning 2007; Lynn 2007; Vlak 2007)

No	Name of Insect Cell Line	Source	Applications	References
1	AC20	*Agallia constricta*	Virus infectivity tests	Chiu and Black 1967
2	IPLB-LD-64	*Lymantria dispar* pupal ovaries	Used to quantitate infectivity	Goodwin *et al.*, 1978
3	IPRI 108	*Malacosoma disstria* larval hemocytes	Nuclear polyhedrosis virus infectivity *in vitro* assay	Volkman and Goldsmith 1982
4	NIH-SaPe-4	*Sarcophaga peregrine*	Used in study of host parasite relationship in insect borne pathogenic microbes	Komano *et al.*, 1987
5	WIV-BS-02	*Biston suppressaria*	Infection studies of *homoceros tubulosa nuclear polyhedrosis virus*	Mitsuhashi 1989
6	FTRS-AoL	*Adoxophyes orana neonate* larvae	Research on viruses; susceptible to insect viruses	Mitsuhashi 1989
7	AS-H 1	*Agrotis segetum*	Research on granulosis virus	Kozlov *et al.*, 1990
8	BTI-EAA	*Estigmene*	Recombinant protein	Hink *et al.*,

		acrea larval hemocytes	production insect cell lines	1991
9	FPMI-CF-1	*Choristoneura fumiferana* midguts	Expression of recombinant proteins using baculovirus vectors	Hink *et al.*, 1991
10	Se6FHA	*Spodoptera exigua*	Useful in nuclear polyhedrosis virus research and in production of recombinant proteins	Hara *et al.*, 1993
11	Sf9 cells	*Spodoptera frugiperda* pupal ovarian tissue	Recombinant protein production using baculovirus and in evaluation of host-virus interaction	Davis *et al.*, 1993, Ma *et al.*, 2014, Wilde *et al.*, 2014
12	APE1	*Antitrogus parvulus*	Development of engineered entomopox viruses as microbial control agents	Fernon *et al.*, 1996
13	IBL-SLO-1A	*Spodoptera litura*	Useful in studies like replication of *S. litura* nuclear polyhedrosis virus in vitro	Shih *et al.*, 1997
14	NIAS-LeSe-11	*Leucania separata* larval fat body	Research on nucleopolyhedrovirus	Yanase *et al.*,1998
15	CSIRO-BCIRL-HP1	*Helicoverpa punctigera* embryos	Useful in virological studies	McIntosh *et al.*, 1999
16	XP-1	*Xylotrechus pyrrhoderus*	Production of insect pathogenic viruses and recombinant proteins	Iwabuchi 1999
17	Lulo	*Lutzomyia longipalpis*	Can be used in vaccines and diagnostic tests	Rey *et al.*, 2000
18	LPC-Aa98-19	*Anacridium aegyptium*	Suitable for virus multiplication and manipulation and also used in biopesticides	Hernandez-Crespo *et al.*, 2000
19	Schneider 2	*Drosophila*	Useful in transfection	Benting *et al.*,

	(S2)	*melanogaster*	research	2000, Suske 2000
20	BCIRL/AMC YAfO(/T)V-CLG	*Anagrapha falcifera* adult ovaries (/testes) fat body	Production of viral pesticides, mainly baculoviruses	Goodman *et al.*, 2001a, b
21	BCIRL/AMC YAgE-CLG	*Anticarsia gemmatalis*	Embryos Production of recombinant proteins and viral pesticides	Goodman *et al.*, 2001b
22	NIV-HA-197	*Helicoverpa armigera* embryonic tissue	Application in the mass production of this baculovirus as a bioinsecticide	Sudeep *et al.*, 2002 a
23	Sf21 cells	*Spodoptera frugiperda* pupal ovarian tissue	Research on baculoviruses and their use for producing recombinant proteins	Chen *et al.*, 2005, Lynn 2003
24	IPLB-Ekx4T	*Ephestia kuehniella* embryos	Useful in biocontrol research and susceptible to nucleopolyhedro viruses	Lynn and Ferkovich 2004
25	AFKM-On-H	*Ostrinia nubilalis*	Useful in virus transfection research	Belloncik *et al.*, 2007
26	RAE25	*Rhipicephalus appendiculatus*	Useful tool in defining the complex nature of the host vector and pathogen relationship	Bell-Sakyi *et al.*, 2007
27	High Five cells	*Trichoplusia ni* cabbage looper ovarian cells	Recombinant protein expression using baculovirus or transfection	Hink 1970, Zhang *et al.*, 2008
28	TI-1 (Thysanoplusia)	*Thysanoplusia intermixta*	Studies of insect pathogenic viruses and baculovirus expression vector system	Hashiyama *et al.*, 2011
29	WIV-BS-481	*Buzura suppressaria* larval hemocytes	Research on baculovirus studies	Grasela *et al.*, 2012
30	BCIRL-Cc-AM	*Cactoblastis cactorum*	Support baculovirus infection and used in	Grasela *et al.*, 2012

			alternative biocontrol method	
31	NN-1	*Nephotettix nigropictus*	Used in phytoreovirus research	Duan and Zhang 2014
32	RIRI-BR1	*Blaps rhynchoptera*	Used as folk medicine in Yunnan Province, China	Zhang *et al.*, 2015
33	KLBIQ-Chsu-I	*Chilo suppressalis*	Production of insect virus expression vector	Liu *et al.*, 2015

Table I: List of the most notable insect cell lines with the source of each and its applications in entomological research

Cell lines can be successfully established from tissues or cells of insect larvae or pupae. The Sf21, Sf9, and the BTI-TN-5B1-4 (or High Five) cells are the most commonly used insect cell lines in research (Table I). These three cell lines are derived from lepidopterans. The Sf9 and Sf21 are from pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), and the High Five cells are from ovarian tissue of the cabbage looper, *Trichoplusia ni* (Hu¨bner). Transformed stable insect cell lines are often from dipteran insects including the fruit flies (Tephritidae, Drosophilidae) and mosquitoes (Culicidae), among which the cell line *Drosophila melanogaster* Schneider 2 (S2), derived from *D. melanogaster* Meigen, is most commonly used in insect cell line research. Insect cell lines are isolated from different tissues and organs (i.e., fat body, embryonic tissues, ovaries, imaginal discs, testis, dorsal vessel, etc.) at different stages of development. Overall, the greatest numbers of insect cell lines have been developed from the embryonic tissues, followed by ovarian tissue; however, in India, the majority of lepidopteran cell lines have been developed from the ovarian tissue, followed by embryonic tissue (Pant *et al.*, 2002).

To date, an average of ~10 billion US dollars is spent per year for synthetic insecticides to control agriculturally and medically important insect pests (Beckmann and Haack 2003; Smagghe *et al.*, 2009). Resistance is the most serious bottleneck in the successful use of pesticides these days. The

extensive use of pesticides has led to the development of resistance in a variety of targeted pest species around the globe. (Tabashnik *et al.,*2009). Essential crop pests, livestock parasites, common urban pests, and disease vectors have, in some instances, developed resistance to such an extent that their control has become extremely challenging. (Van Leeuwen *et al.,* 2010; Gondhalekar *et al.,* 2011; Gill and Garg, 2014). The problems associated with these general toxins, including insect resistance and environmental concerns, have incentivised the development of more insect-specific screening procedures. There is an increasing interest in the development of *in vitro* methods to replace traditional animal toxicity tests for screening purposes. The ultimate goal is to achieve an alternative system that allows rapid testing of candidate ingredients, formulations and finished products and allows accurate prediction of toxic effectiveness at the animal level as a whole. There are some main criteria for the development of an alternative cell-based research procedure.

Culture media for maintaining cell lines are complex combinations of amino acids, salts, vitamins, growth factors, carbohydrates, metabolic precursors, trace elements, and hormones. The requirements for these constituents differ among various cell lines. Carbohydrates are generally provided as glucose, but in a few instances, glucose is substituted with galactose in order to reduce lactic acid accumulation in the cultures. Other carbon sources include some amino acids (especially L-glutamine) and pyruvate. pH is maintained by one or more buffering systems with CO_2/sodium bicarbonate, phosphate, and HEPES (N-[2-hydroxyethyl]piperazine-N0 -[2-ethanesulfonic acid]) as the most commonly used. Commonly used culture media are the Eagle Minimal Medium, Dulbecco's Modified Eagle's Medium, and Roswell Park Memorial Institute Medium (Arora, 2013). Insect cell lines are usually maintained at 26–28^{o}C. Maintenance below the optimal temperature range will yield a decreased cell

growth rate. Above 30^{o}C, cells may become increasingly less viable and may not recover viability even when the temperature is reduced to the optimal range (Drugmand *et al.,* 2012).

Subculturing of the cell line cultures must be performed periodically to retain log phase growth and maximum viability. Anchor-dependent monolayer, or adherent, cell cultures should be subcultured at 100% confluence, typically at a 1:5 dilution (volume of cells to final volume of medium) in order to maintain log phase growth. Suspension cell cultures should be sub-cultured to a concentration of 0.7 x 10^6 to 1.0 x 10^6 cells/ml before reaching a density of 2.0 x 10^6 to 2.5 x 10^6 cells/ml. If surplus cells are available from sub-culturing, those should be preserved in a suitable protective agent (e.g., glycerol, dimethyl sulfoxide) and stored at temperatures below -130^{o}C (cryopreservation) until they are needed for experimentation (ATCC 2012).

Newly established insect cell lines are characterized using the usual parameters of morphology, growth curve, species specificity, karyology, etc. Morphology and viability must be microscopically recorded at regular intervals. The growth medium also must be monitored for microbial contamination, which may be presented as unusual shifts in pH, increased turbidity, or appearance of suspended particles. In addition to daily inspections, it is important to test a sample of the culture regularly for the presence of bacteria, fungi, and mycoplasma. There are several methods that can be used to check for these contaminants (Sudeep *et al.,* 2005a). Cells grown in suspension cultures develop either as single cells or as groups of cells. Viable cells appear round and refractive while dead cells are smaller and darker. Some cells may attach and grow on the inner surface of the culture vessel and appear round or flattened, resulting in some cell lines developing as mixed adherent and suspension cultures. The percentage of these adherent cells varies with conditions and cell density. Cell debris may also be detected

in healthy cell culture populations (ATCC T Animal Cell Culture Guide tips and techniques for continuous cell lines, www. atcc.org). Both trypan blue and erythrosine B stains are used to conduct periodic viability assays to quantify the number of viable cells in a cell culture population. Both stains are excluded by the cell membranes of living cells but are taken up and retained by dead cells, which lack intact cell membranes (Krause *et al.,* 1994).

Establishing insect cell lines for a specific research objective is often a slow and challenging undertaking as primary cell cultures do not develop into cell lines in all cases. Many of the isolated cells take several months to be successfully cultured, and the resulting cells may not be appropriate for the preferred application. Of the approximately 9,50,000 insect species that reportedly exist, we have only 500 insect cell lines from only approximately 100 insect species. Additional challenges to insect cell culture reside with the development of growth media for specific needs. Media required for all the various sources of cells for culture are presently not available and have not been developed.

Development of primary cell lines from midgut cells of stored grain pest *S. oryzae*, the most significant coleopteran pest, have been attempted with no success so far, perhaps due to the lack of a suitable growth medium or suboptimal use of other factors involved in the establishment of the cell cultures (Peters and Black 1970; Peters 1971). However, efforts have been made to initiate cell lines from Thrips and Bugs from other than midgut tissues (Nagata *et al.,* 1997; Kritzman *et al.,* 2002) with limited success as secondary cultures have not been successfully maintained. Establishment and characterization of a novel cell line from midgut tissue of *Helicoverpa armigera* (Lepidoptera: Noctuidae) has been reported by Li *et al.,* (2015b) and other groups (Aljabr *et al.,* 2014) have successfully Established midgut cell culture from *Rhynchophorus ferrugineus* (Olivier) and have assessed the toxicity of different insecticides. Thus to our knowledge, there is a lacuna as

far as stored grain pests are concerned and particularly from the midgut tissue. Hence, the present study is an attempt to isolate, culture and characterization of the insect cell line in order to provide a tool for physiological studies as well as *in vitro* bioassays and insecticide screening studies.

Insect-specific insecticide needs to be developed. For verifying the efficacy of such insect-specific insecticide mass rearing and pure breed of the pest is a prerequisite under controlled conditions to reduce stochastic variation as there cannot be a realistic success in pest monitoring and management without a better understanding of the dynamics of insects' life cycle (Merville *et al.*, 2014). Many workers have delved into the life cycle of *S. oryzae* (Barbuiya *et al.*, 2002; Choudhury and Chakraborty, 2014; Akhter *et al.*, 2017) concerning the generations completed per year (Barbuiya *et al.*, 2002), type of food grain (Singh *et al.*, 2013; Ojo and Omoloye, 2012; 2015 and 2016) and with different agro-climatic conditions (Abass *et al.*, 2014; Farrell, 2018; Ndemera *et al.*, 2018). Host preference is a significant feature of any organism's life history. The entire life cycle is dependent on the suitability of the host. Host preference and host suitability have been studied in *S. oryzae* using different stored grain by Sharma *et al* (2016) where they have focused on different varieties of maize with reference to the stored products. Zunjare *et al.*, 2015 have unravelled the genetics of weevil resistance in maize for adopting a suitable breeding strategy for the development of *S. oryzae* resistant cultivars. Athanassiou *et al.*, (2017) in their studies have proved that the coexistence of the species leads to competition for host preference in laboratory conditions. Previous studies have also reported that the host preference varies based on seed size, germ layer of the seed, moisture and its nutritional values (Ojo and Omoloye, 2016; Akhter *et al.*, 2017). Thus from the ongoing literature survey, it can be seen that there is plethora of information on biology and ecology of *Sitophilous* sp. with different approach, however, with reference to the present inventory where the main focus was

rearing of *S. oryzae* for the development of the cell line and to have an abundant number of adult *S. oryzae* the host preference was also investigated for four stored grains (maize, rice, chickpea, and millet) under laboratory conditions. ***The present study was undertaken to investigate a suitable rearing protocol, life cycle, fecundity rate, and Sex ratio as well as the preference of different stored grains by S. oryzae in control laboratory conditions.***

The results revealed that *S. oryzae* illustrated a definite host preference for the grains viz. maize, rice, chickpea, and millet. The number of larvae that transformed into adults and emerged out from the respective grain culture was lowest (32.5 days) with maize, followed by chickpea (35 days), followed by rice (39 days) and the longest time was that in the millet (43 days). Food is the primary factor that determines the length of the life cycle, difference in the total length is known to be dependent on the richness of the food, nutrients content and the size of the grain (Campbell, 2002; Danho *et al.,* 2002; Subedi *et al.,* 2009; Singh *et al.,* 2013). The least incubation time taken in maize grains can thus be attributed to the free availability of high nutrients from the germ layer of the maize grain. On the other hand, the highest time taken for maturation with millet grain suggests the least selectivity of this grain due to its size. Our study is in accordance with the earlier reported work (Keskin and Ozkaya, 2014; Danho *et al.,* 2015; Ojo and Omoloye, 2016, Akhter *et al.,* 2017; Oloyede-Kamiyo and Adetumbi, 2017) where they have proved the importance of the grain size and oviposition of *S. oryzae* with different grains. Thus the larger size of maize compared to chickpea, rice, and millet most likely offer greater fitness benefits as well as the huge probability of larval survival, larger progeny size, and support for larger numbers of progeny than smaller seeds. Results of fecundity, oviposition and longevity were also parallel with that of the host preference i.e it was maximum in the maize grains (Thakare, 2009; Ojo and Omoloye, 2016 and Akhter *et*

al., 2017). Further, the percentage loss of grain weight was also observed and it was found that the highest loss of grain was in maize (29.88%), followed by chickpea, rice and millet by 26.54%, 22.02%, and 16.10% respectively. Variation in grain loss by *S. oryzae* further confirms the host preference for laying a large number of eggs for the healthy upcoming F1 progeny. Various studies show that the annual loss of stored grain by these pests from 15% to 57% with the preference of different grains to be dependent on the size as well as the nutrient content (Shivakoti and Manandhar, 2000; Upadhyay *et al.,* 2011; Bhandari *et al.,* 2015).

Insects, molds and mites that damage stored grains cause large-deterioration, and low moisture or water activity further develops in situ undesirable metabolites, apart from calorie depletion and selective nutrients. Further, cereals and millets carry only marginal amounts of proteins, however, they are rich in total carbohydrates and calorie contents. The depletion of specific tissues of the seeds such as the germ, bran, and endosperm has their influence on nutritional availability and calorie supply. The findings of the current investigations have disclosed significant changes in the nutritional composition of all the tested grains. A phenomenal decrease in sugars of infested grains was observed. The phenomenal decrease in sugars of infested grains was observed. Sugars are mainly confined to the endosperm of the crop (Singh *et al.,* 2013), the reduction in carbohydrates observed in the maize (37.97%), followed by chickpea (30. 27%), Rice (26.96%) and least in millet (23. 98%) is parallel with the rate of infestation. It was noted that even protein content exhibited a downfall with 13.96% in maize, 11.6% in chickpea, 9.93% in rice and 8.03% in millet. However, the effect was less pronounced compared to carbohydrates which might be because of *S. oryzae* consumes exclusively endosperm, and often leaves the bran intact. These observations are in agreement with previous studies by Bamaiyi *et al.,* (2006) where they

have reported *Callosobruchus maculates* infestation on nutritional loss on stored cowpea grains.

Thus, the study on rearing and host preference of *S. oryzae* concludes, among all the grains tested; maize is the most preferred host for the rearing of *S. oryzae* in the laboratory conditions. All the stages of the life cycle, starting from egg-laying to the emergence were found to be highest in the maize. The study also suggests that maize was rich in carbohydrate that has increased the size, fecundity, and longevity of the insects. Hence, maize is the best suitable grain for the rearing of *S. oryzae* insects.

The establishment of insect cell lines has been the focus of intensive research since the 1980s, which has facilitated the industrial production of proteins, vaccines and insecticides (Ikonomou *et al.,* 2003; Drugmand *et al.,* 2012). During the last four decades, research about insect cell culture has revealed promising outcomes, leading to the development of high-speed screening technologies as a new insect pest management tools (Smagghe *et al.,* 2009; Airenne *et al.,* 2013). There are a marked interest and usage of insect cell culture (AlJabr *et al.,* 2013), primarily due to its application in recombinant biotechnology for the expression of foreign genes and the potential commercial-scale production of the pesticidal baculoviruses (Zitzmann *et al.,* 2017). While cell lines are now yielding new insights into basic cellular processes, other important processes, such as the mechanisms of cellular resistance to insecticide/pesticides is still not well explored (Jarvis, 2009).

Most insect cell lines are usually derived from embryos, ovaries, hemocytes, testis, newly hatched larvae (neonate larvae), pupae, fat body, silk glands and epidermis (Mclntosh *et al.,* 1983; Su *et al.,* 1987; Kolokol'tsova *et al.,* 1995; Shih *et al.,* 1997; Sudeep *et al.,* 2002a, b; Shao *et al.,* 2008; Imanishi *et al.,* 2012; Grasela *et al,* 2012; Goodman *et al.,* 2012; Ding *et al.,* 2013; Zhang *et al.,* 2006, 2014; Zheng *et al.,* 2010, 2014; Hu *et al.,* 2014). Apart

from these body parts, the alimentary canal is one of the most important tissues playing a crucial role in insect physiology. Study on the insect alimentary canal is important as it is the site of digestion, detoxification, and transport as well as has an important role for semiochemical production, which is an imperative process in the insect life cycle (Hall *et al.,* 2002a, 2002b; Nardi *et al.,* 2012). The most popular cell lines originated from the agriculture pests are known to be derived from Noctuid species, for instance, SF 21 and SF9 from *Spodoptera frugiperda* and high five cells from cabbage looper *Trichoplusia ni* (Yu *et al.,* 2016). The use of cell lines for research and commercial applications is currently dominated by three cell lines. Nevertheless, the continued development of new cell cultures from other species is important for the future growth of insect cell studies. The development of cell lines from agricultural pests has been well reported while the development of cell lines from stored grain pests remains elusive. There is a lacuna in the in-depth studies of insect cell lines derived from Coleoptera in general and store grain pests in particular. Taking into consideration the higher degree of species divergence within the order Coleoptera (Hurst *et al.,* 2004, 2007; Hunt *et al.,* 2007) more coleopteran cell lines should be established to enable the study of insects in this order. ***Hence, the second aim of the present study was to isolate, culture and characterize the primary cell line from the midgut tissue of stored grain pest Sitophilus oryzae (SoMG).***

One of the critical factors for the successful initiation of the primary cell line of insects is the optimization of the growth media (Aljabr *et al.,* 2013). Insect cells are readily amenable to suspension culture and the continuous improvement of cell culture media and additives has been reported to contribute and has been a reliable and robust scale-up practice for commercial applications (Agathos, 2007; 2010). In our study, among all the media analyzed, Grace's insect media with an altered concentration of FBS and ecdysone was found to be more suitable and had resulted in optimum

growth of the cells, which is in agreement with the previous study done by Aljar *et al.*, 2013. As reported earlier by Chittranjan *et al.*, (2009) the insect steroid hormone ecdysone triggers, as well as controls cell death and cell survival and, have confirmed the presence of functional pro-survival genes using RNA interference in ecdysone-treated Drosophila l(2)mbn cells. The primary goal of the present work was to initiate and establish a new cell line form the midgut cell; hence the positive role of ecdysone as an additive in the media probably is helping in the survival of the cells. However, RNAi studies will prove the dependency and potential function of ecdysone in cell growth regulation.

Characterizing cell lines based on phenotype or gene expression is a potential authentication technique. After the successful survival of the cells two DNA fragments (COI and 16S rRNA) for cell line identification were used as COI sequences are easier to align and the sequencing quality of 16S rRNA is comparatively higher (Ratnasingham and Hebert, 2007; Lv *et al.*, 2014; Ahmed *et al.*, 2017). The homology sequence database results revealed a 100% similarity between SoMG cell lines and its host *S. oryzae*. Further, it also confirms that DNA barcoding is a strong tool that replaces isoenzyme analysis for the identity of cell line characterization. In addition we had also characterized the cells using the marker genes of cuticle: cup1, cup4, and hmg167 to confirm the origin of the cell line. Expression of all the three genes was found in midgut cells supplied with Grace's Insect media having 25% FBS +10pmol Ecdysone confirming the presence of epidermal cells in the midgut which is in agreement with the studies of Shao *et al.*, (2008) where they have established HaEpi cell line from *H. armigera* and have proved that the cell line is an epidermal cell line based on its unique gene expression pattern.

Karyotyping confirms cell line identity and species of origin. Comparative cytogenetic to derive the phylogenic relationship

among *Sitophilus* sp grain weevils (Coleoptera, Curculionidae, Dryophthorinae) has been studied by Silva *et al.,* (2018) but no studies have dealt with the karyotyping of *S. oryzae* cell line, hence, we have compared and analysed the karyotype obtained from *S. oryzae* insect. Our results are in accordance with the above-described characteristics of autosomes and sex chromosomes of *S. oryzae, S. granarius* and *S. linearis*. Thus it can be concluded that the newly established adult *S. oryzae* midgut cell line has its epidermis roots and the cell line responded well in Grace's insect media with 25% FBS + 10 pmol ecdysone. This opens up a 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.

In-vivo studies on insecticides ' activities are carried so as to evaluate the insecticides ' actual effects by simulating the natural environment, however, some of these assays are labour intensive and time-consuming (Huang *et al.,* 2003). In contrast, *in vitro* studies are becoming an useful assay method in screening of insecticides, not only because they can greatly reduce the screening time but also provide more useful information, such as insecticides mechanisms of action in a rapid and inexpensive manner (Andersen and Krewski, 2009; Zhang *et al.,* 2012). Over the past decades, different industries have demonstrated an increasing interest in the development of *in vitro* methods for the study of insecticides effect. Sf-9, Sf-21, Tn-368, and High-Five are the cell lines most widely used in industrial applications and the number of established insect cell lines has expanded, along with the number of tissue sources. The use of insect cell lines for protein production has evolved from laboratory-scale experimental work to industrial applications. (Elias *et al.,* 2007; Drugmand *et al.,* 2011). The commercially available Sf9 insect cell line derived from pupal ovarian tissue of *Spodoptera frugiperda* is used for *in vitro* assays to estimate the effect of different

insecticides (Lynn, 2002; Ikonomou, 2003; Saito *et al.*, 2005; 2006; Taniai *et al.*, 2006; Szewczyk, 2009; Tebourbi, 2011; Cox, 2012; Ilboudo *et al.*, 2014; Sihler *et al.*, 2018). Organophosphates are agricultural insecticides and are among the most commonly used insecticides in the world (Lerche *et al.*, 2003). Number of organophosphates such as malathion and fenitrothion insecticides have been and are being used against stored grains pests which includes lesser grain weevil, *S. oryzae*, lesser grain borer *Rhyzopertha dominica* (Hooper *et al.*, 2003; Rajendran and Muralidharan, 2005; Nighat *et al.*, 2007;); red flour beetle *Tribolium castaneum*, (Khawaja *et al.*, 2012; Bajracharya *et al.*, 2013).

Thus, after isolating and establishing the origin of SoMG cell line the next aim was to perform the screening of the insecticides, in view of the aim for comparing the newly developed cell line Sf9 cell line was taken as a positive control. Two organophosphates: malathion and fenitrothion were chosen and in an attempt to fulfil the aim, first the IC_{50} of both the organophosphates was obtained and then after the sub-acute dose was taken into consideration for the further studies where, ROS generation, DNA damage, and cell death was checked. For ROS generation-specific genes were taken into consideration and rtPCR was carried out.

In the present study, quantitative estimation for the apoptosis using AO/EB staining method was carried out. The results displayed a significant increase in apoptosis of SoMG and Sf9 cells treated with fenitrothion and malathion and that the fenitrothion shows greater cytotoxic impacts. The cells treated for 96 hrs with sub-lethal concentrations of the selected chemicals reveal that the apoptotic activity in cells treated with HD illustrated greater cytotoxic impacts, with the maximum number of cells undergoing apoptosis. Previous studies have revealed that the dysfunction of mitochondria can induce cell apoptosis via the generation of ROS. Intracellular ROS functions as a trigger of signaling molecules to initiate downstream events in regulating

cell differentiation, cell cycle, and apoptosis. Excess ROS production is associated with mitochondrial Ca^{+2} overload as Ca^{+2} itself can enhance mitochondrial membrane ROS production, eventually resulting in mPTP opening (Sedlc *et al.,* 2010; Aldakkak *et al.,* 2011, Wu *et al.,* 2015). Cytochrome C is typically anchored to the outer surface of the mitochondrial membrane by cardiolipin, which is rich in unsaturated fatty acids (Petrosillo *et al.,* 2003). High level of ROS can cause oxidative damage to cardiolipin, resulting in dissociation of the cytochrome C and subsequent release into the cytosol, which is followed by mitochondrial depolarization (Chiu and Oleinick, 2001). Once Cytochrome C is released into the cytosol, it binds to Apaf-1 to form an apoptosome, resulting in activation of Caspase cascade and cell death (Kim *et al.,* 2019). Thus, our results have suggested that the insecticide mediated toxicity in SoMG cells by the production of surplus ROS can escort cells towards programmed cell death. Moreover, the rate of early and late apoptosis was measured and results explain that the cells treated with HD illustrated significantly increased rate in time and dose-dependent manner suggesting that SoMG was more sensitive towards fenitrothion.

Therefore, together the results of the of MTT assay, AO/EB staining, DAPI, DCFH-DA, ROS gene expression, and FACs analysis suggest that out of the two pesticide screened on SoMG cell line fenitrothion was found to be highly toxic compared to malathion and further it induces the apoptosis through the changes in the mitochondrial membrane potential of cell that leads to formation of ROS (O^-, $H202^-$, NOO^-) leading to the activation of the scavenger molecules like antioxidants (CAT, SOD, GST). These molecules trigger the cascades of events inducing apoptosis inside the cells. Moreover, the cell line developed from the midgut of *S. oryzae* also shows promising results when compared to Sf9. Corroborating our results on SoMG cells, similar studies are performed on Lepidopteron insect cell lines where apoptotic assays were employed using FACs (Huang *et al.,* 2013). So, to have

a holistic view, gene-specific studies on BAX, BCl-2, caspases should be carried out. However, the data is scarce in terms of genes related to initiator caspases and Bcl-2 family and there is a need to identify these genes in insects. Furthermore, as discovered in humans the characterization of apoptosome formation and mechanisms of caspase activation also needs to be elucidated in different members of insects (Yang *et al.*, 2012).

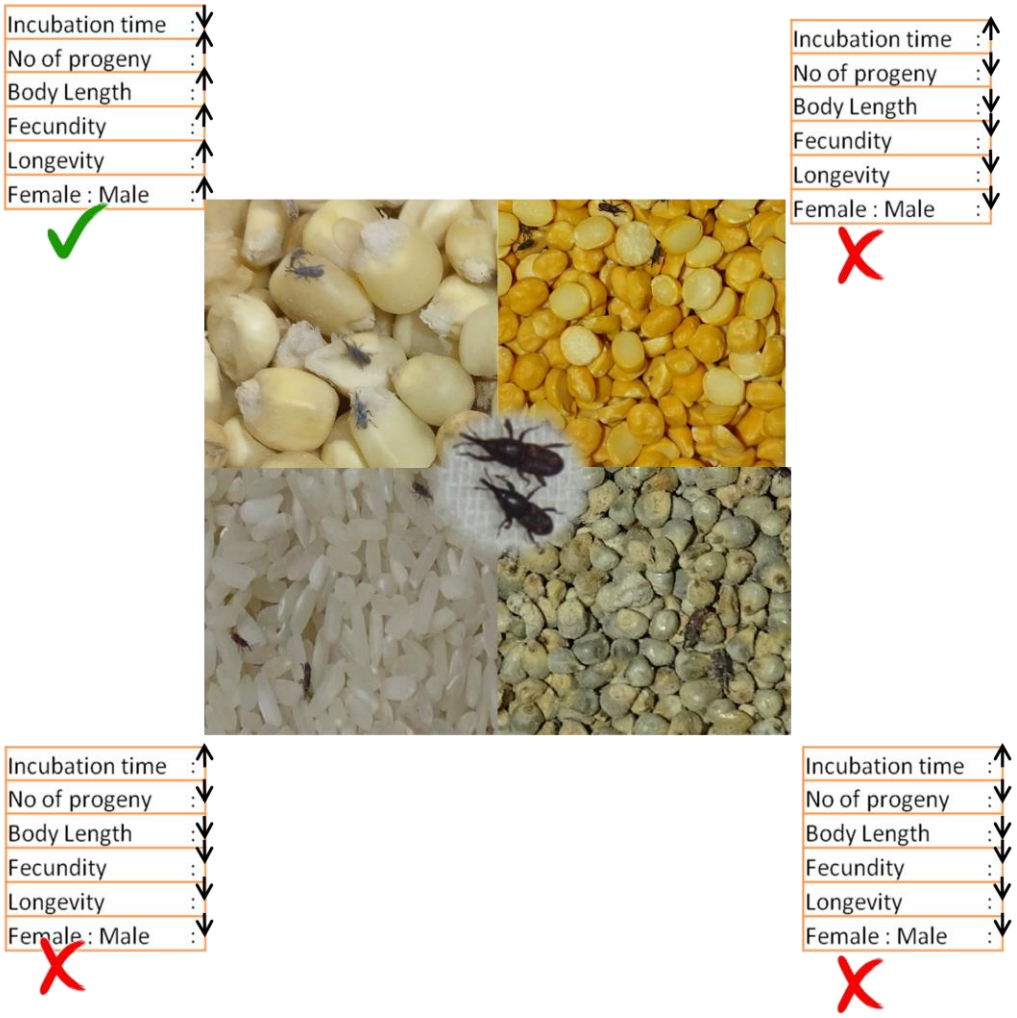

Figure I: The diagram depicting the host preference of *S. oryzae*. Maize grains was found to be most preferred with lowest incubation period giving the highest number of progeny, body length, fecundity, longevity, and female: male ratio compared to rice, chickpea, and millet .

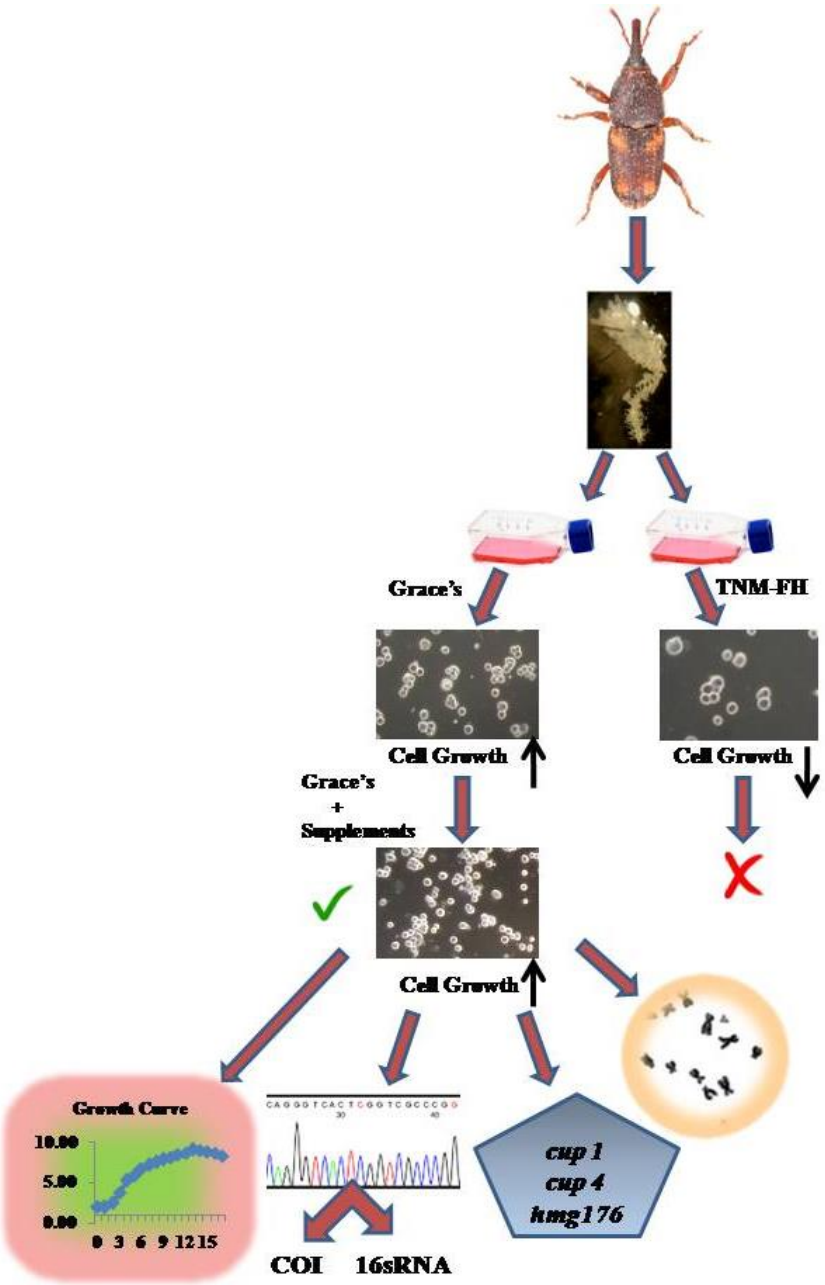

Figure II: Illustration showing the isolation of midgut cells of *S. oryzae* cultured in two insect media. Grace's media with supplements resulted in an appreciable growth of the cells. Molecular characterization of newly developed SoMG cells was confirmed by COI and 16sRNA sequencing. Karyotyping and its epidermal in origin was authenticated by expression of cup1, cup4, and hmg176.

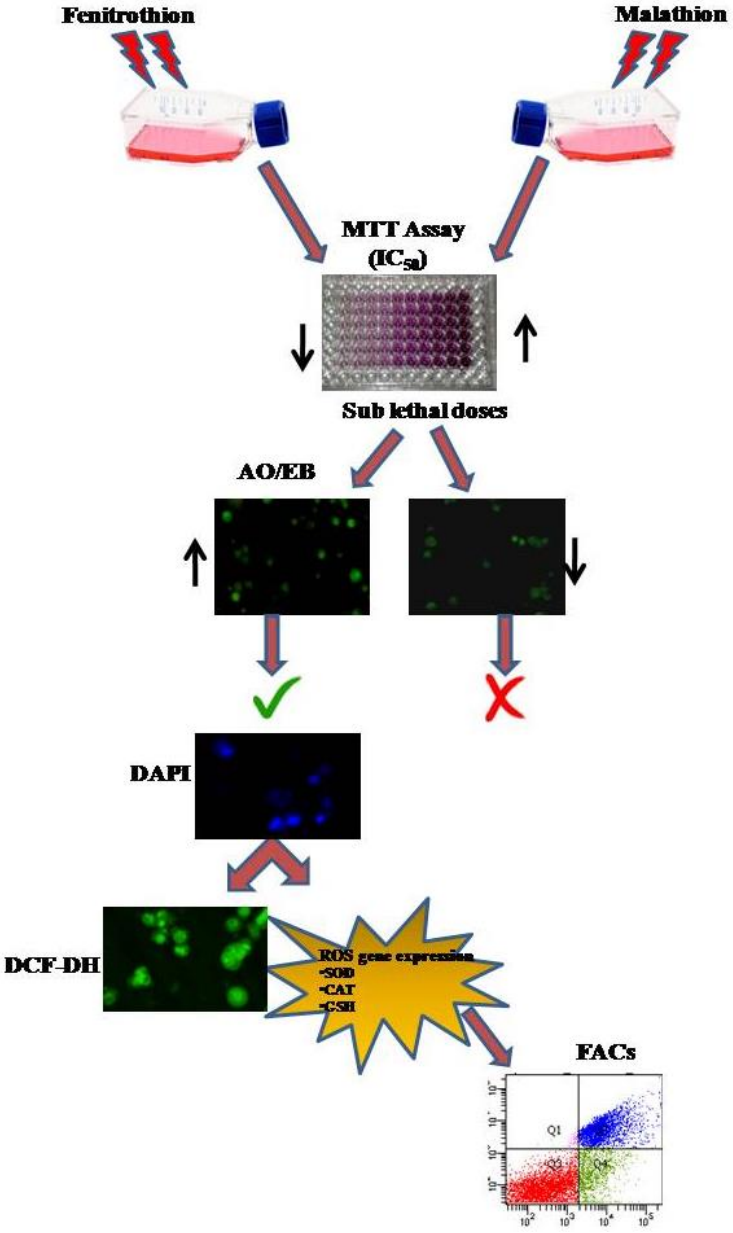

Figure III: Summarizing the results of the toxic potential of organophosphate insecticides - Fenitrothione and Malathion on SoMG primary cell-line. Cytotoxic assay confirmed the Fenitrothion to be highly toxic with increase apoptosis in AO/EB staining. Furthermore, DNA damage was also confirmed by DAPI staining and ROS generation by DCFDA stains. Therefore, activation of antioxidants SOD, CAT and GSH were confirmed by validating the gene expression followed by quantification of apoptosis and live cells by FACs analysis.

Future prospects/Recommendation

- The SoMG cell line obtained from the present work is the first primary cell line developed from the midgut tissue of the stored grain pest *S. oryzae*. The morphological, cytogenetic, and molecular identification is done which has confirmed its origin and also been characterised. However, ***biochemical identification by performing isoenzyme and its next-gen sequencing will further help in authentication and will throw more light its genome characteristics and evolutionary relationships.***
- To check the efficiency of the newly developed SoMG cell line, screening was performed and it did show the same efficiency as that of the Sf9 cell line which was taken as a positive control. The screening test revealed that the cell line generated ROS and the quantitative PCR confirmed the up-regulation of the specific antioxidant genes. ***Thus, the specific signaling pathway and its mechanism will help in understanding the molecular mechanism for the same.***
- Though there is an advancement in terms of insect physiology and mechanism of action of different chemicals *in-vivo*, nonetheless, the studies are scarce in terms of the development of new cell lines from different tissues of insects. Moreover, insect cell lines are important tools in many aspects of virus-related research, including viral propagation and optimization for the development of viral pesticides
- ***We recommend that the cell line SoMG should be immortalised with bacculo-virus or vector expression system that will help in the mass productions of viral pesticide or recombinant proteins.***
- Furthermore, in the future, the way classical insecticides are used will diminish, as new possibilities will be developed. These include the exploitation of newly discovered receptors and pathways such as the eicosanoid system in insect immunity along with the new understanding of resistance. ***Consequently, it is also necessary to study this pathway to elucidate the mechanism of resistance in the cultured midgut cells of S. oryzae***

- As we predict the SoMG cells may have a totipotant nature, ***therefore studies should be more focused on determining the stem cell properties to unravel the mechanism of their resistance towards insecticides.*** This will not only open up new avenues of research but will also increase the understanding of stem cell research in insects in particular and invertebrates in general.
- Adding on, this cell line will also aid in the development of new pesticide targets in the midgut cells of the insects ***and, a detailed study should be attributed to the development of bio-pesticides and synthetic pesticides with its specific targets.***
- Advanced analytical techniques like ***GC-MS, 2D Gel electrophoresis (2D-PAGE) and NMR studies should be employed for the detection of novel proteins. In addition, characterizing its structural and molecular attributes will help us to understand its role in insecticide resistance in SoMG cells.***
- The future holds promise for continued development as seen in recent work on signaling mechanisms, endocrinology and toxicology, and several other aspects of insect cell biology. We hope that present work will provide the foundation for future entomologists to leverage the power of cell cultures to address questions in insect metabolism, vectorial capacity, and adaptation to different stresses and challenges.