7. General Discussion

*Trichogramma* is a parasitoid wasp widely used as a biological control agent against most of the lepidopteran insect pests. It is a potential biocontrol agent in a variety of crops such as cotton, corn, tomatoes, avocados, walnuts, apple, paddy, sugarcane etc., (Hassan, 1993). Sugarcane being one of the important economic crops of India, pest attack is the major threat to the sugarcane cropping system and to agro-economy of India. Biological control of sugarcane pest using *Trichogramma* is one of the efficient methods of pest control in sugarcane.

The main focus of the present research was to characterize and identify the *Trichogramma* populations and to detect the presence of *Wolbachia*, a parthenogenesis inducing α-proteobacterium, because the number of females is proportional to the efficacy of biocontrol system. Since the interactive mechanism between *Wolbachia – Trichogramma* is poorly understood; an attempt was made to unlock the molecular mechanism behind their symbiosis.

National Bureau of Agriculturally Important Insects (NBAII), Bangalore, provided 14 populations of *Trichogramma* collected from various sugarcane ecosystems across India. Initially all the 14 populations of *Trichogramma* were identified using ITS2 primers and genetically characterized using RAPD markers. The ITS2 PCR produced an amplicon of 525-550bp. The ITS2 sequences when searched for their homology at NCBI BLAST revealed that except Andhra Pradesh population which was identified as *Trichogramma pretiosum*, all other populations as *Trichogramma chilonis*. Sequencing of ITS1 and 2 region have been extensively used to examine the taxonomic status of mosquito and to distinguish species and strains among *Gonatocerus morilli* (Xi Li, 2007). From these observations, it is evident that ITS2 regions amplification and sequencing can be effectively used for identification of *Trichogramma spp*. For the genetic diversity estimation among *Trichogramma* populations, out of 40 RAPD markers used, markers which were consistent and reproducible were considered for the analysis. With 8 polymorphic markers, 1389 scorable bands were obtained. A dendrogram constructed based on the bands revealed that the populations formed in the clusters were not in consonance with the geographical collection zones. It was observed that the geographically distant Andhra Pradesh (*T. Pretiosum*) and Haryana (*T. chilonis*) populations were genetically closely
related. It could be a possible reason that they are genetically related introduced species (Sankaran.1974).

After identification and characterization of *Trichogramma* populations, it was important to detect the presence of *Wolbachia*. Bacterial identification in general was done by 16S rRNA gene characterization; however, the *Wolbachia* surface protein has become an efficient and relevant tool for the *Wolbachia* detection (Braig et al., 1998). By amplifying *wsp* region using specific primers, the presence of *Wolbachia* was detected only in *T. pretiosum* from Andhra Pradesh population but not in the native species of *T. chilonis*. The possible reason for this could be that the *T. pretiosum* was an introduced species in sugarcane ecosystem against stem borers (Nagaraja et al., 2008). Hence it was proposed to characterize the *wsp* region reported for all the *Trichogramma* species with reference to the *wsp* of *Wolbachia* present in *Drosophila simulans* and *Nasonia vitripennis*. The phylogenetic estimation of *wsp* sequences majorly clustered into two groups each with 18 and 15 individual sequences. The occurrence of *wsp* of *T. pretiosum* and *T. chilonis* (10) were not distantly separated and hence the horizontal transfer of *Wolbachia* of *T. pretiosum* to non-harboring Indian *T. chilonis* would enhance the *Trichogramma* dependant biocontrol efficacy in Indian cropping system. The *wsp* of *Drosophila* and *Nasonia* were clustered separately suggesting the specificity of *wsp* across species. The exact role of *wsp* in host symbiont interaction remains unknown but the studies shows that the *wsp* region is antigenic and triggers the host immune response (Siozios et al., 2008). The secondary and tertiary structure prediction confirmed the eight β-barrel structure with four extracellular loops. The *wsp* structure is homologous to the NspA outer membrane protein of *Neisseria* spp. The transmembrane prediction of the *wsp* protein sequences based on the hidden Markov model exhibited the eight β-barrel structure with four hydrophilic extracellular loops. When antigenic region was predicted for the *wsp*, it was observed that the antigenic region was extended between 23rd to 43rd amino acid positions which were embedded within the lipid bilayer. This is a possible reason that *Wolbachia* infect the host without eliciting the host immune response.

Since *Wolbachia* was not detected in native *T. chilonis*, the bacterium can be horizontally transferred and for this *Wolbachia* needs to be isolated and purified from a host. The maternally transmitted *Wolbachia* cannot be cultured in an external medium and requires established cell line for culture and maintenance (Munderloh
and Kurtti, 1995). Methods have been developed to culture the bacterium in established cell lines. *Wolbachia* has a broader host cell range and has even cultured in human embryonic lung fibroblast cell lines (Dobson et al., 2002; Fenollar et al., 2003). In the present study, *Wolbachia* was isolated from *T. pretiosum* and the infection in Sf21 ovarian cell lines was successfully established. No major cytological difference was observed at 18 hours after infection but on microscopic observation, presence of granulations was prominent by 24 hours of incubation when compared with the mock infected Sf21 cell line. The presence of bacterium was also confirmed molecularly by *wsp* PCR.

After maintenance of bacterium for few days in Sf21 cell lines, it was observed that the granulation gradually disappeared which hinted the decrease of *Wolbachia* density. To estimate the persistence of *Wolbachia*, the *wsp* was quantified by semi-RT PCR and a growth curve was established. The growth curve displayed the decline of *Wolbachia* density after 5th day of infection and was not detected after 9 days. This observation revealed that *Wolbachia* was either eliminated or killed on prolonged incubation. *Spodoptera* actin specifically developed as a control to study the growth of Sf21 cell lines illustrated the viability of cells. Hence, it has to be confirmed that decrease in *Wolbachia* is not due to the host cell damage but due to host cell response to foreign invasion. Furthermore, the MTT based assay (Fallon and Hellestad, 2008) of metabolic activity of the *Wolbachia* infected and uninfected cells were in consonance with the growth curve developed for *Wolbachia* survival. This strongly suggested that the metabolic activity of the host cells were intact even after *Wolbachia* infection.

It was reported that for horizontal transfer of *Wolbachia* to a new host, the bacterium should not be adapted to the culturing cell line for long duration (McMeniman et al., 2008). Hence, in the present study, when *Wolbachia* was cultured for horizontal transfer to *T. chilonis*, the bacterium was cryopreserved for long term use. The cryopreserved bacterium could induce infection in new Sf21 cells implying the viability of the bacterium after long time storage. A protocol was also developed to isolate *Wolbachia* from the cell lines, and in a study (unpublished) conducted by NBAII, the cryopreserved bacterium was transferred to *T. chilonis* and increased the number of females in population. Hence, the process of multiplying *Wolbachia* in a
cell line and using it for horizontal transfer is an important achievement of the present study.

As discussed above, interactive mechanism being the main focus of the investigation, it was proposed to analyze the biochemical changes occurring during infection. In the previous study, it was observed that *Wolbachia* was expelled from the Sf21 cell on prolonged incubation. Therefore, a GC-MS based metabolic profiling of *Wolbachia* infected and uninfected Sf21 cells were performed. *Wolbachia* infected Sf21 cell lines infected for 24h and 48h were considered along with a mock infected Sf21 cells for comparison. More than thousand peaks were generated for all the sample sets when they were analysed in all possible combinations. It was observed that there was an up-regulation of differentially expressed metabolites in 24 h and 48 h incubated samples; but in Sf21 plain – Sf21 Inf 24h metabolites were seen up-regulated and down-regulated This showed a total imbalance of the metabolic activity during initial hours. The pathways analysis of the differential metabolites proposed that the pathways that comes under carbohydrate metabolism were initially down-regulated but increased after 24h of incubation. Kim and Dang (2005) reported that the apoptosis was directly related to metabolism whereas Pastorino and Hoek (2003) reported that the hexokinase enzyme of glycolysis pathway strongly involved in anti-apoptotic mechanism. In this present investigation, the glycolysis/pentose pathway was up-regulated in and after 24h of incubation. Also, the onset of expression of metabolites involved in drug metabolism showed that the cell releases the stress related metabolites/proteins to defend the foreign body invasion; in this case the *Wolbachia*.

To substantiate the above findings and also the expression of metabolites, expression at the gene level was analyzed. The genetic information of the *Trichogramma* was unavailable except for few ITS sequences and marker genes. The foremost aim was to develop a comprehensive transcriptomic database for *T. pretiosum*. This species was selected because for the reason that this is the only identified *Trichogramma* species among the collected populations that naturally carry *Wolbachia*. This is also one of the species used globally against many insect pests.

Transcriptome database was established for *T. pretiosum* with and without *Wolbachia*. *Wolbachia* was eliminated through antibiotic treatment (Stouthamer et al.,
Although the bacterium was eliminated immediately after treatment, the elimination was made stable by treating the *Trichogramma* lines continuously for subsequent generations.

Illumina based platform is widely used for *de novo* sequencing. The same platform was employed in the present study to sequence *T. pretiosum* as there was no reference sequence available for *Trichogramma*. The sequence assembly generated 34655 transcripts for Control (*T. pretiosum* without *Wolbachia*) and 32781 transcripts for Wild (*T. pretiosum* with *Wolbachia*). The major interest was to analyze the expression of transcripts at immune level. Hence, differential gene expression (DGE) was performed using DESeq tool. From a total of 40287 clustered transcripts, 2145 were only expressed in Control and 1847 were expressed in Wild. There could be a possibility that these genes were specifically expressed in presence or absence of *Wolbachia* and further characterization and validation of these genes may provide more prominent information on the *Wolbachia – Trichogramma* endosymbiosis. The values of q-significant and p-significant were considered which were $\leq 0.05$. In q-significance, total of 76 genes were reported and of which 19 were up-regulated and 57 were down regulated. Whereas in p-significant, a total of 839 genes were differentially expressed in which 134 were up-regulated, 631 down-regulated and 74 neutral transcripts. The major focus of the study was to understand the immune mechanism of *Trichogramma* on *Wolbachia* infection and hence the total clustered transcripts were searched for sequence homology at Insect Innate Immunity Database which is the resource for immune genes reported for *Nasonia*, *Drosophila*, *Apis*, *Anopheles* and *Acyrthosiphon*. The results revealed the role of immune genes in different immune pathways such as Antimicrobial peptides, IMD, Humoral response, Cell cycle regulation, JNK, JAK/STAT, Toll and an undesignated pathway.

Though many transcripts were involved in immune response, only a few genes were differentially expressed. Of the 2577 immune transcripts, only 38 were found to be differentially expressed with 24 up-regulated, 4 neutral and 10 down-regulated. When pathway enrichment analysis was performed, only 23 had significant role in immune pathways. GroEL belongs to the chaperonin family of molecular chaperones, required for proper folding of proteins, is reported in a large number of bacteria (*Zeilstra-Ryalls et al.*, 1991). In eukaryotes, the proteins Hsp60 and Hsp10 are structurally and functionally nearly identical to GroEL and GroES, respectively. In
*Trichogramma* differential immune expression, a homologous gene for *Wolbachia* GroEL and DnaK were expressed at a higher level of 164 and 141 folds respectively. Both these genes were found to involve in JAK/STAT pathway. A high expression level of Ankyrin-like protein which helps in protein-protein interaction and Relish were seen at 18 and 15 folds. A homologous gene to *PPO* which regulates the process of melanization- an innate immune mechanism of melanizing the pathogens and damaged cells - was increased to 14 folds. Melanization is the result of other proteolytic cascade that is induced upon injury. The key enzyme in this cascade is phenoloxidase, which is present as a precursor form, prophenoloxidase in the hemocytes or in the plasma (*Ashida, 1990*). Activation of the phenoloxidase system occurs as a result of a Ca$^{2+}$-requiring cascade involving serine proteases. It can be elicited by microbial components, namely ~-(1,3)-glucan and peptidoglycan, that bind to upstream proteins in the cascade. Yellow which is up-regulated by 3.98 folds is an enzyme in the melanin pathway and downstream of DOPA (intermediate of this pathway), and also is necessary for the male sexual behavior. Dual oxidase is a protein previously shown to kill bacteria and other microbes in fruit flies. In mosquito (*Ha et al., 2009*), they are responsible for forming a fine network of proteins that protects beneficial bacteria and allows them to grow in the gut without activating mosquito immune responses (*Kumar et al., 2010*). They were up-regulated by 3.81 folds. The transcription levels of 18wheeler involved in Toll pathway, a critical component of the humoral immune response, increased after bacterial infection. In *Trichogramma* it was expressed 2.83 folds higher. MYD88 an adapter in Toll signalling pathway was found neutral with two heat shock proteins and an unidentified gene. Serpin that helps in proteolytic processing of neuropeptide precursors, Lachesin with undesignated pathway, an HSP21.4 and Cytochrome P450 - a detoxifying enzyme were down regulated.

The present study also enlighten that genes involved in the stress response were generally over-expressed in presence of *Wolbachia* suggesting that the bacterium has a protective effect on host physiology and immunity or that host compensatory mechanisms have been developed to reduce the harmful impact of the presence of *Wolbachia*. Studies also suggested that endosymbiont tolerance may be achieved either by specific bacterial adaptations or by host measurements shielding the bacterium from the innate host defense mechanisms (*Ratzka et al., 2012*).
Further to this investigation, validation of immune genes will provide in-depth information on host–endosymbiont interaction. Other functional transcripts generated by Trichogramma sequencing can be used to identify any protein ligand interaction which promotes the Wolbachia invasion in the host.

7.1 References


