DEVELOPMENT AND CHARACTERIZATION OF RECOMBINANT PROTEINASE INHIBITOR LOADED VEHICULAR SYSTEMS FOR THE PLANT PROTECTION AGAINST PEST

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SUBMITTED BY

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CERTIFICATE

This is to certify that the work included in the Ph.D. thesis entitled **'Development and characterization of recombinant proteinase inhibitor loaded vehicular systems for the plant protection against pest**' submitted by **Ms. Neha Khandelwal** was carried out by the candidate under my supervision in the Division of Biochemical Sciences, CSIR, National Chemical Laboratory, Pune-411008, India. The material obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION

I hereby declare that the thesis entitled **'Development and characterization of recombinant proteinase inhibitor loaded vehicular systems for the plant protection against pest**' submitted for the degree of Doctor of Philosophy to Savitribai Phule Pune University has not been submitted by me for a degree/diploma to any other university.

Neha Khandelwal
(Research Student)
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Dedicated to My Beloved

Parents

and Family

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Abbreviations

2D- Two Dimension
ACN- Acetonitrile
AD- Artifical diet
apo-TF- Apo-transferrin
APX-Ascorbate peroxidase
Asn- Asparagine
BApNA- N -benzoyl-DL-arginine-p-nitroanilide hydrochloride
BBIs- Bowman-birk Inhibitors
BCME- Bicontinuous microemulsion
BET- Brunauer, Emmett and Teller
BJH- Barret–Joyner–Halenda
CanPIs- *Capsicum annuum* Proteinase Inhibitors
CBB-R250- Commassie brilliat blue R-250
CD- circular dichroism
CI- Chymotrypsin inhibitor
CTAB- Cetyltrimethylammonium bromide
DIGE- Difference gel electrophoresis
DEP- Differential exprespressed protein
DTT- dithiothreitol
Ex- Excitation
FACs- fatty acid-amino acid conjugates
FA- Formic Acid
FTIR- Fourier Transform infra red
g, mg, µg, ng- gram, milligram, microgram, nanogram
GC- Gas Chromatography
Gln- Glutamine
GXCT- Gel x-ray film contact print technique
HSA- Human serum albumin
HGP- Helicoverpa gut proteases
HGPI- Helicoverpa gut protease inhibitor
IC_{50} - Inhibitor concentration at 50% inhibition
IEF- Isoelectric focusing
Ile- Isoleucine
IPG- Immobilized pH gradient
IRD- Inhibitory repeat domain
JA- Jasmonic acid
K- Lysine
Kd- dissociation constant
kDa/kD- kilo Dalton
KOH- Potassium hydroxide
L,mL, µL- liter, milliliter, microliter

LC- Liquid chromatography
LOX- Lipoxygenase
M, mM, µM- molar, miillimolar, micromolar
m, nm, cm- meter, nanometer, centimeter
MALDI-Matrix assisted laser desorption ionization
ME- Microemulsion
MeJa- Methyl Jasmonate
Met- Methionine
MGY- Minimal glycerol media
MM- Minimal Methanol
MS- Mass spectrometry
NaCl- Sodium chloride
NaOH- Sodium hydroxide
NMR- Nuclear magnetic resonance
NPs- nanoparticles
o/w- oil in water
OPDA- 12-oxophytodieonic acid
OS- Oral secretion
PAGE- Polyacrylamide gel electrophoresis
PAL- Phenylalanince Lyase
PALS- Phase analysis light scattering
Pin I- potato type I inhibitor
Pin II- potato type II inhibitor
PIs- Proteinase inhibitors
PLGS- ProteinLynx Global Server
PPO- Polyphenyl Oxidase
Q-TOF- Quadrupole- Time of Flight
R- Arginine
ROS- Reactive oxygen species
SBA- Santa Barbara Amorphous
SDS- sodium dodecyl sulphate
SE- Standard error
SEM- Scanning electron microscopy
SiO_2N- Silica nanospheres
SOD- Superoxide dismutase
TD- Threonine deaminase
TEM- Transmission electron microscopy
TEOS- Tetraethyl orthosilicate
Thr- Threonine
TI- Typsin Inhibitor
TIU- Trypsin inhibitory unit
TOF- Time of flight
Trp- Tryptophan
UV- ultra violet
w/o- water in oil
WIB- Water: Isopropanol: Butanol
WOS-Wounding with oral secretion
WW- wounding with water

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Thesis Abstract

In this study, we have attempted to analyze the induced proteome responses in wild relatives of *Cajanus cajan* and cultivated pigeonpea upon *Helicoverpa armigera* infestation and associated elicitation treatments. Studying these responses is important to comprehend plants ability to deal with pest damage either by tolerance or by producing defensive molecules. We have observed that plant undergoes large scale shifts in metabolism as deciphered from the identification of proteins belonging to several metabolic pathways. Whereas, in a more targeted approach, well-documented plant defensive molecule called serine proteinase inhibitors (PIs) was characterized from a non-host plant *Capsicum annuum*. PIs efficacy and applicability against *Helicoverpa armigera* was evaluated in the external environment by using bicontinuous microemulsion and silica nanosphere based delivery systems. These delivery vehicles offer unprecedented advantages such as i) eco-friendly and cost effectiveness, ii) flexibility to use in contrasting environments iii) targeted and controlled release and iv) plasticity to incorporate active ingredients with different mode of action for enhanced efficacy by delaying pest resistance.

Quantitative proteomic approach to study pigeonpea defenses against Lepidopteran insect pest

Pigeonpea (*Cajanus cajan* L.) is one of the important legumes that suffer severe losses by *Helicoverpa armigera* attack. However, little is known about the induced molecular responses in terms of altered protein level upon insect infestation. Therefore, proteome profiling of *C. cajan* wild relatives (*Flemingia stricta*, *Cajanus platycarpus*, *Cajanus scarabaeoides*) with two cultivated varieties of pigeonpea (Vipula (tolerant) and ICPL 87 (susceptible) were investigated upon elicitation using shot gun proteomics approach. All these plants were subjected to wounding with larval oral secretion and analyzed for differentially expressed proteins. We identified over 40 non-redundant differentially expressed proteins (DEPs) upon elicitations in pigeonpea cultivars and their wild relatives. Most of these DEPs were found to be up regulated in mechanical wounding (WW) and/or wounding with oral secretion (WOS) treatment as compared to their

respective untreated controls. In general, these DEPs belong to physiological processes such as photosynthesis, primary metabolism and energy regulation, signal transduction, genetic information processing, and active oxygen removal. Between the two tested pigeonpea varieties, Vipula displayed significant change in the accumulation of proteins compared to untreated control in response to elicitation. Time course study of WOS induced differential proteome in Vipula was validated using 2D difference gel electrophoresis technique and MALDI-TOF-TOF. Proteins that are differentially expressed could work together to play a major role in the induced defense response and may prove to be the strongest candidates against insect pest for further research in crop protection.

Development and characterization of microemulsion based proteinase inhibitor loaded formulation

Proteinase inhibitor (PI) from *Capsicum annuum* containing four proteinase inhibitory repeat domains (CanPI-7) is known to antagonize insect pest *Helicoverpa armigera* by inhibiting gut proteases leading to its starvation and growth retardation. CanPI-7 was incorporated in bicontinuous microemulsion and explored for their efficacy and stability *in vitro* and *in vivo*. Bicontinuous nature of the microemulsion containing water: 2-propanol: 1-butanol (55:35:10 w/v) was characterized using conductivity measurements and found to be suitable for cargo delivery. Examination of water rich microemulsion on chickpea leaf surface by scanning electron microscopy caused instant surface modifications but does not lead to any significant damage to the chickpea leaf. The water soluble CanPI-7 protein was incorporated in the aqueous domains of the bicontinuous microemulsion, which in turn ensures greater wettability and wider spread on the (hydrophobic) leaf surface as revealed by contact angle measurements. Inhibitory activity of the incorporated CanPI-7 was investigated against trypsin and *Helicoverpa armigera* gut proteases. Interestingly, stability and activity of CanPI-7 was found to be similar to its aqueous counterpart. Significantly, CanPI-7 delivered using the microemulsion on chickpea leaf was not easily washed off the leaf surface and activity inhibition assays indicate a three-fold increase in the leaf retention of the PI. Furthermore, *in vivo* evaluation of incorporated CanPI-7 revealed significant reduction in weight and survival

rate exhibiting adverse effect on the growth and development of the insect. Thus this study presents the potential of bicontinuous microemulsion-based delivery of proteinase inhibitors and/or proteins in the field which can further be extended for broad spectrum application purposes and may prove to be a promising tool for crop protection against insect pests.

Bio-physical evaluation and *in vivo* delivery of plant proteinase inhibitor immobilized on silica nanospheres

Recombinant expression of *Capsicum annuum* proteinase inhibitors (CanPI-13) and its application via synthetic carrier for the crop protection is the prime objective of our study. Herein, we explored proteinase inhibitor peptide immobilization on silica based nanospheres and rods followed by its pH mediated release *in vitro* and *in vivo*. Initial studies suggested silica nanospheres to be a suitable candidate for peptide immobilization. Furthermore, the interactions were characterized biophysically to ascertain their conformational stability and biological activity. Interestingly, bioactive peptide loading at acidic pH on nanospheres was found to be 62% and showed 56% of peptide release at pH 10, simulating gut milieu of the target pest *Helicoverpa armigera*. Additionally, *in vivo* study demonstrated significant reduction in the insect body mass (158 mg) as compared to the control insects (265 mg) on 8th day after feeding with CanPI-13 based silica nanospheres. The study confirms that peptide immobilized silica nanosphere is capable of affecting overall growth and development of the feeding insects, which is known to hamper fecundity and fertility of the insects. Our study illustrates the utility and development of peptide-nanocarrier based platform in delivering diverse biologically active complexes specific to gut pH of *H. armigera.*

Overall, the study emphasizes on elucidating the specific responses of a particular plant-pest pair to get a deeper insight underlying plant mechanism of resistance or tolerance to herbivory. Information obtained can be successfully translated in a more applied way by utilizing interdisciplinary approaches for plant protection.

Chapter 1

Introduction and Review of Literature

Chapter 1

1.1 Sustainable Crop Production: Current Challenges

Global food production has increased substantially in the past half century propelled by constantly expanding population (**Fig. 1.1A**). Advancement in agronomic technologies including crop management has revolutionized traditional practices thereby increasing crop production several fold. Important contributions of these innovative techniques are (i) developing crops with improved genetic potential, disease- and stress- resistant cultivars and improved photosynthetic efficiency, (ii) appropriate use of fertilizers (iii) developing chemical pesticides such as weedicides, insecticides, fungicides and (iv) improved irrigation systems (**Evans, 1993; 1998; Gregory and George, 2011**). Production of cereal crops like wheat, rice and maize has surpassed the production of other food crops. This has caused human diet to become less diverse resulting in micronutrient malnourishment (**White and Broadley, 2009**). Data for prevalence of undernourishment in India as well as the world testifies the above scenario (**Fig. 1**).

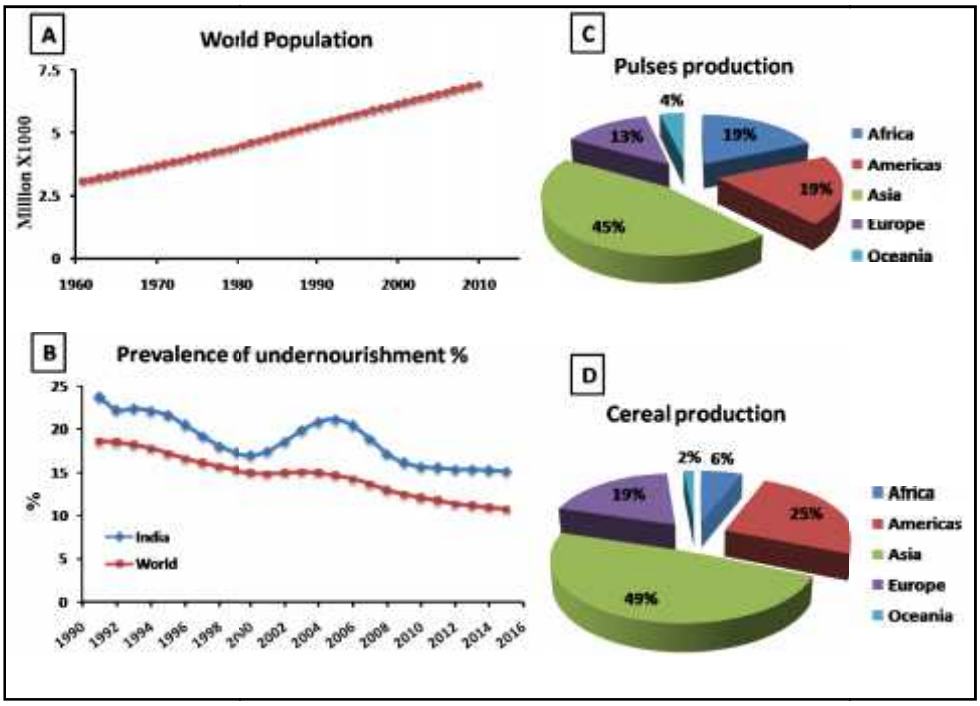


Figure 1.1: Graph showing [A] Increase in world population from 1960 to 2010, [B] Prevalence of undernourishment in world- and Indian scenario; Pie chart showing

worldwide distribution of production of [C] pulses, and [D] cereals (Data obtained from FAOSTAT; http://faostat3.fao.org/home/E)

The increase in global food production exerted major environmental impacts jeopardizing biodiversity and enhancing greenhouse gas emissions (**Tilman *et al.*, 2011**). In addition, increasing use of chemical pesticides and fertilizers has threatened terrestrial and marine ecosystem/s. This raises concern to develop sustainable measures to achieve greater yield while keeping a check on environmental impact by implementing advanced agronomical approaches.

1.2 Biotic Stress with Focus on Herbivore Invasion

Farmers in tropical developing countries have suffered crop losses due to biotic stress accounting upto 50% against 25 to 30% in Europe and U.S.A. respectively (**Yudelman *et al.*, 1998**). Several researchers have focused their attention towards insect pest attacks which is currently responsible for the global crop loss causing at an average of 15%. This has raised an alarm to safeguard crops by controlling pest population, (**Maxmen, 2013 Agriculture and drought outlook**). Among many, Lepidopterans (moths, butterflies) are the third largest order of class insecta (**Capinera, 2008**) causing most of the damage in their caterpillar stage by chewing off the plant parts unlike moth, a nectar feeding stage, example, *Helicoverpa armigera*, which is one of the major threat and is the focus of our study.

1.2.1 Strategies to Control Pest Attack

In the past 50 years with increase in agriculture land, frequent herbivore invasion have resulted in huge productivity loss (**Huis, 1989**). Increasing number of non-native, invasive pests are introduced to new regions challenging food security and exerting an economic burden. Thus, for the crop protection several strategies were adopted.

Among various strategies (**Fig. 1.2**), chemical control of pests has emerged as a revolutionary approach, which has contributed in increasing yield by warding off damaging pests (**Muthomi *et al.*, 2007**). Use of insect- targeting pesticides is significant at global level (**Fig. 1.3A**); in India, insecticides alone share the major percentage of pesticides (**Fig. 1.3B**). Synthetic organic pesticides such as organophosphates, carbamates, synthetic pyrethroids and neonicotinoids were developed, of which most of them affect insect nervous system *via* physical contact, inhalation or ingestion. However, their persistence in environment and toxicity to non- target species cannot be overlooked. Moreover, their extensive application in the crop fields has led insects to develop resistance to chemical pesticides over a period of time.

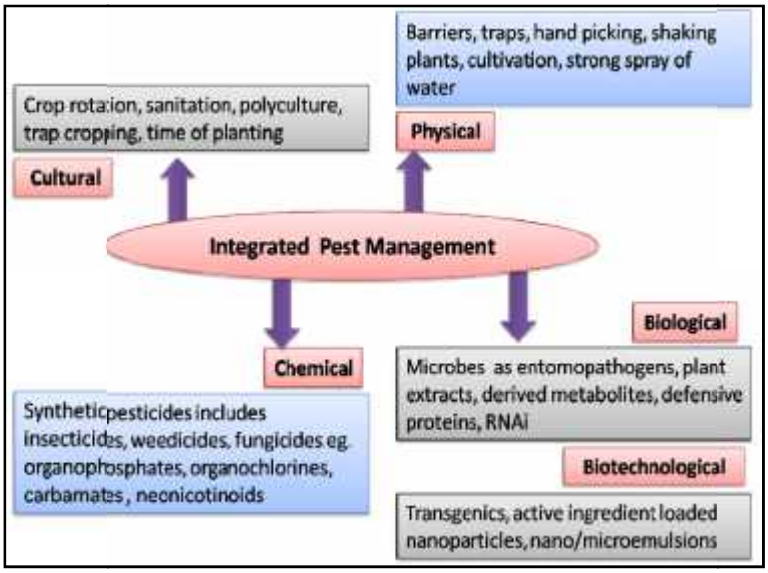


Figure 1.2: Schematic representation of various strategies to control pest attack

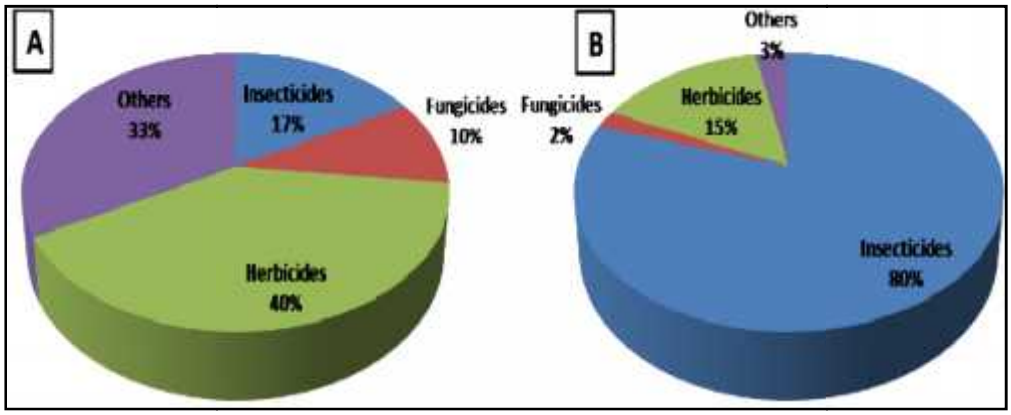


Figure 1.3: Pie-chart showing percentage use of pesticides [A] globally, and [B] India (Data obtained from De *et al.,* 2014, SpringerBriefs in Molecular Science)

Realizing its harmful effects on human health and ecosystem, researchers seek for integrated pest management strategies that offer sustainable solution for pest control. Naturally available plant- derived compounds such as neem, garlic, pepper etc. were traditionally accepted as insect deterrants. Similarly, use of pheromones provided an alternative approach towards an environmentally benign solution over chemical methods. However, their implementation on a large-scale requires combination of advanced biological and chemical tools. For this, potential of various compounds from plants and microbial systems for their effective role against insects was anticipated. These natural systems were identified and screened for their stability and efficacy in diverse environment, which was further blended with synthetic chemicals or compounds to form biopesticides. Biopesticides offer an important alternative to existing pest- control strategies utilizing integrated approach towards crop protection (**Seiber *et al.*, 2014**).

1.2.2 *Helicoverpa armigera* an Agriculturally Important Pest

H. armigera (Lepidopteran: Noctuidae) is a well known devastating polyphagous pest targeting broad spectrum of plants. It possesses properties such as high reproductive capacity, short life cycle and high mobility that contribute to its polyphagy nature. According to a recent report, geographical span covered by this insect pest is enlarging and lately its invasion into South and Central America was reported (**Kriticos, 2015**).

Considering its rapid global invasion, an immediate action towards its control demands to investigate its physiology at temporal and spatial level, to understand its specific behaviour and resistance mechanisms. *H. armigera* has developed a complex system of gut milieu, armed with digestive proteases with varying specificities. This ability of insect allows them to adapt to a wide range of host plants that differ quantitatively and qualitatively in their protein content. (**Patankar *et al.*, 2001; Srinivasan *et al.*, 2006**). Proteases that are known to inhabit the gut of insects are trypsins, chymotrypsins, elastases, cathepsin-B like proteases, aminopeptidases and

carboxypeptidases (**Chougule *et al.*, 2005**). Digestive system of insect predominantly consist of serine proteases with at least 21 and 14 trypsin and chymotrypsin types, active at alkaline pH (**Chikate *et al.*, 2013**). It is found that presence of divergent proteases is required to deal with complex plant protein in a tissue- specific manner. In addition, various strategies are adopted by insects to deal with pesticides by modifying their physiological status and carrying out functions such as sequestration, excretion, producing detoxifying enzymes and target site mutation (**Dawkar *et al.*, 2013**). This has enabled *H. armigera* to attain broad-spectrum pesticide resistance while extending its global reach. Thus, a constant exploration is required to identify new molecules which are cost- effective and environmentally- compatible.

1.3 Plant- Insect Interaction: Co-evolutionary Arms Race

Plants being sedentary in nature interact with components of microbiome and macrobiome. The latter constitute herbivores, predators and pollinators (**Dicke and Baldwin, 2010; Whitham *et al.*, 2006**). Plant- interactions with insects, represents a classical example of chemically mediated communication and forms one of the dominant associations on terrestrial ecosystem. This dynamic and complex relationship influences the plant to undergo phenotypical changes which ultimately are responsible to shape the composition of insect community at different trophic levels such as attracting herbivorous natural enemies. Thus, understanding this relation presents a wide scope of investigation from gene- evolution to community- interaction stretching through signaling crosstalk, metabolite/protein changes and multi- species communication (**Stam *et al.*, 2014; Mishra *et al.*, 2015**). Several studies are focused to understand this complexity between individual plant and insect species to study underlying mechanism of host selection and plant resistance or tolerance. Based on the theory of co-evolution, put forth by Ehrlich and Raven (**1964**), plants evolved to limit pest damage by producing plethora of compounds exerting toxic, repellent or anti-nutritive effects on insects. Whereas insects co-evolved to develop mechanisms in order to avoid, metabolize, sequester, excrete or detoxify plant defense system. For example, a single change of residue in Na-K ATPases can lead to lowered insensitivity to cardenolides, a toxic derivative of plant (**Dobler *et al.*, 2012**). Highly alkaline pH in the midgut also enables insects to adapt or metabolize

plant defense compounds such as β- glucosidases (**Vetter, 2000**). Thus co-evolution of plants and insects has led to their speciation over an extended time scale, under the influence of climate, geographical location and species distribution (**Kasting and Catling, 2003**).

1.3.1 Plants Defense Responses: Fitness and Survival

Integrated pest management serves to develop strategies to control pest population by the blend of conventional breeding techniques with contemporary biological methods for developing improved cultivars. One of the strategies involves screening of pest- resistant and/or tolerant traits to facilitate increase in crop yield. This has incited researchers to understand the molecular intricacy of plant responses elicited upon insect attack, which forms the basis of resistant or tolerant traits (**Kerchev *et al.*, 2012**). Plants defensive system is comprised of constitutive and inducible mode of responses, which may have direct or indirect effect on attackers. Direct effects include first line of defense consisting of specialized morphological features and deterrents, which restricts insects from damaging plant tissue. Examples are thorns, spines, hairs, wax, resin and lignin (**Fernandes, 1994**). Direct defenses are also mediated by some toxic chemical induced by plants which have repellant, toxic and anti- metabolic effect on insect, hampering its growth and development (**Fig. 1.4**). These toxic compounds mainly comprise secondary metabolites such as phenolics, terpenoids, anthocyanins, alkaloids, quinones, proteinase inhibitors (PIs) etc. (**War *et al.*, 2012; Hanley *et al.*, 2007**). Herbivore- induced plants emit concoction of volatiles that attract predators, thereby indirectly providing protection to the plant by feasting on herbivores. Compared to constitutive expression, defense responses induced upon herbivore damage seems to be more durable and provides plasticity to plant defense at low metabolic cost (**Howe and Jander, 2008**).

1.3.2 Secondary Metabolism: Route to Plant Defense

Transient accumulation of reactive oxygen species (ROS) in local and systemic tissue is the primary response of plant upon damage. Studies elucidating plant defense regulatory mechanism have established the significant role of plant hormones such as jasmonic acid

(JA) (**Creelman and Mullet, 1997**). JA is the product of octadecanoid pathway, which triggers a signal cascade for the production of defense compounds upon mechanical wounding or larval feeding. Compounds derived from linolenic acid, such as 12-oxophytodieonic acid (OPDA) and methyl jasmonate (MeJa) are also involved in mediating this response. This signaling mechanism leads to the production of secondary metabolites that are actively involved in plant defense. Phenolics are the largest group of secondary metabolic products and studied widely in relation with host- plant resistance (**War *et al.*, 2012**). Lignin, a phenolic heteropolymer reduces the feeding by herbivores and upregulation of lignin- related genes are documented (**Barakat *et al.*, 2010**). Peroxidase and polyphenol oxidase are the enzymes that catalyse oxidation of phenols. **Bhongwong *et al***., (**2009**) suggests that quinones inhibit protein digestion in *H. armigera.* Flavonoids are other set of compounds studied in connection with plant- insect interaction. Isoflavonoids, example, Judaicin and maackiain, isolated from wild chickpea relatives showed antagonistic effect on *H. armigera* (**Renwick *et al.*, 2001**). JA- mediated activation of PI gene has been identified as a significant event in plant defense whose function is to interfere with the insect protein digestion, thereby, arresting its growth and development.

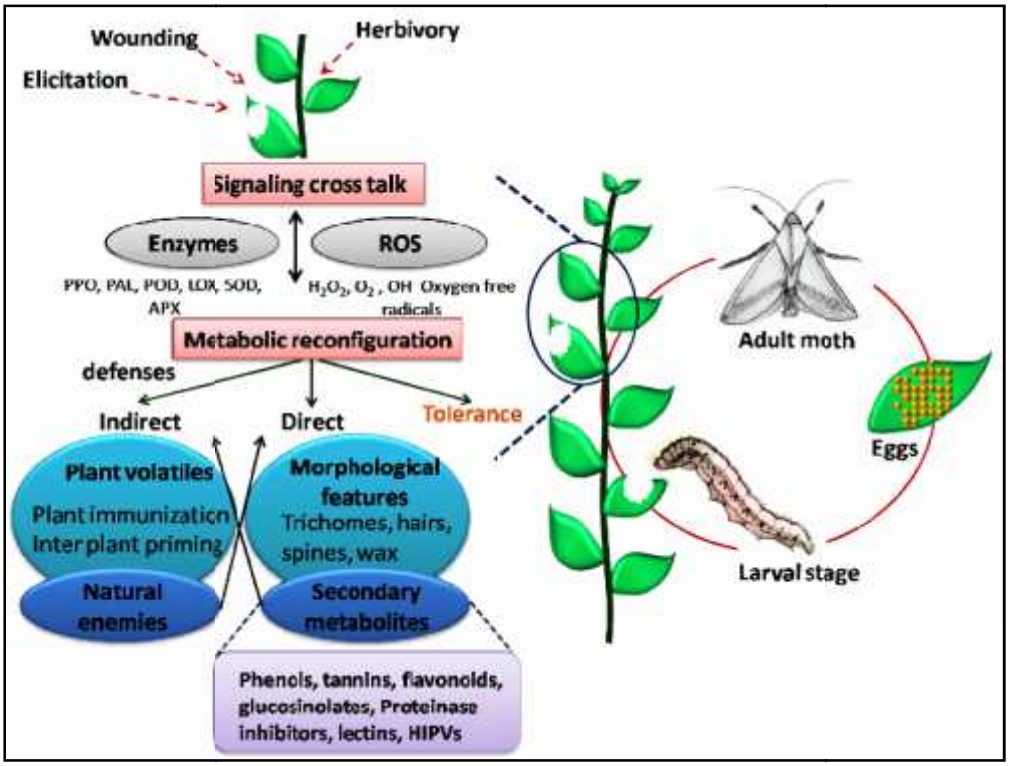


Figure 1.4: Figure depicting *H. armigera* passing through adult moth, eggs, and larval stages. Larvae feeding on plants along with wounding and other elicitation treatments can induce signal cross talk with the production of phenylpropanoids and octadeconoids. In addition, ROS pathways get activated. Althogether, plants carry out metabolic reconfiguration which includes defense (direct and indirect) and tolerance. (PPO, polyphenyl oxidase; PAL, phenylalanine lyase; POD, peroxidase; LOX, lipoxygenase; SOD, superoxide dismutase; APX- ascorbate peroxidase.

1.3.3 Primary Metabolism Reconfiguration Upon Herbivory

In addition to the production of defense related compounds, plant exhibits vast changes in primary metabolism upon herbivory (**Giri *et al.*, 2006**). Primary metabolic changes largely focus on carbohydrate and amino acid reconfiguration, which forms the backbone of defense compounds. Carbohydrate synthesis is carried out *via* photosynthesis in green plants, which shows large-scale metabolic changes upon herbivory. Increase in photosynthesis can be seen under two conditions: (i) when carbohydrates are required for the synthesis of defense metabolites, to compensate for the loss due to damaged tissue

and/or (ii) insect mediated manipulation of metabolism to obtain more carbon from feeding tissues. However, there are myriad evidences which justify decrease in photosynthesis upon herbivory, as plants use resources for synthesizing defensive compounds by compromising photosynthesis (**Zhou *et al.*, 2015**). Several studies have reported that

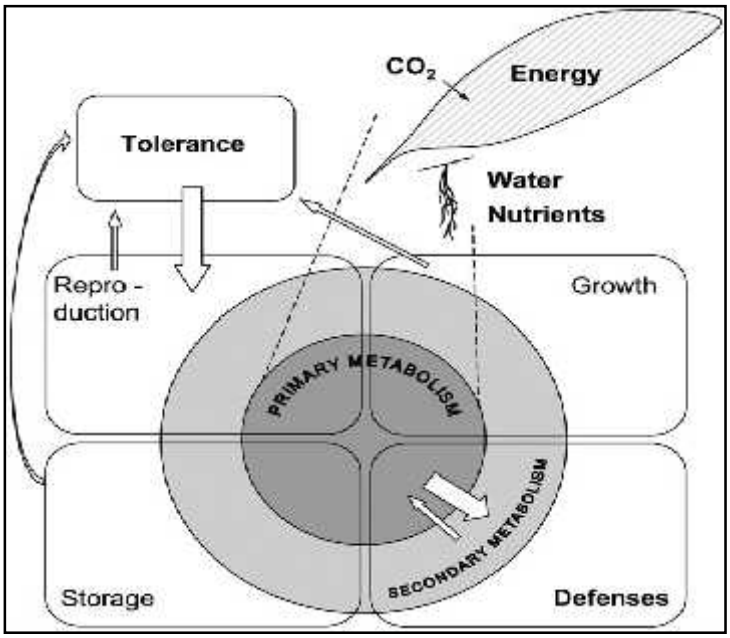


Figure 1.5: Basic functions in plants such as growth and development, reproduction and storage are the consequences of primary metabolic processes. During defense change in pimary metabolic resource allocation could lead to tolerance. Secondary metabolism, majorly involved in plant defense depends on energy and resources from primary metabolic pathway. Parts of primary metabolism play role in plant defense (Adpoted from Schwachtje and Bladwin, 2008).

expression of genes involved in photosynthesis display downregulation by mechanical wounding, larval feeding, insect oviposition and exposure to volatiles (**Coppola *et al.*, 2013; Giri *et al*, 2006; Reymond *et al.*, 2004**). On the contrary, few reports suggested up regulation of photosynthesis related gene, for instance, in wheat and barley against Russian wheat aphids (*Diuraphis noxia*) (**Botha *et al.*, 2006; Gutsche *et al.*, 2009**).

Another important primary metabolic pathway, which involves amino acid metabolism gets altered upon insect infestation. Amino acid production is found to be up-regulated and supposed to have multiple implications such as (i) as growth limiting by blocking essential amino acid availability, as shown by the upregulation Threonine deaminase (TD) gene, a committed step in the biosynthesis of Isoleucine (Ile) which catabolizes essential amino acid threonine (Thr) in insect gut (**Chen *et al.*, 2005**), (ii) act as precursor for the synthesis of defense metabolites. For instance increased expression of methionine (Met) and tryptophan (Trp) related genes contributed for the greater accumulation of glucosinolates in relation to herbivory (**Appel *et al.*, 2014**) and (iii) nitrogen assimilation into Glutamine (Gln), Glutamate (Glu), Asparatate (Asp), and Asparagine (Asn) and its transport to the other parts of the plant. Increased transcript expression of amino acid metabolism related genes upon elicitation are reported including Glu synthase, Gln synthetase (**Divol *et al.*, 2005; Voelckel *et al.*, 2004**). It is also likely that increase in free amino acids is induced by insects to obtain nitrogen to fulfil the requirement for their own physiological process. Thus, it is suggested that plant diverts primary metabolite based resources to attain tolerance or resistance from insects, which are pre-requisite to study in order to develop cultivars with pest resistant traits (**Fig. 1.5**).

1.4 Pigeonpea (*Cajanus cajan* (L.) Millisp): Molecular Perspective for Defense Against Insects

Pigeonpea is considered an important source of dietary essential minerals, Vitamin B and protein especially for vegetarian population and a valuable source of income for millions of poor farmers in developing countries. Low diversity in primary gene pool poses restrictions towards crop breeding programs to develop cultivars with improved traits. Previous investigations examining elicited responses of pigeonpea, revealed enhanced antioxidant activity and induction of various defense- related proteins such as polyphenol oxidases, lipoxygenases, phenylalanine ammonia lyase, tyrosine ammonia lyase, PIs, leucineaminopeptidases, etc. (**Padul *et al.*, 2012; Kaur *et al.*, 2015; Lomate *et al.*, 2013**). Induction of PIs was observed after wounding and MeJa treatment in pigeonpea local and systemic leaves. However, lack of strong expression or low diversity of defense

compounds has resulted in insects to adapt and feed voraciously on this legume. Studies suggests that wild relatives of pigeonpea represent a vast source of untapped genetic resources which demonstrates source of resistance, however limited research in this aspect is available in pigeonpea at the molecular level (**Mallikarjuna *et al.*, 2007**). Thus, considering the importance of pigeonpea in terms of food security and economical development, there is a necessity to explore potential candidates which can confer resistance from *H. armigera* feeding.

1.4.1 Cultivated and wild relatives: Resistance *versus* Susceptibility

Pigeonpea crop endure severe losses in grain yield by *H. armigera* infestation. Cultivated pigeonpea exhibited low to moderate resistance against *H. armigera* (**Reed and Lateef, 1990**). Wild relatives of pigeonpea are shown to be an important source of resistance. However, transfer of traits from wild relative to cultivated variety through a distant gene pool poses difficulty in breeding. This can be addressed by gene isolation and cloning methods through gene insertion into cultivated variety (**Parde *et al.*, 2012**). Screening of pigeonpea wild relatives for the presence of proteinase inhibitors have shown significant activity against *H. armigera* **(Parde *et al.*, 2012)**. Another study revealed that trichomes on pods surface of several wild relatives contain antifeedant compounds that have lowered *H. armigera* feeding to a significant level. Feeding habits of insect also varies with changes in the chemical composition of the pod surface and trichome density in cultivated and wild accessions (**Sujana *et al.*, 2012**).

1.4.2 Induced plant molecular responses: “Omics” approach

Studies elucidating plant defense responses upon herbivory are focused to understand the dynamics of transcriptional changes, differential protein accumulation, and metabolic profiling using advanced “Omics” technologies (**Bykova, 2015**). Analysing transcriptome changes is the first step to decipher molecular basis of plant defense responses **(Singh *et al.*, 2008; Schenk *et al.*, 2000; Reymond *et al.*, 2000)**. Differential transcript abundance could also be identified between insect feeding and wounding revealing insect specific transcriptional changes or genes responsive to fatty acid amino acid conjugates found in

the oral secretion of insect (**Halitschke *et al.*, 2003**). Interestingly, transcriptional changes of specific genes cannot be correlated with their proteins levels, which imply that transcript abundance or repression does not necessarily lead to the respective protein accumulation or degradation. Thus it is important to find an actual proteomic change that is responsible to shape plant phenotype under specific condition.

Most of the proteomic study is based on mass spectrometry (MS) analysis, which is a powerful technique to determine qualitative and quantitative information about the entire protein set in a cell/tissue. High throughput proteome profiling using 2-dimensional gel electrophoresis (2D) techniques coupled with MS analyses have een employed in several plant defense related studies (**Hue *et al.*, 2015**). This has gained substantive importance in plant defense research to elucidate treatment specific responses. In general, proteomic investigation conducted in different plants in response to herbivory revealed large scale metabolic changes by identifying proteins involved in active oxygen removal, photosynthesis, amino acid metabolism, defense signal transduction and many more (**Giri *et al*., 2006; Collins *et al*., 2010; Fan *et al*., 2012; Zhang *et al.*, 2015**).

Plant system is largely complemented by the production of secondary metabolites that play direct and indirect role in defense system. Advancement in metabolite profiling methods and combinatorial protocols have generated enormous amount of information related to plant metabolomics. Some of these techniques are gas chromatography-MS (GC-MS), liquid chromatography (LC-MS) and nuclear magnetic resonance (NMR). Generation of secondary metabolites and their instrumental role in limiting pest attack by exerting deterrent, toxic and anti-nutritive effect is evident in various studies. Some important examples of plant secondary metabolites and their role are discussed in above section (secondary metabolism: route to plant defense) (**Barah and Bones, 2015**).

1.5 Plant Proteinase inhibitors (PIs): Innate Defensive Strategy

PIs are commonly present in plant kingdom and generally found in storage tissue such as seeds and tubers. Majority of the PIs studied, belongs to solanaceae, fabacae and poaceae families (**Richardson, 1991**). Occurrence of PI in plants regulates protease activity,

however, their defense-related function against herbivores was also recognized. A major breakthrough in the field of plant defenses occurred by the pioneer work of Prof. Clearance Ryan (Special issue 7 of Phytochemistry 2008, Volume 69) through the identification of PIs expressed locally and systemically in tomato and potato plants as a response to Lepidopteran feeding (**Green and Ryan, 1972**). PIs display defensive capability though disruption or inhibition of protease activity in insect midgut. Protein indigestion leads to non-availability of free amino acids creating burden on insect's digestive physiology, ultimately hampering its growth and development. Being a single gene product, introducing PI gene for developing transgenic lines, provides more practical advantages over multigene families (**Dunse *et al.*, 2010**). One of the important advantages of utilizing PI in insect control measures is its low selection pressure on pests, thereby reducing chances of attaining resistance to a considerable time.

1.5.1 Plant PIs: Inhibitory activity towards various proteases

PIs on the basis of their specificity towards mechanistic class of proteases are categorized into serine, cysteine, asparatic and metalloprotease PIs (**Terra and Ferreira, 1994**). This specificity is based on the presence of respective amino acid at the reaction centre. Among these, defensive role of serine PIs were well documented for their inhibitory activity against serine proteases. Serine proteases inhabit lepidopteran insect alkaline gut milieu, majorly constituting trypsin, chymotrypsin and elastases. Serine PIs are divided into four major types depending on their occurrence, sequence and functional analysis viz. bowman-birk inhibitors (BBIs), kunitz type, squash family and wound inducible PIs; potato type I inhibitor (Pin I) and potato type II inhibitor (Pin II) (**Ryan, 1990**). Wound inducible PIs received significant attention in plant defenses as their production is largely determined specifically by herbivory and wounding (**Green and Ryan, 1972**). Transgenic cotton expressing both type I and type II inhibitor, obtained from *S. tuberosum* and *N. alata* respectively, confer high resistance to cotton plants against *H. armigera* (**Dunse *et al.*, 2010**). In particular, Pin II type PIs has gained enormous attraction to determine its role in limiting pest population owing to their structural and functional diversity. Thus, it is interesting to understand existing diversity of PIs, which can be explored for their potential utilization in host plant resistance.

1.5.2 *Capsicum annuum* Pin II PIs: Structural and functional aspects

Previous investigations have examined the susceptibility of host plants to insect attack, thus wild relatives or non-host plants offer an attractive approach to elucidate naturally occurring candidates for host plant resistance (**Giri *et al.*, 1998; Harsulkar *et al.* 1999**). *C. annuum* is a non preferred host of insect *H. armigera* which was extensively studied for the presence of different PI isoforms. CanPIs consist of functional domains ranging from 1 to 4 IRDs connected together by protease sensitive linker region (**Fig. 1.6**). This multidomain precursor protein generates mature single form of ~55 amino acid long functional IRD. This structural feature was validated by interaction of *H. armigera* gut proteases (HGP) with different isoforms of CanPIs generating IRDs (**Mishra *et al.*, 2010**). Linker sequences are small with few amino acids sequences (QRNAK, EENAE, EASAE, EGNAE, EETQK) that expose the protease-processing site. Differential accumulation of CanPIs was monitored in different elicitation treatments such as aphid infestation, wounding and wounding with oral secretion suggests their specific activity profile in specific treatments (**Mishra *et al.*, 2012**). It is interesting to see that the unique number of CanPI transcripts is reported to be 47, of which 9 genes contain 4-IRDs, 3-IRD are present in 20 genes, 15 contains 2-IRD whereas only 3 gene contains single domain (**Shin *et al.*,2001; Kim *et al.*, 2001; Tamhane *et al.*, 2009; Mishra *et al.*, 2012**). These 47 genes are formed by the different combination of 28 unique IRDs out of which 7 are chymotrypsin specific whereas 21 are showing trypsin inhibitor effect. CanPIs shows high homology with only 4% variation in the sequences, which is majorly due to the amino acid a difference in individual IRDs. Partial repeat sequences at the C- and N-terminal regions is typical in Pin II type of solanacae family thus forming clasped bracelet like structure, however, it is lacking in CanPIs and hence it forms a separate cluster among Pin II PIs of Solanaceae. Arising of diversity in CanPIs in regard with number of inhibitory repeats, differential expression and protease specificity represent important evolutionary phenomena and signify its importance in plant defense system.

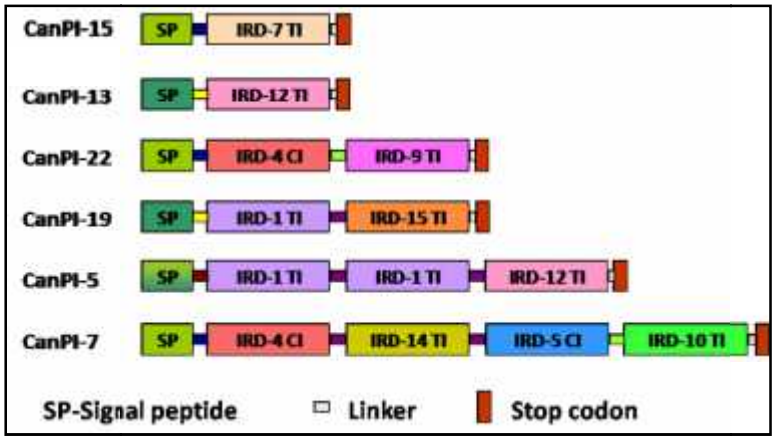


Figure 1.6: Structural representation of 1 to 4 IRDs of CanPIs (Adopted from Mishra *et al*., 2010)

1.5.2 Inhibitory activity of CanPIs on *H. armigera*

Inhibitory potential of CanPIs were tested against *H. armigera in vitro* and *in vivo* showing significant effect in reducing insect growth and delaying pupation. Egg laying capacity was also reported to be adversely affected after CanPI feeding (**Tamhane *et al.*, 2005**). Six recombinant CanPIs namely CanPI-15 and CanPI-13 carrying 1-IRD; CanPI-22 and CanPI-19 are 2-IRD proteins CanPI-5 (3-IRD) and CanPI-7 (4-IRD) were tested *in vitro* for their efficacy against trypsin and chymotrypsin and *Helicoverpa* gut proteases (**Fig. 1.7A**). Qualitative assessment of inhibitory activity of CanPI-5 and CanPI-7 was established by feeding studies and determined inhibition of gut proteases (**Fig. 1.7B**). Studies demonstrated CanPI-7 exhibited highest and differential inhibitory activity against proteases as compared to other CanPIs (**Mishra *et al.*, 2010**). These investigations have set an important paradigm for the contemporary research to utilize PI in transgenics or formulations for their enhanced applicability and efficacy in natural environment. Transgenics offer a viable alternative strategy to chemical pesticides but due to stringent regulatory issues another sustainable approach could be the development of pesticidal formulation. Development of biopesticidal formulations provides more easy and practical approach as it provides flexibility to changes in composition as per requirement. For example, syngeristic effects can be obtained by incorporating more than one active ingredient for broad-spectrum specificity of PIs. Furthermore, to delay the

resistant obtained by insect pests, molecules specific to one insect but with different mode of action can be used together.

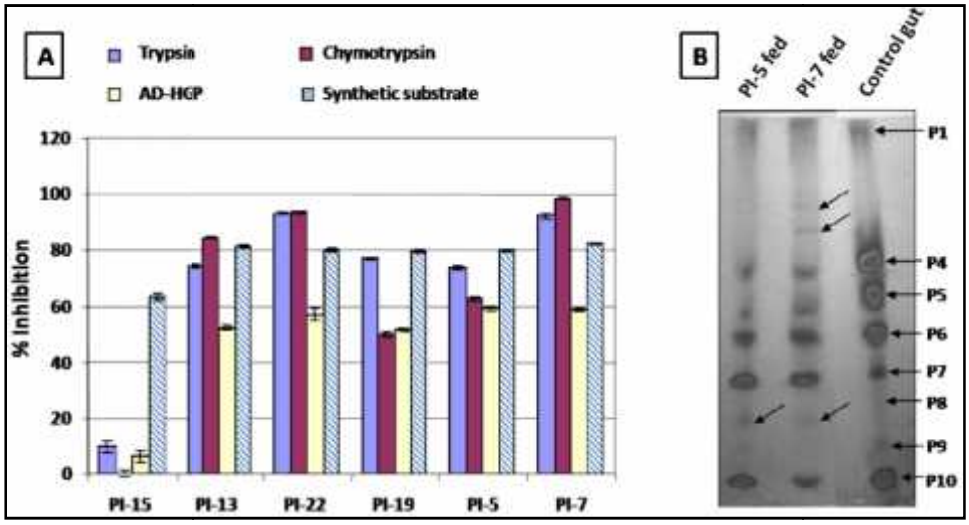


Figure 1.7: [A] Inhibitory activity of CanPIs against various HGP and commercial proteases, [B] protease profiling of PI fed insect gut revealing inhibition of number of proteases bands (Adopted from Mishra *et al.,* 2010).

1.6 Biopesticides Formulation

For successful application of the bio-actives, it is desirable that the pesticide formulations must include the following traits, *viz*. (i) improved activity, (ii) increased spreading and retention ability on plant surfaces, (iii) stability in the external environmental milieu and (iv) ease of handling (**Woods, 2003**). Formulations are prepared in different ways by assessing various physico-chemical properties of bioactive agents. In several instances there is a practical difficulty in applying or spreading the active ingredients directly for effective insect control. Furthermore, active ingredients may be chemically unstable, difficult to handle and may have short shelf life. These 'active ingredients' are generally formulated using other excipients which provides a protective shield and may act as adjuvants. Generally, these formulations are differentiated by physical classes e.g. emulsions, ready to use aerosols, smoke or fog generators, microencapsulated materials, pastes, gels and other injectable baits. Pesticide formulation may consist of (i) pesticide active ingredients that control the target pest, (ii) the vehicle, e.g. organic solvent or

mineral clay, (iii) surface-active ingredients including stickers and spreaders (e.g. emulsifiable concentrates) and (iv) other ingredients, such as stabilizers, dyes, and chemicals to enhance the pest control activity. Water insoluble pesticides are formulated using microencapsulation and nano-emulsion formulation methods (**Wang *et al.*, 2007**). A pesticide delivery system requires blend of active ingredient, surfactant and water or adjuvant.

1.6.1 Examples of plant derived compounds as active ingredients

Over the past decades, plant derived compounds and extracts have been investigated for their ability to control insect pests. However, only limited plant products are registered despite of intensive research on their identification (**Table 1**). Neem (*Azadirachtaindica*) based products such as neem seed extract, azadirachtins, pyrethrin and limonene are amongst the widely accepted botanical insecticides (Isman *et al*., 1990). Azadirachtin, a triterpenoid isolated from neem, interferes with embryonic development of *Spodoptera frugiperda* (**Correia *et al.*, 2013**) and *Helicoverpa armigera* shown to be highly susceptible to the neem seed extract (**Bhushan *et al.*, 2011**). In another example, a citrus extract consisting **δ**-limonene was formulated as an emulsion that demonstrated up to 100% mortalityto mealybug and scale insects (**Hollingsworth, 2005**). Some of the pesticides based on the plant essential oils and plant-derived compounds hold special status of 'exempted active ingredients'. They have reduced risk to the environment and are exempted from registration and extensive toxicological and environmental tests. A few examples are: cinnamon, citronella, lemon grass, garlic, thyme, pure compounds like eugenol and citric acid (**Cantrell *et al.*, 2012**). Though many of them act as natural repellents against various arthropods, our focus here is on those compounds that have efficacy for use in insect control in crops.

Table 1.1: Some examples of formulations of plant derived compounds and their target pest.

Class	**Plant insecticidal compounds/ Mode of action**	**Details of formulations**	**Effective against**	**References**
Monoterpenoids	A- pinene linalool/ Antifeedant Limonene/ Neurotoxic	Adsorb on Silica nanoparticle Emulsion 1% limonene, 0.75% APSA-80, 0.1% Silwet L-77	*Spodopteralitura* *Achaea janata* (Lepidopterans) *Pseudococcuslongispinus* *Aleurodicus disperses* (Hemipterans)	Rani *et al*., 2014 Hollingsworth, 2005
Tetranortriterpenoids	Azadirachtin/ Molt disruptant Antifeedant	Nanoformulation in colloidal suspension neem oil 2% (w/v), neem extract 0.5% (w/v), Span 60 0.5% (w/v), polymer (poly(e-caprolactone))0.5% (w/v), and Tween80 0.5% (w/v)	*Plutellaxylostella* (Lepidoptera)	Forim *et al* 2013
Alkaloids	Nicotine/ Neurotoxic mode of action, blocks ca^{++} ion channels	Nicotine dispersions (3 wt % sodium caseinate, 20 vol % nicotine oleate)	Aphids (Hemiptera)	Casanova *et al*., 2002

1.7 Nano or Micro Based Pesticides

Nanotechnology refers to a technique involving synthesis, engineering, modification and implementation of materials or solutions having particles or droplet size ideally below 100 nm (**Khandare *et al.*, 2012**). Use of nano-pesticides is well regarded with the availability of nano-emulsions, nano-capsules or nanoparticles that offer unprecedented advantages over the conventional delivery systems by offering wide range of solubility, thermal stability, biodegradability, large surface area and permeability (**Kah and Hofmann, 2014; Bergeson, 2010**).

1.7.1 Microemulsions Formulations

Due to the differential solubility of active ingredients, emulsions are prepared by dispersing liquid droplets (as a dispersed phase) into another liquid as a continuous phase (e.g. oil/water or water/oil emulsion). This system could be further stabilized by adding surface-active ingredients (**Sathishkumar *et al.*, 2008**). Such formulations are routinely used with certain modifications depending on the environment, crop, target insect pests and their feeding habits. Formulation development is currently progressing with objectives of high loading of active ingredients and for wide spectrum applications resulting in greater efficacy and applicability against target insect pests. Towards this, microemulsions, microcapsules and nanoparticles are promising delivery systems for cargo transport.

Micro/nano-emulsion-based pesticide formulations have advantages over the conventional pesticidal emulsions due to their long-term thermodynamic stability and small droplet size (**Wang *et al.*, 2007**). Altogether these systems possess wide ability for solubilisation allowing loading of different polar and non-polar compounds in an acceptable range **(Sathishkumar *et al.*, 2008**). Thus, it is possible to design microemulsions complementary to the chemical bio-actives. This will aid in effective loading, enhancing efficacy, greater stability and improved uptake of the target molecule. An example include incorporating cyhalothrin, an organic pesticide in microemulsion to develop formulation (**Zhao *et al.*, 2009**). Such systems can be efficiently utilized and

modified to contain biologically active ingredients and might prove to be a promising approach. On the other hand, encapsulation of pesticidal biomolecules in microemulsion has not been largely explored. However, such microemulsions can have immense potential in the agriculture industry owing to its increased dispersion over larger contact area, wettability and enhanced penetration properties (**Paul and Moulik, 2001**). While bioformulations intend to cause no harmful effect on the environment, the possibility of phytotoxic effect on the target crops after multiple applications must be recognized.

1.7.2 Nanoparticles as Advanced Delivery Systems

Nanoscience and its transformation into nanotechnology platforms are anticipated to offer better solutions for targeted delivery. Towards this, both organic and inorganic components have been widely explored in fabricating nano-sized systems, deliberating the prudent applications in engineering and biomedical sciences. In general, nanosystems offer several tasks in parallel including loading of cargo molecules, either small or large, through the conjugation process (**Khandare *et al*., 2012**). Methods such as encapsulation, covalent bonding, adherence and adsorption, render significant impact on the 'smart' delivery approach to achieve controlled payload release (**Ghormade *et al.*, 2011**). Conversely, inorganic nanomaterials, e.g. iron oxide, silica, and calcium phosphate nanoparticles have generated great interest in this field as they offer unprecedented opportunities in delivering bio-actives (**Debnath *et al.*, 2012; Banerjee *et al*., 2015**).

1.7.2.1 Inorganic Pesticide Delivery Systems: Emphasis on Silica Based Materials

Inorganic micro and nano particle based formulations for pesticide delivery have attracted great attention over the last few years as they offer high stability, chemical versatility and biocompatibility (**Ghormade *et al*., 2011**). Amongst inorganic materials, silica nanoparticles have been the most widely explored material for the pesticide delivery as they offer inimitable advantages. They possess several unique features *viz*. physical and chemical stability, tunable pore sizes, high surface area and well-defined surface properties making them ideal for hosting guest molecules of various sizes, shapes and functionalities (**Ghormade *et al*., 2011**). In addition, they provide stability to bio-

active molecules from chemical to physical parameters such as temperature, moisture, plant's chemical secretions, soil pH and composition These characteristics must be considered for the efficient delivery of many peptide and protein molecules.

Debnath *et al*., (**201**1) reported that treating insects with silica nanoparticles (15 to 30 nm) resulted in higher mortality than when the insect were treated with bulk silica. This study revealed that surface functionalized silica nanoparticles i.e., hydrophobic, hydrophilic or lipophilic coatings) have differential effect on *Sitophilus oryzae* mortality. In another study efficiency of silica nanoparticles capped with 3-Mercaptopropyltriethoxysilane and hexamethyldisilazane was assessed against *Spodoptera litura* (**Debnath *et al*., 2012**). Significant nanocide activity was found in terms of larval mortality when fed with functionalized silica nanoparticles.

Silica nanoparticles have also been used for delivery of pesticides with short half-life. For example, avermectin an insect chloride channel inhibitor that blocks neurotransmission shows a major problem in the field due to UV inactivation. Li *et al*., (**2007**) investigated porous hollow silica nanoparticles to deliver avermectin. These nanoparticles not only protected avermectin from UV degradation by encapsulation but also controlled its slow release. Furthermore, the nanopesticide formulation exhibited a sustained-release pattern for nearly 30 days (**Li *et al*., 2007**).

Advances in formulation technologies have contributed to the successful development of nano or micro based formulations and imparted enhance activity, greater stability of the compounds and biodegradability. However, choosing an ideal nanomaterial or formulation for field application is critical. Materials that are biocompatible, nontoxic and biodegradable are preferable and should be researched. It is very important to study both the short-term and long-term effects of the formulations on plant physiology to avoid adverse effects by integrating current agricultural technology with advancements in nanotechnology; improved crop productivity can be achieved while minimizing environmental and human health effects.

Organization of thesis

Based on the findings of previous studies pertaining to insect pest interaction and available information on their utilization for plant protection, I have organized my thesis into three chapters which in general discuss plant defensive compounds and their utilization for susceptible plant protection. Extensive literature survey was carried out which forms the foundation of given objectives. This is followed with general discussions along with its possible future implications.

Chapter 2: Quantitative proteomic approach to study pigeonpea defenses against Lepidopteran insect pest

This chapter deals in understanding proteome profiling of *C. cajan* wild relatives (*Flemingia stricta*, *Cajanus platycarpus*, *Cajanus scarabaeoides*) with two cultivated varieties of pigeonpea (Vipula (tolerant) and ICPL 87 (susceptible) upon elicitation using shotgun proteomics approach. Proteins identified were differentially accumulated in the pigeonpea plants and belong to physiological processes such as photosynthesis, primary metabolism and energy regulation, signal transduction, genetic information processing, and active oxygen removal.

Chapter 3: Development and characterization of microemulsion based proteinase inhibitor loaded formulation

In this section, we have explored the potential of proteinase inhibitor for its efficacy and applicability in outer environment. Bicontinuous microemulsion based delivery system was utilized for the protein loading which exhibit enhanced retention of proteinase inhibitor on the leaf surface.

Chapter 4: Bio-physical evaluation and *in vivo* delivery of plant proteinase inhibitor immobilized on silica nanospheres

Herein, we explored PI peptide immobilization on silica based nanospheres and rods followed by its pH mediated release *in vitro* and *in vivo*. Immobilized PI molecule tested

against *Helicoverpa armigera*. The study confirms that peptide immobilized silica nanosphere is capable of affecting overall growth and development of the feeding insects, which is known to hamper fecundity and fertility of the insects.

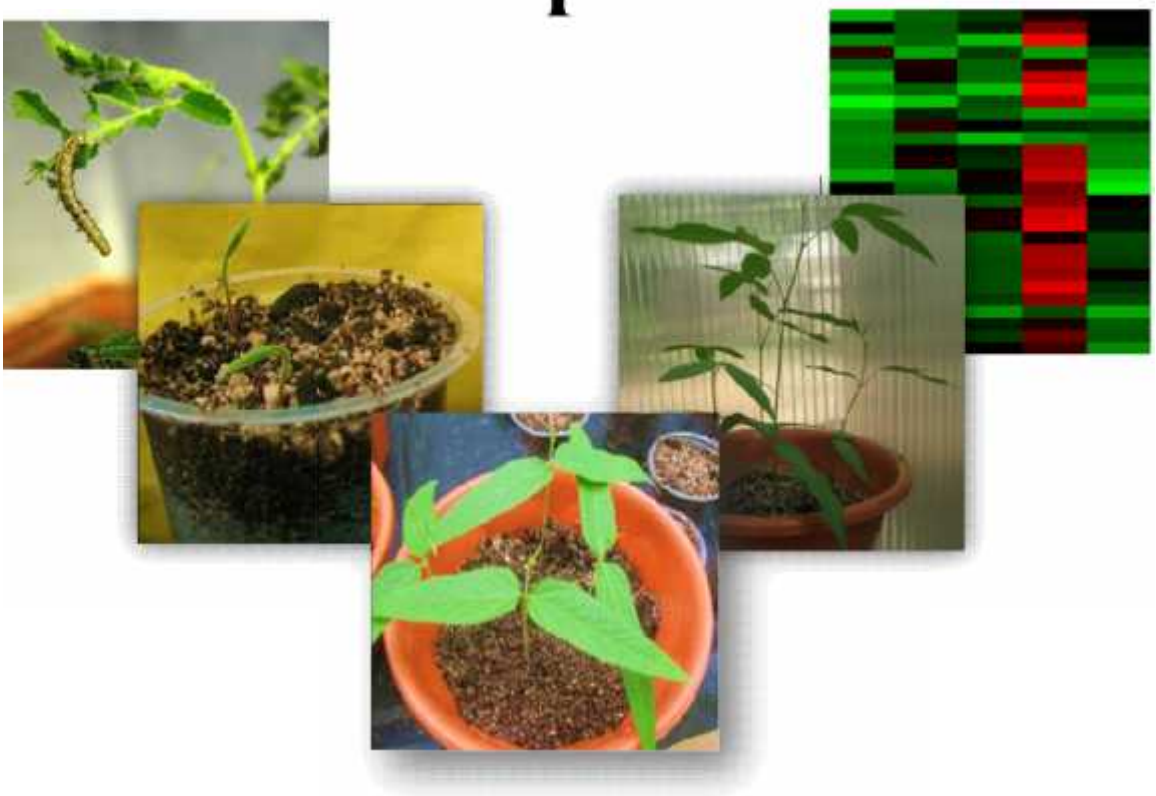
Chapter 5: General discussion and future outlook

In this section, several aspects of plant tolerance/resistance mechanism have been discussed along with the potential role PIs in conferring resistance against pest. Obtained information can be translated in a more applied way by employing interdisciplinary approaches. This requires further characterization of defensive molecules for their enhanced applicability and efficacy at the field level.

Bibliography

Curriculum Vitae

Chapter 2



Quantitative proteomic approach to study pigeonpea defenses against Lepidopteran insect pest

Chapter 2: Quantitative proteomic approach to study pigeonpea defenses against Lepidopteran insect pest

2.1 Introduction

Studies examining plant resistance have demonstrated that damage induced responses are more durable and beneficial to the plant fitness as compared to the constitutive expression (**Agrawal, 1998**). Induced resistance is closely associated with the reallocation of carbon and nitrogen sources that form the precursor for defensive metabolites. Such resource partitioning occurs among primary and secondary metabolism which is largely focused on the fate of carbohydrates and amino acids (**Zhou *et al*., 2015**) that act as source of energy as well as are involved in biosynthesis of defensive secondary metabolites. Hence, it is clear that to allow metabolic reconfiguration, plant undergoes large scale transcriptional and translational regulation in response to elicitation (**Hermsmeier *et al*., 2001; Giri *et al*., 2006; Singh *et al*., 2008**). Recent advances in technologies have empowered us to characterize large-scale transcriptome, proteome and metabolome of several herbivore affected plants leading to discovery of plausible defensive compounds. The promising role of these functional molecules that predominantly exerts repellent, toxic and anti-metabolic effects on herbivores is largely evident (**Padul *et al*., 2012; Singh *et al*., 2013**). In addition, plant also adapts with a tolerate mechanism by repairing damaged tissue with the involvement of increased photosynthetic efficiency and compensatory growth (**Karban and Baldwin, 1997; Kessler and Balwin, 2002**). Thus, a particular plant-insect system presents an intricate and dynamic interaction which is highly specific and provides a scope to decipher the degree of plant tolerance or resistance by studying their metabolic changes upon elicitation. Production of reactive oxygen species (ROS), proteinase inhibitors (PIs), threonine deaminases, alkaloids, phenolic compunds and terpenoids etc, are few of the well-studied metabolites in connection with induced responses in several plants such as *Arabidopsis*, tomato, soybean, and tobacco, and needs to be extended for several othercrops (**Lin *et al*., 1990; Bi *et al*., 1994; Stotz *et al*., 2000; Wu *et al*., 2007; Attaran *et al*., 2009; Fan *et al*., 2012; Kaur *et al*., 2014**).

Pigeonpea (*Cajanus cajan* L. Millisp.) is one of the important leguminous plant and is the staple crop along with major source of protein in human diet. It supports the livelihood of millions of farmers in Asia, Africa, South and Central America and Carribean (**Mula and Saxena, 2010**). Pigeonpea cultivation in challenging environments exposes it to biotic and abiotic stresses resulting into severe yield losses. *Helicoverpa armigera* pest attack has been considered as a major threat to pigeonpea yield. Furthermore, low genetic diversity in the primary gene pool poses restriction in conducting effective crop breeding programs to develop cultivars with improved defense traits (**Bohra *et al.*, 2010**). Despite the importance of this crop in food security and economic development, it lacks intensive scientific effort and detailed study on interaction of *H. armigera* and pigeonpea at the molecular level. Previous investigations in *C. cajan* have revealed enhanced antioxidant activity and induction of various defensive proteins elicited by herbivory such as polyphenol oxidases, lipoxygenases, phenylalanine ammonia lyase, tyrosine ammonia lyase, proteinase inhibitors, leucine aminopeptidases (**Padul *et al.*, 2012; Lomate *et al.*, 2013; Kaur *et al.*, 2015**). It can, therefore, be assumed that due to lack of strong expression or low diversity of defensive compounds have caused the insects to adapt and feed voraciously. Potential of wild relatives which act as an importance source of resistance can be screened for desirable traits for their further implementation in classical breeding or genetic engineering (**Mallikarjuna *et al.*, 2010**). For an example, a specific sesquiterpene pathway gene from wild tomato was able to induce herbivore resistance in cultivated tomato (**Bleekera *et al.*, 2012**). Similarly, proteinase inhibitors from wild accessions of pigeonpea demonstrated inhibitory effect on *H. armigera* growth and development (**Parde *et al.*, 2012**). In another study pod surface exudates of wild relatives of pigeonpea were tested for their antagonistic effect on *H. armigera* (**Sujana *et al.*, 2012**).

In this study we attempted to provide an overview of proteomic changes in wild relatives and cultivated varieties of pigeonpea upon elicitation treatments specific to *Helicoverpa armigera*. Wild relatives namely, *F. stricta*, *C. platycarpus*, *C. scarabaeoides* belongs to quaternary, tertiary and secondary, genepool respectively. While two cultivars that differ in their tolerance to insect damge are Vipula (tolerant) and

ICPL-87 (susceptible). New advancement in mass spectrometric analyses have made it plausible to carry out protein profiling on a large-scale and is been employed in various plant stress related studies (**Salekdeh *et al*., 2002; Lee *et al*., 2004; Dawkar *et al*., 2013**). Using similar approach, we demonstrate the changes in protein level in pattern wheel punctured leaves followed by oral secretion application (WOS) in these wild relatives and cultivated pigeonpea cultivars. Furthermore, in-depth time based analysis in Vipula was carried out using 2-dimensional difference gel electrophoresis (2D DIGE) by subjecting it with several elicitation treatments *viz*. methyl jasmonate (MeJA), WOS and larval feeding. MeJA is a volatile form of jasmonic acid which triggers defense signaling cascade and plays central role in inducing defense related proteins such as PIs (**Sasaki-Sekimoto *et al*., 2005**). Transcription and proteomic studies revealed that MeJA play instrumental role in plant defense mechanism as shown in *Arabidopsis*, *Nicotiana* and maize exhibiting significant alteration at gene and its functional unit (**Heidel and Bladwin 2004; Sekimoto *et al*., 2005; Zhan *et al*., 2015**). On the other hand, Lepidopteran oral secretion contains specific fatty acid-amino acid conjugates (FACs) which activate insect responsive genes (**Halitschke *et al*., 2003**). These elicitation treatments were used in several studies to decipher changes in protein levels in order to understand plant induced mechanisms to limit the pest population or tolerate pest attack by reconfiguring inherent resources. Thus, using mass spectrometry approach, we present an overview of pigeonpea defense responses specific to *H.armigera*.

2.2 Results and Discussion

2.2.1 Protein Identification through Mass Spectrometry

In this study, shot gun proteomic approach, which utilizes LC-MS/MS was used to analyze complex mixture of peptides. Peptide matching was carried out against Viridiplantae using Uniprot databank (www.uniprot.org). NanoLC-MS/MS data was processed using ProteinLynx Global SERVER (PLGSv2.5.1) for the protein quantitation to determine their up and down regulation with their respective control. Expression analysis parameters were set to filter only those proteins present in all the three biological replicates and yielded over 200 DEPs in *F. stricta*, *C. platycarpus*, *C. scarabaeoides*,

Vipula and ICPL 87. Unique proteins were sorted among the identified proteins to remove uncharacterized and redundant proteins that were manually curated on the basis of highest score. Thus number of DEPs were identified in *F. stricta* (71), *C. platycarpus* (53), *C. scarabaeoides* (46), Vipula (34) and ICPL 87 (45). Identified proteins from all the plants were categorized for their involvement in different biological process as per the gene ontology (GO) criteria. Most represented categories were protein-chromophore linkage with molecular function of ATP binding, metal ion binding and chlorophyll binding, energy metabolism (ATP synthesis and hydrolysis), photosynthesis, carbon fixation, glycolytic process (**Fig. 2.1**).Only one of the wild relatives, *F. stricta*, revealed proteins related to biotic and abiotic stress response in biological process. Over 18% of the proteins were uncharacterized for any function. These variations in wild and cultivated plants in terms of differential protein levels and identification of species specific proteins could be an indicative of differential regulatory mechanism upon elicitation. In 2D DIGE analysis, peptide mass fingerprints of selected tryptic digested proteins were determined by matrix-assisted laser desorption ionization- time of flight (MALDI-TOF) spectra. Peptides were matched with NCBI nr database entry of the National Centre of Biotechnology Information (NCBI) and the search program used was Mascot with a *P*- <0.05. A detailed information of these proteins such as accession numbers to identify their putative function, identification scores, number of matching peptides, molecular masses and isoelectric point along with sequence coverage and peptide sequences were presented (**Table 2.1**).

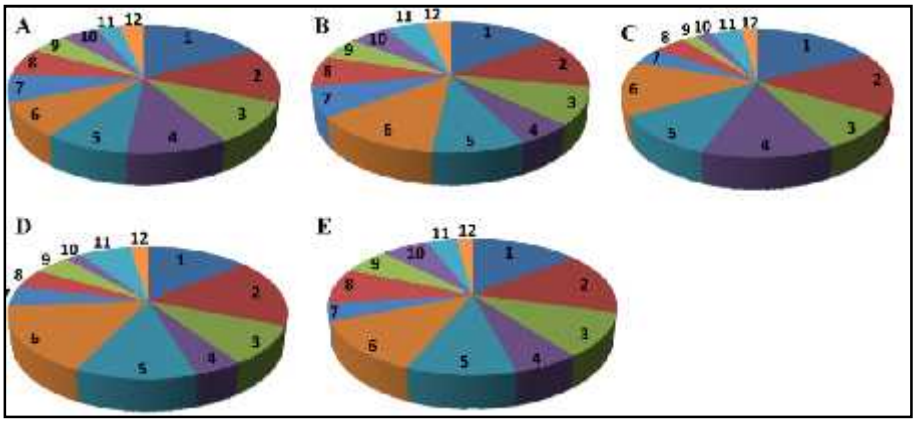


Figure 2.1: WOS elicited proteins were categorized as their biological process identified in all the wild and cultivated plants [A] *F. stricta* [B[*C. platycarpus* [C] *C. scarabaeoides* [D] *C. cajan* (vipula) [E] *C. cajan* (ICPL 87). Highly represented categories are: 1. Protein-chromophore linkage [GO:0018298], 2. ATP synthesis coupled proton transport [GO:00159860], 3. Photosynthesis, light harvesting [GO:0009765], 4. Photosynthetic electron transport in photosystem II [GO:0009772], 5. ATP hydrolysis coupled proton transport [GO:0015991], 6. Photosynthesis [GO:0015979], 7. Glycolytic process [GO:0006096], 8. Photorespiration [GO:0009853], 9. Carbohydrate metabolic process [GO:0005975], 10. Reductive pentose-phosphate cycle [GO:0019253], 11. Carbon fixation [GO:0015977], 12. Carbon utilization [GO:0015976].

Table 2.1: Protein spots accumulated differentially in *C. cajan* after oral secretion treatment were identified by MALDI-TOF against NCBI green plant database.

Spot No.	Accession	Protein	MALDI score/No.of peptide	Th Mw/pI	Observed Mw/pI	Sequence coverage (%)	Sequence
25	gi|125573095	hypothetical protein OsJ_04535 [Oryza sativa Japonica Group]	51/1	29.68/10.3	180/6.2	8	M.SSWNSPYYDTSSYGAGSGGGGGGGR.R
51	gi|8134568	Full=5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	194	85/3	120/7.7	4	K.YLFAGVVDGR.N R.IPPTEELADR.I K.YGAGIGPGVYDIHSPR.I
78/79	gi|115420	NADP-dependent malic enzyme	112/2 143/2	65.2/6.4	97.4/7.5	3 3	K.SIQVIVVTDGER.I K.LLNDEFYIGLR.Q
84	gi|224140653	predicted protein [Populus trichocarpa]	59/1	61.3/5.4	100/5.6	2	K.LPSHYLVSPPEIDR.T
88	gi|2506277	RuBisCO large subunit-binding protein subunit beta, chloroplastic	384/4	63.2/5.8	100/4.4	9	K.VVAAGANPVLITR.G R.DLINILEDAIR.S K.AAVEEGIVVGGGCTLLR.L K.SQYLDDIAILTGGTVIR.E
99	gi|169794058	ATP synthase CF1 alpha subunit	291/4	55.6/5.2	82/4.0	10	R.LIESPAPGIISR.R R.IAQIPVSEAYLGR.V

							K.ASSVAQVVTTLQER.G K.IVNTGTVLQVGDGIA R.I
102/299	gi|1000936	Ribulosebisphosphate carboxylase	177/278/1	42.3/6.6	70/6.631/7.3	62	R.ELTLGFVDLLR.D K.TFQGPPHGIQVER.D
103	No significant match				70/6.1		
105	gi|11587	unnamed protein product	70/1	47.5/6.3	70/6.5	3	K.TFQGPPHGIQVER.D
106	gi|502163420	BAHD acyltransferase DCR-like	63	49.9/5.8	66/5.8	3	K.VYDIDFGWGKPEIVR.S
108	gi|168312	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	304/4	53.1/6.2	66.3/6.7	9	K.DTDILAAFR.V R.VALEACVKAR.N R.EITLGFVDLLR.D K.TFQGPPHGIQVER.D
109	gi|1304257	ribulose-1,5-bisphosphate carboxylase large subunit	292/4	51.5/7.2	66.3/6.7	9	K.DTDILAAFR.V R.DNGLLLHIHR.A R.ELTLGFVDLLR.D K.TFQGPPHGIQVER.D
111	gi|40647581	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	445/6	52.5/6.4	66.3/7.0	13	K.DTDILAAFR.V R.VALEACVKAR.N R.DNGLLLHIHR.A R.EITLGFVDLLR.D K.LTYYTPQYQTK.D K.TFQGPPHGIQVER.D
112	gi|1000936	ribulosebisphosphate carboxylase	259/3	42.3/6.6	66.3/7.5	8	R.DNGLLLHIHR.A R.ELTLGFVDLLR.D K.TFQGPPHGIQVER.D
118	gi|6688796	Ribulose-1,5-bisphosphate carboxylase/oxygenase	165/3	51.7/6.2	66.3/7.3	7	R.DNGLLLHIHR.A R.DIDLGFVDLLR.X K.TFQGPPHGIQVER.D
119	gi|16215	catalase	210/3	57.1/6.7	66.3/7.9	7	R.APGVQTPVIVR.F R.DEEVNYFPSR.Y

							K.TWPEDILPLQPVGR.M
121	gi|303842	beta-tubulin	261/3	50.4/4.7	70.3/3.9	9	R.FPGQLNSDLR.K R.INVYYNEASGGR.Y K.GHYTEGAELIDSVLD VVR.K
123	gi|17026394	UDP-glucose pyrophosphorylase	272/3	51.6/6.0	70.3/4.1	9	K.GGTLISYEGR.V K.VLQLETAAGAAIR.F K.ATSDLLLVQSDLYTL EDGFVIR.N
124	gi|114421	Full=ATP synthase subunit beta, mitochondrial	197/2	59.9/5.9	70.3/4.3	5	R.VLNTGSPITVPVGR.A R.FTQANSEVSALLGR.I
131/132/133/134	gi|3850914	ATP synthase beta subunit	801/7 164/2 530/6	52.8/5.0	66.3/4.8 66.3/5.3 66.3/4.6 66.3/5.0	22 6 8	K.AHGGVSVFGGVGER. T R.IVGEEHYETAQR.V R.FVQAGSEVSALLGR.M K.VALVYGQMNEPPGA R.M R.DVNEQDVLLFIDNIFR. F R.IFNVLGEPIDNLGPVD TR.T K.ELQDIIAILGLDELSEE DR.L K.GIYPAVDPLDSTSTMLQPR.I
161	gi|127046	S-adenosylmethionine synthase 2	45/1	43.6/5.5	59/5.6	3	R.FVIGGPHGDAGLTGR. K
165/171	gi|255641658	unknown	109/2 126/2	42.0/5.2	60/4.3 57/4.4	7 7	K.AEYDESGPSIVHR.K K.SYELPDGQVITIGDER. F
167/168	gi|7960277	ribulose bisphosphate carboxylase activase B	378/4 333/3	48.0/6.9	59/4.1	14 11	R.EGPPTFDQPK.M K.GLAYDISDDQQDITR.

							G R.LVDTFPGQSIDFFGALR.A R.VPIIVTGNDFSTLYAPLIR.D
176	gi|13877511	glutamine synthetase precursor	166/2	47.9/6.7	61/4.4	6	R.HKDHISAYGEGNER.R K.IIAEYIWIGGTGIDVR.S
199	gi|120666	Glyceraldehyde-3-phosphate dehydrogenase	326/2	36.7/8.3	55/8.0	10	R.VPTVDVSVVDLTVR.L K.GILGYTEDDVVSTDFVGDSR.S
214	No significant Match				47/4.9		
227	gi|224061310	predicted protein	299/3	35.7/8.5	45/6.2	12	R.LFGVTTLDVVR.A K.ALEGADVVIIPAGVPR.K K.NGVEEVLGLGPLSDFEK.E
237	gi|22633	fructose-bisphosphate aldolase	100/2	42.7/7.5	45//5.2	5	R.SAAYYQQGAR.F R.LASIGLENTEANR.Q
285	No significant match				36.5/6.4		
298	gi|9587205	LHCII type I chlorophyll a/b-binding protein	114/2	28.0/5.1	31/4.6	11	K.SVSSGSPWYGPDR.V R.WAMLGALGCVFPELLAR.N
312	gi|131899	Ribulose bisphosphate carboxylase large chain	225/3	52.1/6.1	27/6.6	7	R.DNGLLLHIHR.A R.EITLGFVDLLR.D K.DDENVNSQPFMR.W
313	gi|6525083	Ribulose-1,5-bisphosphate carboxylase/oxygenase	310/4	50.1/6.6	27/6.9	10	R.VALEACVKAR.N R.DNGLLLHIHR.A

		large subunit					R.EITLGFVDLLR.D R.LSGGDHVHSGTVVGK .L
330	No significant match				21.5/4.4		
334	gi|120532	Ferritin-1, chloroplastic	67/1	28.2/5.7	25.5/4.9	4	K.GHGVWHFDQR.L
442	gi|42541536	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	48/1	48.8/6.7	14/3.7	2	K.DTDIIAAFR.M

2.2.2 Differential Accumulation of Proteins in Wild Relatives and Cultivated Pigeonpeas

Analysis of DEPs in WOS and WW treated tissues demonstrated significant upregulation in plants treated with oral secretion. A similar but relatively lower level of protein was found in the WW treatment compared to the WOS treatment (**Fig. 2.2**). This difference was previously explained by various transcriptomic studies that have revealed specific gene expression upon WW, however, unique plant responses to insect elicitors were observed (**Halitschke *et al*., 2003; Hermsmeier *et al*., 2001; Schittko *et al*., 2001; Halitschke *et al*., 2001; Hui *et al*., 2003;**). Proteins identified were categorized into several groups such as photosynthesis and photorespiration, transcription and translation related, signal transduction pathway, genetic information processing, carbohydrate and energy metabolism and other metabolic pathways (**Table 2.2**).

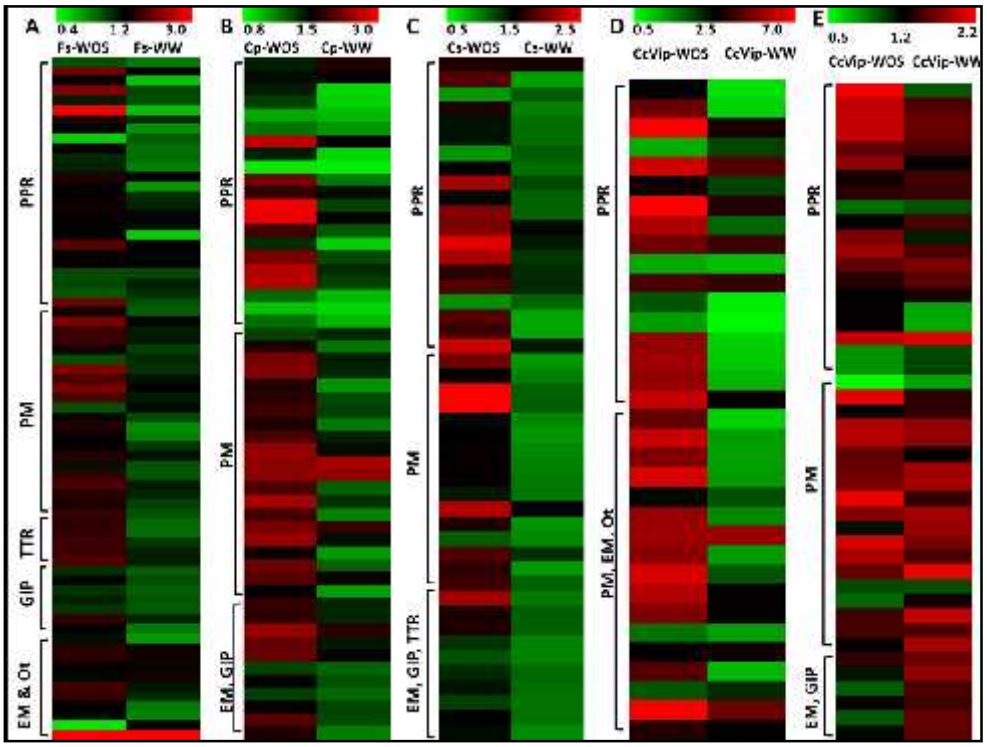


Figure 2.2: Differentially expressed proteins are broadly categorized into PPR, photosynthesis and photorespiration related; PM, primary metabolism; TTR, transcription

and translation related; GIP, Genetic information processing; EM, energy metabolism and OT, others for their comparative analysis in WOS and WW elicitation for [A] *F. stricta* [B] *C. platycarpus* [C] *C. scarabaeoides* [D] *C. cajan* (vipula) [E] *C. cajan* (ICPL 87).

Analysis of DEPs revealed 28 proteins common to all the WOS treated plants that were involved in photosynthesis and photorespiration related pathway and primary metabolic pathway (**Figure 2.3A**). Interestingly, some proteins were unique to *F. stricta* and could not be identified in other plants such as probable mediator of RNA polymerase II subunit, methionine synthase, chlorophyll a b binding protein and 14-3-3 like protein A. Several photosynthetic proteins displayed differential accumulation in WOS treated plants such as rubisco activase (RCA) was upregulated (4.2) in Vipula and downregulated in ICPL 87 (0.6). Oxygen evolving enhancer protein (OEE) was found to be downregulated in *F. stricta* and *C. platycarpus*. Similarly, light harvesting complex II (LHCII) protein and ribulose bisphosphate carboxylase (RuBPase) large/small chain displayed significant higher accumulation in Vipula. Proteins belonging to primary metabolic processes also displayed differential expression in WOS treated plants such as ATP synthase subunit alpha/beta, malate dehydrogense (MDH), peroxisomalglycolate oxidase (GOX), phosphoglycerate kinase (PGK) and carbonic anhydrases was significantly over expressed in shoot tissues of Vipula (**Figure 2.3B**). Vipula exhibited greater upregulation of proteins upon insect damage or elicitation.

Table 2.2: List of the proteins identified in wild and cultivated Pigeonpea by LC-MS/MS

Description	**Mw (Kda)/ Theoretical pI**	**Fs w1 Score Fold change/P value**	**Cp w2 Score Fold change/P value**	**Cs w3 Score Fold change/P value**	**Cc Vip Score/P value**	**Cc ICPL Score/P value**
Photosynthesis and Photorespiration related						
Alpha form rubisco activase	52.2/5.8	↓13697 0.9/0.2	↑16395 1.4/1.0	↓22166 1.6/1.0	↑7267 2.5/1.0	↑12103 2.1/1.0
Beta form rubisco activase	48.5/7.0	↑24160 2.0/1.0	↑24022 1.4/0.62	↑27312 1.8/1.0	↑13327 4.4/1.0	↓20276 1.9/1.0
Chlorophyll a b binding protein	27.8/4.9	↑61058 1.2/1.0	↑42908 1.4/1.0	↓114132 0.9/0.0	↑35006 8.3/1.0	↑30461 1.9/1.0
Chlorophyll a b binding protein 2 chloroplastic	27.1/5.8	↑3688 2.1/1.0	_	_	_	_
Chlorophyll a b binding protein 3 chloroplastic	27.8/5.3	↑35625 1.1/1.0	↑40606 1.3/1.0	↑66955 0.9/0.08	_	↓24715 1.9/1.0
Chlorophyll a b binding protein type II	28.5/5.3	↑8794 2.9/1.0	↑3737 1.0/0.6	↑9470 1.6/1.0	↑1962 1.0/0.75	↑6468 1.5/1.0
Cytochrome b6 f complex iron sulfur subunit	24.1/8.3	_	↑6656 1.1/0.93	↑18432 1.1/0.91	↑10658 6.1/1.0	↑7305 1.7/1.0
Cytochrome f	35.2/9.1	↑5826 1.4/1.0	↑9846 2.6/1.0	↑7141 1..4/1.0	↑10387 2.8/1.0	↓11582 1.2/0.09
Light harvesting complex II protein	27.8/5.1	↑47445 1.1/1.0	↑50216 1.4/1.0	↓89968 0.8/0.02	↑34647 7.9/1.0	↑39638 1.3/1.0
Oxygen evolving enhancer protein 2	28.5/7.9	↓/4633 0.5/0.0	↓9846 0.8/0.0	↑7141 1.5/1.0	↑10387 5.6/1.0	↓11237 0.9/0.3
Photosystem I iron sulfur	9.0/6.8	↑18918	↑7609	↑15545	↑4345	_

center		1.2/0.97	2.2/1.0	2.1/1.0	4.4/1.0	
Photosystem I subunit PsaD	22.9/9.9	↑13654 1.0/0.7	↑16681 1.8/1.0	↑9934 1.4/1.0	↑8409 1.0/1.0	↑11870 1.2/1.0
Photosystem I subunit VII Fragment	5.9/7.5	↑11417 1.0/0.72	_	_	_	_
Photosystem II CP43 reaction center protein	51.8/6.7	↑9039 1.4/1.0	↑8813 2.9/1.0	↑8808 2.0/1.0	↑2408 3.8/1.0	_
Photosystem II CP47 reaction center protein	55.9/6.2	↑7251 1.2/1.0	↑6157 2.8/1.0	↑5515 1.9/1.0	↑4219 1.7/1.0	↑4692 1.7/1.0
Photosystem II protein D1	38.9/4.9	↑1888 1.2/1.0	_	↑1533 1.7/1.0	_	_
Photosystem II protein D1 Fragment	38.7/5.1	↑1441 1.2/1.0		↑1038 1.8/1.0	_	
Photosystem II D2 protein	39.5/5.2	↑27287 1.3/1.0	↑17515 1.8/1.0	↑11441 2.3/1.0	_	↑14089 1.8/1.0
Photosystem II D2 protein Fragment		↑16193 1.2/1.0	↑6710 1.6/1.0	↑6710 2.1/1.0	_	↑9281 1.5/1.0
Photosystem II type I chlorophyll a b binding protein	27.9/5.0	↑51165 1.2/1.0	↑40606 1.3/1.0	↓95183 0.9/0.14	↑31902 1.1/1.0 _	↑24715 1.3/1.0
Putative rubisco subunit binding protein alpha subunit	20.3/4.4	↑1345 1.8/1.0	_	_	_	↑1914 1.2/0.98
Ribulose 1 5 bisphosphate carboxylase oxygenase large subunit Fragment	52.3/6.1	↑189807 1.3/1.0	↑126967 2.2/1.0	↑211445 1.8/1.0	↑93368 5.1/1.0	↑126654 1.2/1.0
Ribulose bisphosphate carboxylase large chain	52.5/6.0	↑237323 1.2/1.0	↑158027 2.5/1.0	↑177026 1.7/1.0	↑66314 5.3/1.0	↑151118 1.2/1.0
Ribulose bisphosphate carboxylase large chain Fragment	14.0/4.4	_	↑108750 2.5/1.0	_	↑45217 5.1/1.0	_
Ribulose bisphosphate	19.9/8.8	↓5729	↑16220	↑2566	↑1388	↑1749

carboxylase small chain		0.9/0.6	1.2/0.61	2.3/1.0	5.9/1.0	1.9/1.0
Ribulose bisphosphate carboxylase small chain 1 chloroplastic	20.0/8.9	↓9204 0.9/0.35	↓10164 0.9/0.24	_	_	↓1753 0.8/0.11
Ribulose bisphosphate carboxylase small chain 4 chloroplastic	20.0/8.9	↓5729 0.9/0.16	↑5300 1.1/0.66	_	_	↓1613 0.8/0.07
Rubisco activase	48.2/6.2	↑33644 2.0/1.0	↑18495 1.3/1.0	39365 1.9/1.0	↑14025 4.2/1.0	↓18941 0.6/0.0
Primary metabolic pathway						
Aminomethyltransferase	44.3/8.5	↑2601 1.3/0.99	_	_	_	↑1696 1.2/0.95
ATP synthase beta subunit Fragment	50.3/5.1	↑6296 2.0/1.0	↑11924 1.7/1.0	↑5662 1.5/1.0	↑6159 4.7/1.0	↑11871 2.1/1.0
ATP synthase subunit alpha	55.7/5.0	↑7656 1.6/1.0	↑10424 2.2/1.0	↑7495 2.5/1.0	↑6537 6.0/1.0	↑11675 1.9/1.0
ATP synthase subunit alpha chloroplastic	55.6/5.0	↑11601 1.6/1.0	↑14108 2.2/1.0	↑10802 2.5/1.0	↑9794 6.3/1.0	↑8063 1.9/1.0
ATP synthase subunit beta	51.8/5.8	↑10089 1.4/1.0	↑14155 1.7/1.0	↑8370 1.4/1.0	↑8022 6.0/1.0	↑15625 1.5/1.0
ATP synthase subunit beta chloroplastic	53.6/5.1	↓43493 0.9/0.14	↑49288 1.7/1.0	↑47744 1.5/1.0	↑29516 5.2/1.0	↑43242 1.6/1.0
ATP synthase subunit beta Fragment	50.6/5.1	↑66797 2.1/1.0	↑69914 1.8/1.0	↑75907 1.5/1.0	↑50468 5.3/1.0	↑68048 1.6/1.0
ATP synthase subunit gamma chloroplastic Fragment	13.0/4.6	↑1778 1.7/1.0	_	_	_	_
AtpD Fragment	16.7/6.2	↑6296 2.0/1.0	↑11924 1.7/1.0	↑5867 1.5/1.0	↑6159 5.0/1.0	↑11871 2.1/1.0
F1 beta subunit of ATP synthase Fragment	9.5/9.6	↑20693 1.7/1.0	↑19323 1.9/1.0	↑22989 1.5/1.0	↑10667 6.3/1.0	↑13887 1.7/1.0
Fructose bisphosphate aldolase	38.4/6.3	↓31446 0.9/0.12	↑20581 2.3/1.0	↑28943 1.3/1.0	↑12609 5.6/1.0	↑16229 1.1/0.99

Glutamine synthetase	38.9/5.3	_	↑13192 2.3/1.0	↑11654 2.2/1.0	_	↑3889 1.8/1.0
Glutamine synthetase Fragment	46.4/7.7	_	↑9442 2.3/1.0	_	_	↑3146 2.0/1.0
Glyceraldehyde 3 phosphate dehydrogenase	46.7/7.5	↓12850 1.3/0.99	↑7972 1.9/1.0	↑15828 1.6/1.0	↑4500 4.8/1.0	↑13221 1.6/1.0
Malate dehydrogenase	36.1/8.2	↑6996 1.4/1.0	↑16976 2.4/1.0	↑17841 1.1/0.86	↑13488 1.5/1.0	↑18796 1.0/0.56
Malate dehydrogenase fragment	9.1/9.6	↑2604 1.2/1.0	_	_	_	↓6231 0.9/0.33
Methionine synthase	84.2/5.8	↑2810 1.2/0.98	_	_	_	_
Peroxisomal glycolate oxidase	40.7/9.4	↑21814 1.5/1.0	↑13510 2.2/1.0	↑18387 1.8/1.0	6159 2.6/1.0	↑5525 1.4/1.0
Phosphoglycerate kinase	50.2/8.7	↑8608 1.1/0.99	↑20416 2.3/1.0	↑21536 1.6/1.0	↑14884 4.0/1.0	↑20243 1.4/1.0
Phosphoglycerate kinase fragment	25.2/9.7	↑5694 1.5/1.0	↑3493 1.5/1.0	_	_	_
Phosphoribulokinase	45.2/5.6	↑1626 1.7/1.0	↑6838 2.1/1.0	_	↑3567 1.7/1.0	↑7058 1.2/1.0
Serine hydroxymethyltransferase	57.3/9.0	↑3319 1.5/1.0	↑2486 2.0/1.0	↑2268 1.7/1.0	_	_
Triosephosphate isomerase	32.5/6.2	↑2394 1.4/1.0	↑4179 1.5/1.0	_	_	_
Transcription and translation related						
Elongation factor Tu	52.1/6.2	↑3400 1.6/1.0	↑5031 1.8/1.0	↑4219 1.4/1.0	_	_
Elongation factor Tu chloroplastic	52.0/6.2	↑3418 1.5/1.0	↑3513 1.7/1.0	↑3438 1.5/1.0	_	_
Elongation factor Tu Fragment	37.9/4.8	↑1945 1.5/1.0	↑2620 2.4/1.0	_	_	_
Probable mediator of RNA polymerase II	71.1/5.1	↑1673 1.6/1.0	_	_	_	_

transcription subunit 37c						
Probable mediator of RNA polymerase II transcription subunit 37e	71.3/5.0	↑1626 1.6/1.0		_	_	_
Translation elongation factor TU Fragment	27.2/4.9	↑2680 1.8/1.0	↑2658 2.1/1.0	_	_	_
Genetic information processing						
BiP	73.5/4.9	↑1833 1.0/0.63	_	↑842 1.3/0.94	_	_
BiP isoform A	73.2/4.9	↑1509 1.1/0.91	_	↑860 1.4/0.99	_	_
BiP isoform B	73.4/4.9	↑1922 1.0/0.65	_	_	_	_
BiP isoform D Fragment	54.2/7.2	↑1865 1.0/0.82	_	_	_	_
Endoplasmic reticulum HSC70 cognate binding protein	73.5/5.0	↑1923 1.0/0.62	_	↑842 1.2/0.92	_	_
Heat shock protein 70	70.7/4.9	↑2417 1.6/1.0	↑1791 2.1/1.0	↑1548 1.2/0.98	_	
70 kDa Heat shock protein Fragment	61.4/4.9		_	↑2910 1.1/0.93	_	↑740 1.4/1.0
Signal transduction						
14 3 3 like Protein A	29.0/4.5	↑4010 1.2/0.92	_	_	_	_
14 3 3 Protein Fragment	7.5/3.8	↑5051 1.1/0.79	_	_	_	_
Carbon and Energy metabolism						
Catalase	44.6/6.1	↑5159 1.5/1.0	↑8043 1.5/1.0	↑4654 1.6/1.0	_	↓3362 0.9/0.44
Catalase 4	56.7/6.8	↑3256 1.5/1.0	↑6435 1.5/1.0	↑4654 1.6/1.0	_	↓2710 0.9/0.42
Catalse 1 2	56.8/6.8s	_	↑2748	_	_	↑1939

			1.3/0.98			1.1/0.74
Catalase fragment	35.7/6.4	_	↑3242 1.3/1.0	_	_	↑1220 1.1/0.75
Carbonic anhydrase	35.3/6.3	↑19826 1.2/1.0	↑21628 2.0/1.0	↑29318 2.1/1.0	↑16726 3.1/1.0	↑24811 1.1/0.99
Carbonic anhydrase Fragment	23.9/8.2	↑3367 1.1/0.92	↑4220 1.7/1.0	_	_	_
Others						
Actin 1		↑1046 1.6/1.0	_	_	_	_
Actin fragment		↑2920 1.6/1.0	_	_	_	_
SGF 14a		↑4830 1.2/0.92	_	_	_	_
SGF 14h		↑9176 1.1/0.97	_	_	_	_
TO48 2 fragment		↓5981 0.4/0.0	_	_	_	_
TO99 a1 fragment		↑10340 7.4/1.0	_	_	↑4364 12.4/0.89	_

Differential accumulation of pigeonpea proteins are indicated by up and down arrows after oral secretion treatment with their score and P values in Fs w1, Cp w2, Cs w3, CcVip and CcICPL. The protein names were identified against a customized database comprising of total uniprot protein list of *Glycine max*, *Cajanus cajan* and "stress related proteins". n/a indicates p values and fold changes are not applicable; ‾ is represented for the proteins that were not identified. P values are indicated as per the PLGS; P value ≤0.05 is significantly downregulated, and P value ≥0.95 is significantly upregulated.

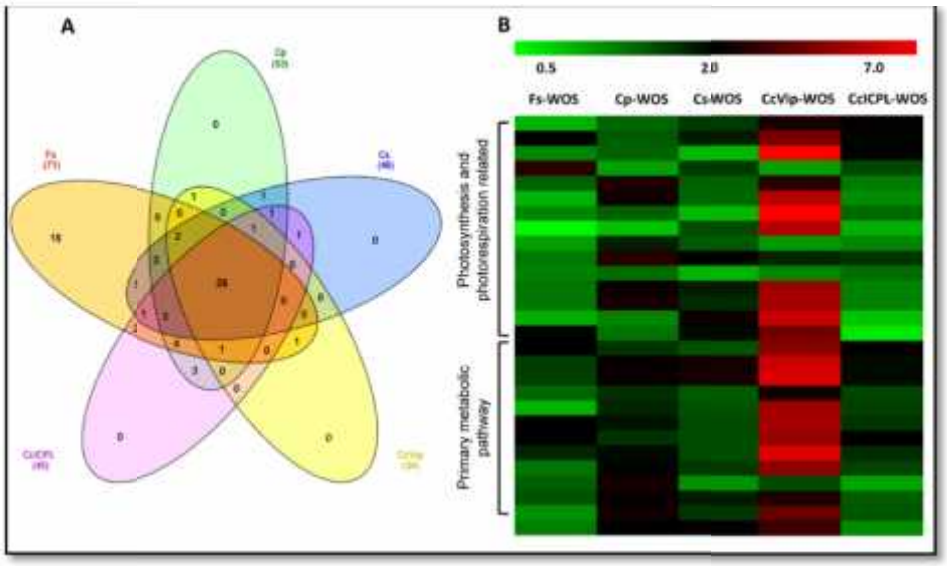


Figure 2.3: [A] Venn diagram summarizing the differentially expressed proteins between OS elicited wild relatives and cultivated varieties, created via InteractiVenn online tool [B] A heat map was generated for the commonly present proteins that are differentially expressed in all the OS elicited leaf tissue of wild and cultivated pigeonpea, followed by their categorization in photosynthesis and photorespiration and primary metabolic pathway.

Differential expression analysis exposed various uncharacterized proteins which were subjected to GO investigation revealed stress related categories such as jasmonic acid signalling, detection of biotic stimulus, MAPK cascade, and hydrogen peroxide regulatory mechanism in addition to their involvement in photosynthesis and primary metabolic processes (**Fig. 2.4 A, B**). In addition, Bancel et al (2015) explained that the occurrence of unidentified proteins is due to no match of tandem mass spectrometry generated peptides to the proteins in the database of closest species. As single amino acid change in the peptide can change its mass and resulting fragmentation pattern (**Bancel et al., 2015**). Thus it can be assumed that proteins which are still not characterized could have essential role in defense responses which require more focus in further studies.

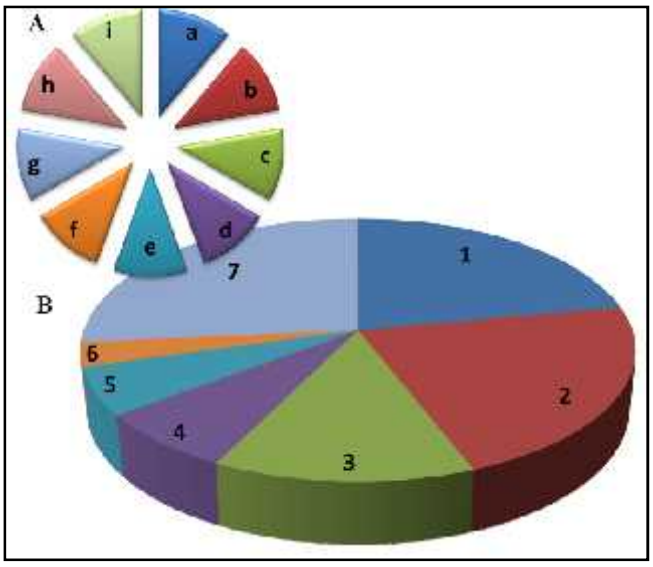


Figure 2.4: Categorization of WOS elicited uncharacterized proteins in their biological processes [A] Figure shows biological processes of the uncharacterized proteins showing exactly similar pattern in wild *F. stricta* , *C. platycarpus*, *C. scarabaeoides* and cultivated plants *C. cajan* ,vipula and ICPL 87 and hence represented by one pie chart biological processes are: a) Defense response to fungus [GO:0050832], b) Detection of biotic stimulus [GO:0009595], c) Jasmonic acid mediated signaling pathway [GO:0009867], d) MAPK cascade [GO:0000165], e) Negative regulation of defense response [GO:0031348], f) Protein targeting to membrane [GO:0006612], g) Regulation of hydrogen peroxide metabolic process [GO:0010310], h) Regulation of multi-organism process [GO:0043900], i) Regulation of plant-type hypersensitive response [GO:0010363]; [B] uncharacterized proteins showing biological processes pertaining to: 1. Photosynthesis, light harvesting [GO:0009765], 2. Protein-chromophore linkage [GO:0018298], 3. Photosynthesis [GO:0015979], 4. Photosystem II stabilization [GO:0042549], 5. ATP synthesis coupled proton transport [GO:0015986], 6. Carbon fixation [GO:0015977], 7. Others shown using an example of one plant, *F. stricta*

2.2.3 Patterns of Protein Accumulation Upon Elicitation in Pigeonpea by 2D DIGE

Vipula, a tolerant cultivar against *H. armigera* of pigeonpea showed greater fold changes as compared to the susceptible and wild relatives of pigeonpea. Hence, to validate LC-MS/MS data, Vipula plants were subjected to four elicitation treatments

including insect feeding, MeJA spray, WOS and WW while untreated plants were used as respective control. In total 42 protein spots were identified on 2D DIGE (**Figure 2.5**).

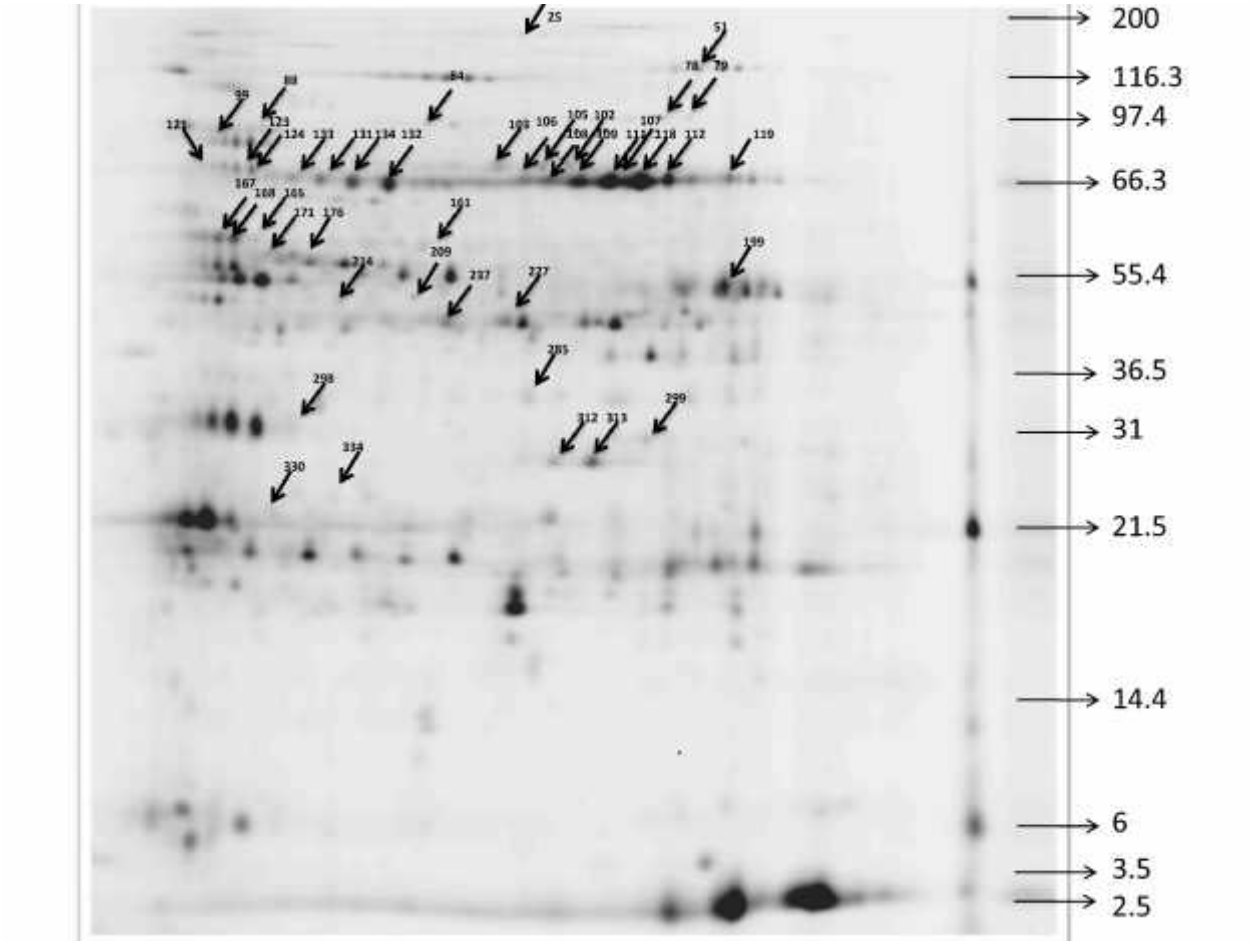


Figure 2.5: A representative image of 2D DIGE gel showing protein spots differentially accumulated in pigeonpea plant aerial shoot upon induction.

Spots showing over 1.5-fold changes were identified by MALDI-TOF-TOF. Of 42 proteins, 26 were upregulated upon insect feeding, 22 in MeJA, 16 in WOS and 3 in WW. Among these, 9, 10 and 4 proteins were unique to larval feeding, MeJA and WOS treatments, respectively (**Figure 2.6A**). Interestingly (spot 51, 99 and 78/79 corresponding to 5-methyl tetrahydropteroyl triglutamate-homocysteine methyltransferase, ATP synthase CF1 alpha subunit, and NADP dependent malic enzyme were identified from Vipula shoot tissue exposed to with larval infestation but could not be detected in other elicitation treatments (**Table 2.3**). Conversely spot 25-OSJ_04535 a hypothetical protein, spot 176-glutamine synthetase precursor, spot 123-

UDP glucose phosphorylase and spot 334-ferritin-1, choloroplastic were detected in WOS but not upon larval feeding. Catalase (spot 119) and tubulin (spot 121) were present in both the treatments of which catalase showed almost equal accumulation but tubulin was more abundant in WOS. Protein spots of Rubisco were detected in all the four treatments, however, the protein spots corresponding to its subunits were found to be differentially accumulated as shown in Table 2. Larval damaged and MeJA leaves shared protein spots of BAHD acyl transferase DCR like - (spot 106), ATP synthases - (spot 124) and glyceraldehyde-3-phosphate dehydrogenase - (spot 199). Fructose bisphosphatealdolase and S-adenosylmethionine synthase 2 corresponding to (spot 237 and spot 161) were significantly upregulated only in MeJA. Another 4 protein spots (131 to 134) were uniquely over expressed in MeJA that were identified as ATP synthase beta subunits. Protein spot 298, LHCII type I chlorophyll a b binding protein was found commonly in MeJA and WOS with greater upregulation in latter treatment. These differential responses suggested specificity of plant molecular responses to various elicitation treatments. GO analysis of all the characterized proteins were carried out revealing their involvement in photosynthesis and primary metabolic processes (**Figure 2.6B**).

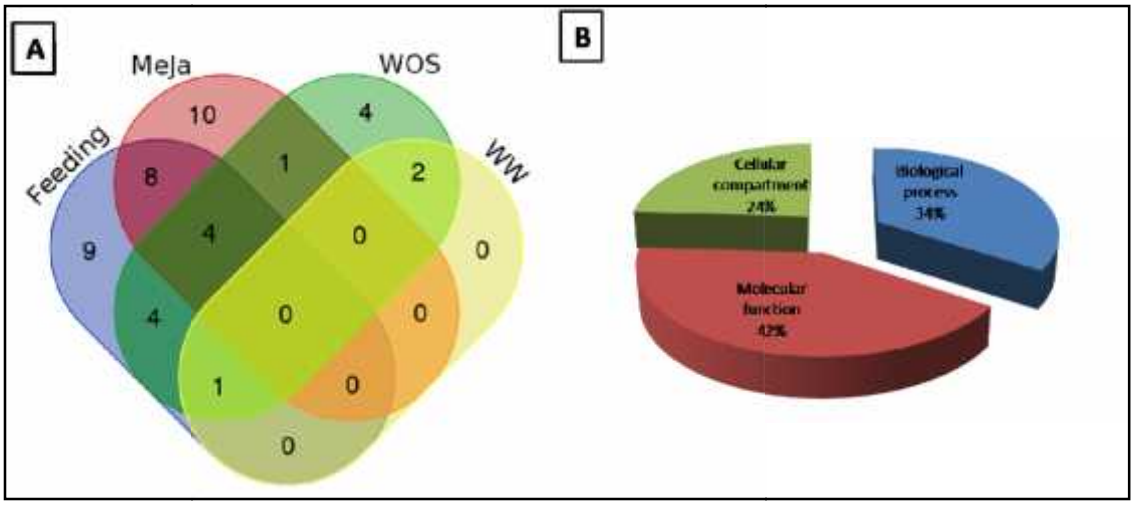


Figure 2.6: [A] Venn diagram to compare the differential expressed proteins among different treatments [C] Gene ontology categorization of total proteins identified by 2D DIGE technique.

Table 2.3: Protein spots accumulated differentially in treatments are shown here along with their p values; '–' is represented for the proteins that were not identified.

Spot No.	Accession No.	Name of Protein	Feeding Fold change/ P value	MeJA Fold change/ P value	W+OS Fold change / P value	W+W Fold change/ P value
25	gi|125573095	hypothetical protein OsJ_04535 [Oryza sativa Japonica Group]	_	_	3.6/0.2	1.6/0.3
51	gi|8134568	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	1.6/0.005	_	_	_
78/79	gi|115420	NADP-dependent malic enezyme	1.6/0.006 1.7/0.001	_	_	_
84	gi|224140653	predicted protein [Populus trichocarpa]	_	1.8/0.04	_	_
88	gi|2506277	RuBisCO large subunit-binding protein subunit beta, chloroplastic	_	1.5/0.02		
99	gi|169794058	ATP synthase CF1 alpha subunit	1.5/0.01	_	_	_
102	gi|1000936	Ribulosebisphosphate carboxylase	1.5/0.005	2.1/0.02	_	_
103	No significant match		1.7/0.0006	1.5/0.02		
105	gi|11587	unnamed protein product	1.8/.001	1.9/0.03	_	_
106	gi|502163420	BAHD acyltransferase DCR-like	1.7/0.007	1.6/0.01	_	_
108	gi|168312	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	1.6/0.01	1.8/0.004	_	_
109	gi|1304257	ribulose-1,5-bisphosphate carboxylase large subunit	1.7/0.02	1.9/0.01	1.6/0.1	_
111	gi|40647581	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	2.3/0.002	2.1/0.02	1.8/0.007	_
112	gi|1000936	ribulosebisphosphate carboxylase	2.1/0.008	1.6/0.008	1.7/0.03	_
118	gi|6688796	Ribulose-1,5-bisphosphate carboxylase/oxygenase	3.0/0.001	1.6/0.03	1.7/0.04	_
119	gi|16215	catalase	1.5/0.04	_	1.5/0.04	_
121	gi|303842	beta-tubulin	1.5/0.07	_	2.6/0.04	_
123	gi|17026394	UDP-glucose pyrophosphorylase	_	_	1.5/0.01	_
124	gi|114421	Full=ATP synthase subunit beta, mitochondrial	1.5/0.005	1.5/0.001	_	_
131/132/133/134	gi|3850914	ATP synthase beta subunit	_	1.5/0.003 1.9/0.0006	_	_

				1.6/0.009 1.9/0.00007		
161	gi|127046	S-adenosylmethionine synthase 2	_	1.5/0.0007	_	_
165	gi|255641658	unknown	1.5/0.001	_	_	_
167/168	gi|7960277	ribulose bisphosphate carboxylase activase B	1.7/0.002 1.6/0.001	_	_	_
171	gi|255641658	unknown	_	1.5/0.001	_	_
176	gi|13877511	glutamine synthetase precursor	_	_	1.5/0.01	1.6/0.04
199	gi|120666	Glyceraldehyde-3-phosphate dehydrogenase	1.8/0.01	1.5/0.02	_	_
214	No significant Match		1.6/0.01			
227	gi|224061310	predicted protein		1.5/0.001		
237	gi|22633	fructose-bisphosphate aldolase	_	1.5/0.0006	_	_
285	No significant match		_	_	3.7/0.01	_
298	gi|9587205	LHCII type I chlorophyll a/b-binding protein	_	2.0/0.02	2.7/0.09	_
299	gi|1000936	Ribulosebisphosphate carboxylase	1.7/0.0004	_	1.7/0.07	1.6/0.2
312	gi|131899	Ribulose bisphosphate carboxylase large chain	3.0/0.006	_	1.8/0.1	_
313	gi|6525083	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	2.5/0.02	_	1.8/0.009	_
330	No significant match		_	_	1.7/0.07	_
334	gi|120532	Ferritin-1, chloroplastic	_	_	2.0/0.01	_
442	gi|42541536	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	_	_	1.5/0.01	_

2.2.4 Potential Defensive Role of the OS Elicited Proteins

Proteins that were upregulated belonged to photosynthesis and primary metabolism pathways. We identified RCA that was differentially accumulated in wild and cultivated pigeonpea plants. RCA is a specific catalytic chaperone that regulates Rubisco function of carbon assimilation by changing its activation state (**Spreitzer *et al*., 2002; Portis *et al*., 2003**). Two isofoms of RCA, α-RCA and β-RCA were also

differentially accumulated at different levels in the leaf tissue. These two isoforms were involved in ATP hydrolysis and Rubisco activation in soybean (**Yin *et al*., 2010**) which shared the closest sequence similarity with pigeonpea (**Varshney *et al*., 2012**). Upregulation of α-RCA isoform was shown in maize and rice upon heat stress. Studies have shown reduction in RuBPCase activity in *rca*-silenced plants leads to decreased reduction in assimilation of nitrate. Glutamine synthetase play important role in nitrogen assimilation and its upregulation was detected only in *C. platycarpus, C. scarabaeoides,* ICPL 87, suggesting their importance in efficient nitrogen utilization (**Miflin and Habash 2002**). However, their regulatory mechanism in OS elicited plants needs to be further studied and its substantial alteration could be an indication of its significant role in plant performance during herbivore attack. Results showed that RuBPCase large chain was over expressed in all the OS elicited plants.

Other photosynthetic protein such as chlorophyll a b binding protein and LHCII showed upregulation in *C. scarabaeoides*. OEE2, another photosynthetic protein was significantly down regulated in *F. srticta* and *C. platycarpus* and notably upregulated in *C. scarabaeoides* and Vipula. These changes might be enabling plants to tolerate herbivore attack by augmented photosynthesis and achieve compensatory growth employed together as a tolerance mechanism against herbivore infestation (**Gómez *et al*., 2012**). ATP synthase α and β subunit exhibited altered level in OS elicited tissues. ATP synthase functions to enrich cellular energy by producing ATP from ADP and phosphate. Increased in ATP synthase was evident upon induction by herbivore as shown in induced soybean tissues upon interacting with cotton worm (*Prodenia litura*) (**Fan at al. 2012**). As herbivore attack induces reconfiguration of resources and over expression of other enzymes, proteins and metabolites which require more energy to carry out these changes might act as a protective mechanism from insect attack (**Fan at al. 2012**). Other elicitated proteins upon OS treatment in pigeonpea were also detected upon herbivory in soybean belong to ROS and primary metabolism pathway, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), malate dehydrogenase (MDH) and phosphoglycerate kinase (PGK). GAPDH enzyme play catalytic role in glycolysis and suggested to be involved in ROS signaling in response to elicitation. Other studies reported that induction of GAPDH was increased in tobacco and chickpea transcripts under the influence of herbivory (**Giri *et al*.,**

2006; Singh *et al*., 2008). MDH is known to possess catalytic properties for reducing oxaloacetate to malate using NADPH and thereby regulating H_2O_2 levels in the cell is known (**Heyno *et al*., 2014**). Another protein identified as PGK is involved in generating ATP from ADP and is employed by the pathways such as glycolysis, gluconeogenesis and photosynthesis (**Banks *et al*., 1979**). Increased abundance of PGK was observed in salt stress but its role in herbivory needs to be studied further (**Manaa *et al*., 2011**). Other two proteins that were differentially accumulated in OS elicited plants were peroxisomal glycolate oxidase (GOX) and carbonic anhydrase. Both the enzymes though are unrelated, act antagonistically to each other. GOX is involved in the generation of H_2O_2 during photorespiration while catalyzing conversion of glycolate residing in peroxisomes to glyoxylate. However, carbonic anhydrase is known to limit oxidative stress and shown to be upregulated in resistant line of Arabidopsis against herbivory by *Plutella xylostella* (**Nyathi *et al*., 2006; Collins *et al*., 2010**). Enzymes involved in the generating and scavenging ROS showed differential accumulation which might suggest their involvement in enabling plants to tolerate or resist stress condition.

2.2.5 Comparison of Proteins Identified in LC-MS/MS and 2D DIGE

Several proteins accumulated at various levels in wild and cultivated pigeonpea. As cultivated varitety of *C. cajan*, Vipula showed upregulation in initial analysis, we used various time points upon elicitation and 2D DIGE approach followed by protein identification using MALDI-TOF-TOF to validate our data. Four treatments *viz.*, larval feeding, MeJA, WOS, WW were given to the plants and protein spots were analysed in shoot tissues harvested at different time points 24, 48 and 96 h. Only those spots were considered which showed upregulation $\geq$1.5-fold. Likewise identification of various 2D DIGE protein spots were revealed proteins corresponding to RuBisCO subunits, GAPDH, catalases, glutamine synthetase precursor, ATP synthase and its subunits, RCA and chlorophyll a b binding protein. Occurrence of upregulated spots varied in different treatments and time points. For instance various protein spots identified as RuBisCo and its subunits in larval feeding at 24 and 96 h. Similarly, GAPDH was detected in feeding and MeJA at 24and 96h, respectively but not found to be upregulated in WOS treatment. Additional proteins, which were not

detected in earlier analysis but found to be upregulated in specific treatments in 2D DIGE are also shown in the Table2. Proteins identified as homocysteine methyl transfease and *S*-adenosyl methionine synthase are involved in amino acid metabolism. These proteins were identified to be upregulated in *Nicotiana attenuata* upon interaction with *Manduca sexta* and suggested that they might have role in disturbing amino acid availability to the herbivore (**Giri *et al*., 2006**). Malic enzyme which showed upregulation at later time pointwas only detected in larval fed tissue catalyzes conversion of malate to pyruvate. Studies have reported its induction in poplar and rice after herbivory and suggested their possible role in regulating cellular redox state (**Schaaf *et al*., 1995; Pavel *et al*., 2012**). OS elicited and larval feeding leaves of Vipula showed over expression of proteins identified as catalase and β-tubulin. Catalase was also detected to be differentially expressed in other wild relatives as well as ICPL87 and plays protective role in plants by catalyzing decomposition of hydrogen peroxide. β-tubulin is one of the constitutively expressed protein but their differential expression was evident in soybean treated with elicitors to induce defense responses (**Hermsmeier *et al*., 2001**). Ferritin, belong to the family of iron storage protein was up regulated only in OS elicited tissue and been known to provide protection against oxidative stress (**Briat *et al*., 2010**). These proteins in concert might exert a significant effect on plant protection by induced resistance.

2.3 Conclusion

In this study, we investigated the proteomic changes in wild and cultivated pigeonpea plants elicited by various herbivore related treatments. Considering a common notion of wild relatives of pigeonpea being the significant source of resistance, it was observed that OS elicited greater abundance of several proteins as compared to its control. Plant undergoes a large-scale transcript changes producing proteins which might be playing significant role in plant tolerance. In this study, we identified various oxidative stress limiting proteins which help to maintain redox state of cell and protect nearby cells. Secondly, increasing photosynthetic efficiency combined with tissue regrowth post damage is the suggestive to restrict the herbivore attack by enhanced tolerance. LC-MS/MS protein identification was validated by more targeted approach, wherein Vipula was exposed to different elicitation treatments at different

time points. 2D DIGE analysis revealed several proteins were upregulated, many of which were identified to be consistent with previous study. This study provides a further scope to carry out proteomic study of wild relatives at various time points to understand the pattern of protein accumulation. Transcript profiling will further contribute to associate the link between induced transcript profile and actual protein expression.

2.4 Materials and Methods

2.4.1 Plant Material and Elicitors Preparation

Pigeonpea wild relatives, *C. scarabaeoides*, *C. platycarpus* and *F. stricta* were obtained from International crop research institute of the semi-arid tropics (ICRISAT), Hyderabad and cultivated varieties of *C.cajan* (Vipula and ICPL87) were obtained from Mahatma phule krishi vidyapeeth (MPKV), Rahuri. All the plants were grown in growth chamber for 40-50 days post germination with 16/8h (light/dark) photoperiod at 25^{o}C. These plants were subjected to mechanical wounding with and without larval oral secretion application. Fourth instar larvae fed on chickpea based artificial diet were used to collect oral spit which was diluted (1:5) using deionised water before instant application on plant wounds. Water application was also carried out after wounding which serves as a control of oral secretion. In addition, non wounded plants were also kept as control. In a separate experiment, Vipula variety of pigeonpea was grown in green house conditions maintained at 25^{o}C and 16/8h (light/dark) photoperiod with light supplied by mixed fluorescent-incandescent lamps at an intensity of 200 µmol quanta/m^{2} per second. 40-50 days old plants were elicited by insect feeding, Wounding with insects oral secretion, wounding with water and methyl jasmonate sprays with their respective controls. Plants were mechanically wounded and oral secretion, diluted to 1:5 was instantly applied. Methyl jasmonate was procured from Sigma-Aldrich Inc., Ontario CAN, plants were treated with a foliar spray of 100 µM methyl jasmonate (MeJA) in 0.01% ethanol and a control solution.

2.4.2 Plants elicitation

H. armigera insects were reared in laboratory conditions and fed on chickpea based artificial diet. Newly hatched neonates were allowed to feed on this diet till 2nd-3rd instar stage following which they were subjected to starvation for 3 to 4 hrs and then

left on pigeonpea leaves. 6 insects were left on each plant pot which was then covered by a percolated bag to avoid insect escape as well as facilitate gaseous exchange. Insects were allowed to feed for 24h before harvesting tissue at different time points 24, 48 and 96 h. For another treatment, oral secretion of 3^{rd}-4^{th} instar larvae were collected and snap freezed and stored at -80^{o}C for further use. Plants were also subjected to mechanical wounding using fabric pattern wheel and wounded along the leaf with 6 serrations per leaf. Tissues were harvested at three time points as above. Diluted oral secretion solution was applied on the wounds to mimic insect infestation. MeJA solution was sprayed on the plants and was kept in a separate chamber for 24 h to avoid volatile contamination to other plants. In a separated experiment, wild relatives and cultivated varieties of pigeonpea leaves were mechanically wounded to which diluted oral secretion was applied and a control treatment of wounding with water. Leaves were wounded along the leaf followed by further serrations on either side of the midrib at every hour till 4 hours after which tissue was harvested and snap frozen for further analysis.

2.4.3 Protein extraction, 2D DIGE gel and image analysis

Proteins were extracted by acetone/trichloroacetic acid method using 200 mg of freeze dried shoot tissue powder. Tissue was suspended in 20 mL 10% TCA/acetone solution and kept at -20^{o}C for overnight. Proteins were precipitated by centrifugation at 15000 × *g* for 10 min at 4^{o}C. Pellet was washed in 10 mL ice-chilled acetone four to five times by centrifugation at 15000 × *g* for 10 min at 4^{o}C until no chlorophyll was present in the acetone. Protein pellet was dried *in vacuo* and suspended in DIGE rehydration buffer (pH 10) containing (7M urea, 2M thiourea, 4% w/v CHAPS, 1% PI mix (GE Health-care)). Contaminants were removed from protein sample by using Clean-Up kit of GE Health-care and further solubilized in DIGE buffer. pH of protein solution was adjusted to 8.5 by adding 10 pH DIGE buffer in small aliquots and quantified using 2D- Quant kit (GE Health-care). 400 pmol/µL working stock of fluorescent CyDye (GE Health-care) solution was prepared by adding DMF and added to 50µg protein solution for labeling as per the instruction. Among all samples, half of the protein samples were labeled with Cy2 dye and half with Cy5. Internal standard was prepared by adding 25µg of all the protein samples and labeled with Cy2. Protein samples were prepared to run of 2D gels and each sample was prepared

containing protein mixture of sample 1 labeled with Cy3, sample 2 labeled with Cy5 and 50 µg of Cy2 labeled internal standard and so on. These protein mixtures were first separated by IEF at 20^{o}C using active rehydration five step protocol: 30V for 10 h, 500V to 500vhr, 1000V to 800Vhr, 8000V to 13,500Vhr, 8000V to 20,000Vhr, by loading samples on 18-cm, 3-10 pH non linear strip (GE Health-care). Following IEF, strips were equilibrated in freshly added 1% DTT containing SDS equilibration buffer (6M urea, 75mM Tris-cl pH8.8, 29.3% Glycerol (v/v), 2% SDS (w/v)) for 15 mins followed by strip equilibration in 2.5% iodoacetamide for another 15 min. Equilibrated strips were loaded on 12% acrylamide gel for second dimension separation on a constant watt which was set at 1watt/gel. After separation gels were scanned on DIGE scanner generating three images of each gel pertaining to for eg. sample 1, sample 2 and internal standard. Gels were further analysed on ProgenesisSameSpots software which revealed normalized volumes of all the spots detected and proceeded further for fold change calculation.

2.4.4 Spot digestion, in solution digestion, and protein analysis

Protein spots that were significantly upregulated ($P<0.05$) were manually cut from the preparative gels using gel picking tools (Harris Uni-Core^{TM}, with 2-mm and 0.75-mm diameter). Preparative gels only differs from analytic gels in two ways viz. 500 µg unlabeled protein was used for each preparative gel and secondly, gels were fixed in 10% acetic acid and 40% ethanol post run for 30 min followed by Coomassie blue G-250 staining for overnight. Trypsin digestion of spots and identification was carried out by Proteomic laboratory, University of York, according to **Ferry *et al.*, 2011**.

For in solution digestion experiment, TCA/acetone precipitation was carried out as detailed above and pellet was resuspended in 5 mL extraction buffer (0.1M Tris, 30 % sucrose, 50mM EDTA, 1% SDS (Sodium dodoceyl sulphate), 8% PVP (Polyvinyl pyrrolidone), 2% β- mercaptoethanol, pH adjusted to 8 by adding HCl). Protein samples were vortexed vigorously till pellet gets dissolved and centrifuged at 10000 RPM for 30 min at 4^{o}C. Debris was discarded and supernatant was treated with Trissaturated phenol in 1:1 ratio. Phenolic phase was removed and more extraction buffer without PVP was added in 1:1 ratio and centrifuges again. For protein precipitation, 3.5 volumes of chilled 0.1M ammonium acetate dissolved in methanol was added to the phenolic phase and kept at -20^{o}C for overnight. Solution was then

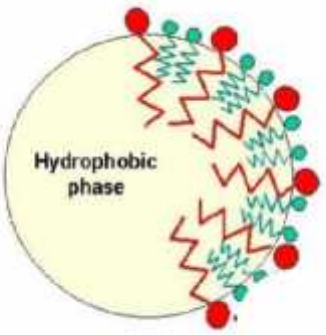
centrifuged at 8000 RPM for 15 min. and pellet was washed 2-3 times with chilled methanol and kept for drying. Dried pellet was treated with 20 µg surfactant *Rapi*Gest^{TM}SF (Waters). Protein volume was then made up to 200µL and kept for mild shaking at 37^{o}C to allow the pellet getting dissolved in surfactant. This was further centrifuged and supernatant was quantified by Bradford estimation. 100µg of protein was heat treated at 80^{o}C for 15 min after which 5 µL DTT (15.43 mg in 1 mL 5 mM Ammonium bicarbonate) was added and kept at 60^{o}C for 15 mins. Solution was further treated with 5µL iodoacetamide (36.95 mg in 1mL 5mM ammonium bicarbonate) and kept in dark for 30min. For trypsin digestion, 5 µg Promega trypsin was added to 100 µg protein and kept at 30^{o}C for overnight passed through proteins were treated mM and 20 µg trypsin was added and kept overnight. Reaction was stopped by incubating digested protein sample at 37^{o}C for one hour after adding 2 µL formic acid. Sample was centrifuges at 10000 x g for 10-15 min at 4^{o}C and supernatant containing peptide mixture was subjected to LC-MS/MS.

2.4.5 Identification of Proteins Using Mass Spectrometry

For LC-MS/MS, peptide mixture was diluted in 3% ACN containing 0.1% FA in 1:3 ratio and subjected to mass spectrometric analysis. 100 fmol of Saccharomyces enolase was used as an internal standard. Mass spectra were acquired in three technical replicates by using NanoAcquity UPLC system coupled to SYNAPT-HDMS (Waters Corporation, Milford, MA). The binary solvent system comprised of mobile phase A constituting 99.9% water and 0.1% formic acid and mobile phase B having 99.9% acetonitrile and 0.1% formic acid. Peptides injected were first subjected to concentration and desalting by passing through a symmetry C18 trapping column (180 µm x 2 cm) (Waters Corporation) using 0.1% mobile phase B at a flow rate of 5 µL/min. nano-LC separation was carried out using a BEH-C18 (1.7 µm x 75 µm x 250 mm) column (Waters Corporation) and peptides separation was achieved by applying a linear gradient from 3 to 40% B for 95 min at 250nL/min. Eluted peptides were transferred to NanoLockSpray ion source of mass spectrometer. MS run was performed in positive V-mode at a resolution of about 9000 full width half maximum (FWHM). MS/MS spectra was collected at 0.7-s intervalsin the range of 50-2000 m/z with alternating low (4eV) and high (15 – 40eV) collision energy. 600 fm/µL human Glu-fibrinopeptide B (m/z 785.8426)in 0.1% formic acid/acetonitrile (1:1 v/v) was

infused at a flow rate of 0.15 µL/min through the reference Nano Lock Spray source every 30s to compensate for the mass shifts in the MS and MS/MS fragmentation mode due to temperature fluctuations. MS data were collected by MassLynx Version 4.0 software. ProteinLynx Global Server Browser Version2.5.1 software (both from Waters) was used for further dataprocessing, identification, and database searches. The MS/MS data were searched against uniprot customized database containing protein sequences reported from *Cajanus* and *Glycine* along with stress responsive proteins. Search parameters uses one missed cleavage and fixed modification of carbamidomethylation of cysteins, and the oxidation of Met as variable modification. In another experiment, typtic digested protein spots were analysed on MALDI-TOF-TOF and peptide mass fingerprints were matched with NCBInr database using organism name Viridiplantae and the search engine was MASCOT. The search parameters used carbamidomethyl (C) as a fixed modification, oxidation (M) as a variable modification, and one missed cleavage.

Chapter 3



Development and characterization of microemulsion based proteinase inhibitor loaded formulation

Chapter 3: Development and characterization of microemulsion based proteinase inhibitor loaded formulation

3.1 Introduction

Enormous amount of resources are directed towards managing insect pests, which are considered as one of the major biotic factors contributing to reduced crop yields (**Mahr and Ridgway, 1993**). Conventional chemical pesticides are increasingly becoming ineffective due to resurgence of resistant pests (**Ahmad, 2007**). These pesticides also pose serious threat to environment and pose ecological hazard. During the last decade development of insect resistant transgenic plants were seen as important tool towards increased crop yields at relatively lower operating costs on pest management (**Christou *et al.*, 2006**). However, the emergence of resistance by insects and genetic modification of edible crops have limitation of acceptability, ethical and other regulatory issues (**Lövei and Arpaia, 2005; Lewis *et al.*, 1997**). Additionally, development of transgenics for a wide range of host plants is also a challenging and demanding task (**Sharma and Ortiz, 2000**). Moreover, insect co-evolution renders various pest controlling strategies to become ineffective thereby demanding a need for simultaneous exploration of novel plant protection strategies.

Plant proteinase inhibitors (PIs) are important naturally occurring compounds that have been recognized in the form of plant defense molecule(s) acting against insect pests (**Green and Ryan, 1972; Broadway and Duffey, 1986**). Several studies have revealed efficiency of PIs in retarding growth and development of *Helicoverpa armigera*, a polyphagous and devastating pest of more than 200 crop plants worldwide **(Harsulkar *et al.*, 1999; Tamhane *et al.*, 2005)**. PIs display inhibitory effect on insect gut proteinases leading to non availability of free amino acid thus creating a metabolic burden leading to no further feeding. In this study, PIs from *Capsicum annuum* were explored for their anti-metabolic effect having 1- to 4-inhibitory repeat domains (IRDs) specific for trypsin or chymotrypsin. Efficacy of PI was previously evaluated by introducing PI transgenics or feeding assays through PI incorporated artificial diet but have not been used as a spray formulation (**Dunse *et al.*, 2010; Joshi *et al.*, 2014; Tamhane *et al.*, 2007**). It can be assumed that with the readily available recombinant techniques for the large scale protein production, direct administration of CanPI-7 on plant surfaces may prove to be an efficient, eco-friendly

and practicable alternative to chemical pesticides and plant genetic modification approaches. However, efficient application of PI on leaf surfaces would require the use of a delivery vehicle, to achieve greater stability, efficacy and applicability.

Microemulsions (MEs) are thermodynamically stable isotropic mixture of water, oil and surfactant/co-surfactant with a diameter in the range of 10-140 nm (**Rao *et al.*, 2009**). These systems have been known to preserve the stability and enzymatic activity of protein and have also been previously explored for drug delivery applications (**Guto and Rusling, 2005; Heuschkel and Neubert, 2008; Lawrence and Rees, 2000; Kreilgaard *et al.*, 2000**). Change in the water and oil ratio allow microemulsion to pass through three systems viz. oil in water (o/w), water in oil (w/o) and bicontinuous microemulsions (BCMEs). In particular, BCMEs have both oil and water as a continuous domains interdispersed together with a surfactant at the interface. They possess wide range of solubilizing ability and a high capacity to load active ingredients of either polarity than in water and oil alone and thus offer an attractive delivery system (**Satishkumar *et al.*, 2008**). Additionally, features like low interfacial tension, fine droplet size and ability to disperse over a larger contact area can make them suitable delivery vehicles to achieve greater surface penetration (**Paul and Moulik, 2001**). Thus their use in field applications can be called as a viable approach alternative to synthetic pesticides. They are also simple to prepare using components that are environmentally compatible and provides flexibility to modify the system by changing its composition for enhanced suitability and applicability in desired environment. In general, penetration properties of BCME and their wide ranging solubilizing ability make them an ecologically benign system for the encapsulation of protein to achieve efficient delivery.

This study demonstrates the use of BCME in combination with recombinant CanPI-7 to evaluate its release in the crop plant for their protection against *H. armigera*. BCME phase was characterized by conductivity measurements and the stability of CanPI-7 was assessed in BCME by activity studies. BCME media delivered greater wettability and spreadability on leaf surfaces enabling encapsulated protein to cover greater surface area on the leaf. *In vitro* studies showed BCME encapsulated proteinase inhibitory activity against trypsin and *Helicoverpa* gut proteases (HGP). Importantly, similar results were demonstrated by feeding studies

revealing significant reduction in growth and development along with inhibition of gut proteases.

3.2 Results and Discussion

3.2.1. Preparation of BCMEs

Various microemulsions with apparent bicontinuous domains were prepared whose compositions are given in the table (**Table 3.1**). In general, three categories of the microemulsion systems were chosen to be tested on plant leaf surfaces: (i) the water, 1-pentanol, and tetradecane bicontinuous microemulsion with the ionic surfactant cetyltrimethylammonium bromide (CTAB BCME) that was reported previously for retaining protein stability and activity (**Guto and Rusling, 2005**); (ii) 1-propanol, water, soyabean lecithin, and limonene bicontinuous microemulsion (lecithin BCME), of which the latter two components were derived from plant (**Papadimitriou *et al.*, 2008**); and finally (iii) 1-butanol and water stabilized by either Tween 80 (Tween 80 BCME) or 2-propanol (WIB BCME), that were chosen for their high water content (**Gu *et al.*, 2006; Wang *et al.*, 2008**). Except for CTAB and 2-propanol, the rest of the ingredients in all the four microemulsions were from the approved list of EPA for their environmental safety.

Table 3.1: Composition of BCMEs along with their ratios.

CTAB BCME	CTAB: 1-Pentanol: Water: Tetradecane (17.5: 35: 35: 12.5)
Lecithin BCME	Lecithin: 1-Propanol: Water: Limonene (19:19:5:57)
Tween 80 BCME	Tween 80: 1-Butanol: Water (35:10:55)
WIB BCME	Water: Isopropanol: Butanol (55:35:10)

3.2.2 BCME Effect on Chickpea and Tomato Leaf Surfaces

It is of primary relevance to screen chemical components of any pesticidal formulation to be environmentally compatible and examine their effect on plants. Selected BCMEs was assessed for their consequence on crop leaf surfaces initially by visual inspection (**Fig. 3.1**). Images were captured for the effect of BCMEs tested on chickpea and tomato and compared with control leaves (**Fig. 3.1A, F**). BCMEs used were CTAB BCME (**Fig. 3.1B, G**), lecithin BCME (**Fig. 3.1C, H**), Tween 80 BCME (**Fig. 3.1D, I**) and WIB BCME (**Fig. 3.1E, J**). CTAB, lecithin and Tween 80 BCMEs started showing their effect on plants in just few hours after application by causing leaf damage in the form of darkening, curling, dehydration and wilting of the leaves. On the other hand, application of WIB BCME to both chickpea and tomato leaf surface shows its uniform spreading and rapid evaporation, leaving no visible changes on the leaf surface or plant development. These features promote WIB BCME for its utilization as a delivery media by CanPI-7 incorporation to be used for further investigations. Presence of relatively high alcohol content in the WIB BCME, it is also essential to understand the effect of individual components on the leaf surface. Treatment of 1-butanol (100%) caused immediate darkening and eventual wilting of the leaflets. Whereas, application of 2-propanol did not yield any significant visual change in the leaf. In addition, individual components were applied at the concentrations present in WIB BCME and reveal no noticeable damage to the leaf. This can possibly be attributed to the fact that the WIB BCME has higher water content in comparison with the other systems evaluated. However, Tween 80 BCME, also having higher water content, but its causal effect in the form of leaf curling is due to the presence of surfactant in the microemulsion.

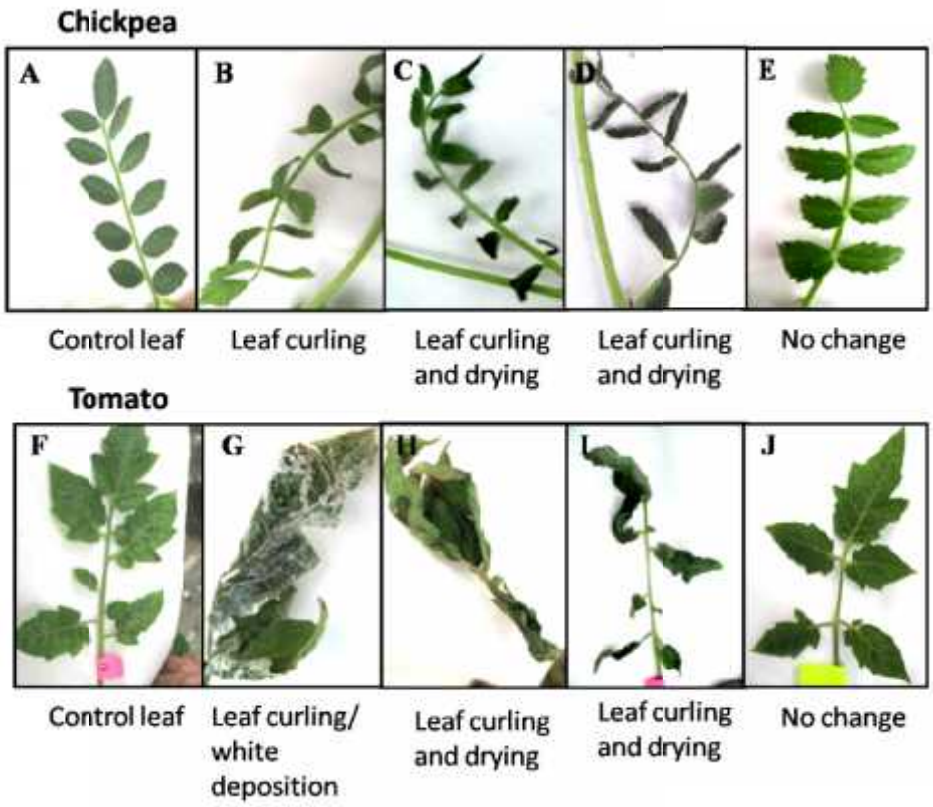


Figure 3.1: BCME sprayed chickpea and tomato leaves were compared between [A, F] Control leaf and leaves sprayed with respective BCMEs [B, G] CTAB [C, H] Lecithin BCME, [D, I] Tween 80 and [E, J] WIB.

3.2.3 Inhibitory Activity of CanPI-7 in BCMEs

To evaluate the retention of CanPI-7 activity in BCMEs, protease inhibition assays were conducted. The percentage protease inhibitor activity of CanPI-7 incorporated in all the BCMEs was determined spectrophotometrically. N -benzoyl-DL-arginine-p-nitroanilide hydrochloride (BApNA) was used as a substrate for trypsin and HGP to measure the inhibition of CanPI-7. The amide bond in BApNA is digested by proteases to yield N-α-benzoyl-DLarginine and p-nitroaniline (**Erlanger *et al.*, 1961**). The formation of p-nitroaniline, a yellow colored product, is proportional to the amount and activity of the protease present. Inhibition of the protease activity by CanPI-7 results in the decrease of spectral absorbance of the product formed. Protease inhibitor activity was assessed by colorimetric test in all the four BCMEs containing 1.5 µg CanPI-7 along with substrate and trypsin enzyme. Results revealed that the activity of PI was maintained in CTAB, Tween 80 and WIB based BCME but no

activity can be detected in Lecithin BCME. However, as spray of other BCMEs showed negative effect on plant surfaces, only WIB was used for further study. Around 90% inhibitory activity was observed in WIB BCME which was significantly higher than other BCMEs (**Fig. 3.2A**). Various range of CanPI-7 concentration was used against trypsin and HGP to compare their activity in WIB and buffer. At low concentration (0.3µg), PI showed higher inhibition of trypsin in WIB media (**Fig. 3.2B**). However at high CanPI -7 concentration, the activity difference in WIB and buffer was not significant.

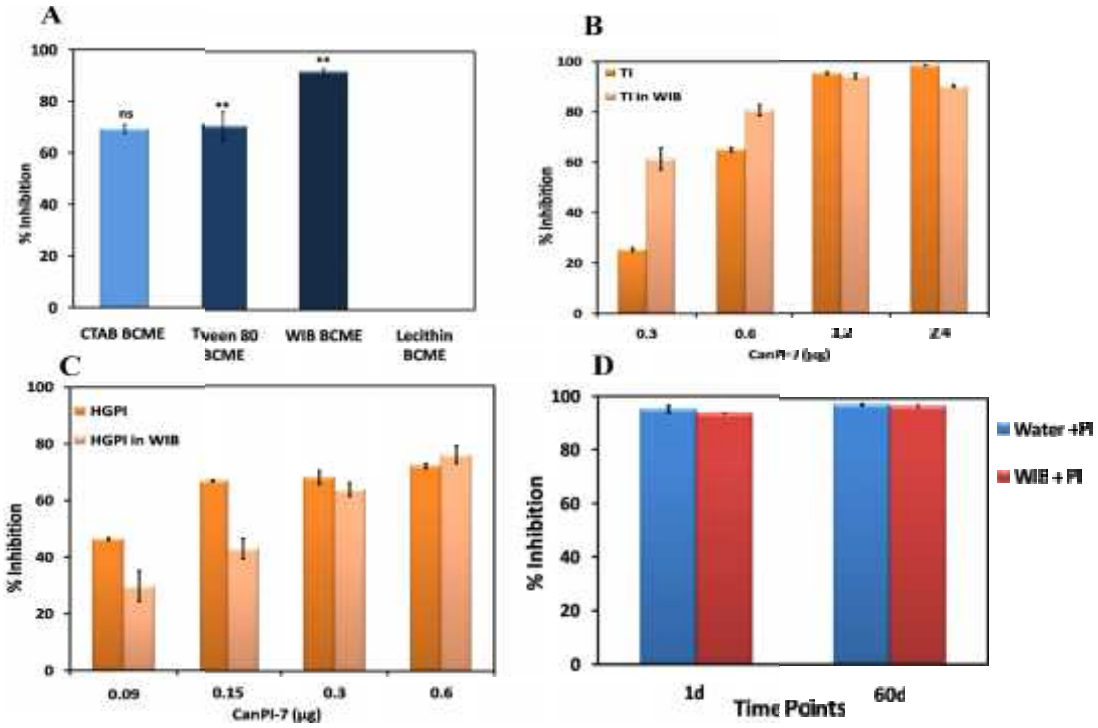


Figure 3.2: Figure shows activity of proteinase inhibitor in BCME(s) [A] % inhibition of proteinase inhibitor incorporated in all the four BCMEs were calculated (B,C) Increasing range of concentration of CanPI-7 was used to assess its activity against trypsin and HGP in WIB media [D] Stability determination by inhibitory activity of CanPI-7 at two intervals (1 d and 60 d) in water and WIB system, Standard error is calculated for n=3

HGPI activity was also assessed by taking ranges of CanPI-7 concentrations (**Fig. 3.2D**). At lowest concentration (0.09 µg) the inhibitory activity was seen more in buffer than WIB. However, with increasing concentration, the difference in inhibitory activity against trypsin and HGP in buffer and WIB was decreased and

hence can be assumed that the presence of solvents and surfactant in WIB is not interfering with the PI activity and thus can be characterized further for application purposes. It was observed that almost 90% of trypsin inhibition (TI) and 60% of the *Helicoverpa* gut protease inhibition (HGPI) were obtained by CanPI-7 in both water and WIB BCME-based assay. Low percentage of inhibition of HGP than trypsin can be explained by the existence of complex protease pool in the gut milieu which may have different specificities (**Mishra *et al.*, 2012**). This suggests that CanPI-7 was stable and active in the WIB BCME microstructure. However, it becomes important to also assess stability of protein incorporated in non native environment for longer duration to determine their shelf life which is an important feature for any formulation preparation. Stability of CanPI-7 was measured in terms of inhibitory activity after 60 days of their preparation and was found be same as compared to its activity to the first day of preparation (**Fig. 3.2E**). In addition, CanPI-7 formulation was also visually monitored for no phase changes in the microemulsion till 6 months stored at 25^{o}C. These results established the applicability of PI formulation on large scale using minimal resources for its delivery and achieving maximum efficacy.

3.2.4 WIB characterization

Phase diagram of the WIB BCME was previously investigated, and the bicontinuous region was reported to occur between 50–60% of water (**Wang *et al.*, 2008**). This information was used for the synthesis of WIB BCME system for CanPI-7 incorporation. Contact angle (θ) is defined as the angle formed at the intersection of the liquid curvature with the interface of the leaf surface and air. The ability of a liquid drop to wet and spread on a specific surface is indicated by values of θ <90^{o}. Values higher than 90^{o} indicates poor wettability and spreadability. Equal volumes of WIB BCME and water were placed on chickpea leaves and the contact angle measurements were taken. The contact angle values for the water and WIB BCME was observed to be 103.3^{o} and 31.4^{o} (**Fig. 3.3A**). Thus, it is established that WIB BCME enables greater spreadability on the hydrophobic leaf surfaces and lead to the deposition of active ingredient covering larger surface.

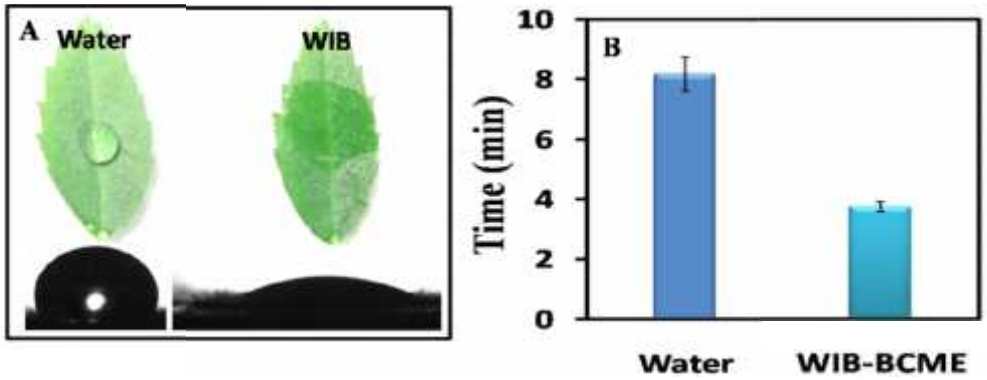


Figure 3.3: [A] Contact angle measurements were performed to evaluate the spreadability and wettability of water (103.3^{o}±0.75) and WIB BCME (31.4^{o}±1.71) on chickpea leaf and their images are given; [C] Evaporation efficiency of WIB was compared with water over time.

Time taken for complete evaporation of WIB was compared with water at 25^{o} C. It is essential to monitor evaporation of pesticide droplets on target crops to control their efficiency under varying conditions of humidity and temperature. Application of foliar pesticides and spray treatments needs to be optimized for their longer retention on target crops. Reduction in evaporation rate also facilitates greater absorption or penetration of active ingredient by the plant. Additionally, with decreased evaporation rate, possible runoff of large droplets can also be expected (**Ytl *et al.*, 2008**). Evaporation of 4 mg (w/v) of WIB and water was compared on chickpea leaf demonstrating faster evaporation of WIB which occurred in 4 min as compared to water (8 min) due to the presence of only 55% water in WIB (**Fig. 3.3B**). Thus to increase the evaporation rate, microemulsion media can be optimized by the addition of adjuvants to aid greater retention.

3.2.5 Study of Leaf Morphology Post WIB Application

To investigate the leaf morphological changes at microscopic level upon WIB application, environmental scanning electron microscopy (E-SEM) was performed (**Fig. 3.4**). The E-SEM images were captured for the control leaves (**Fig. 3.4A**) and WIB spotted leaves exposed to microscopic analysis at different time points after WIB application on the adaxial surface of the leaflet. Leaf structure displayed shriveling at 5 min (**Fig. 3.4B**). Whereas, at 24 h time point, the structure of the leaf returned in its normal state (**Fig. 3.4C**). It is likely that plant structure was regained

due to alleged rehydration of the epidermal cells to maintain turgidity. It is also likely that through these interactions and initial morphological changes, the WIB BCME is able to deliver the CanPI-7 in the trans-laminar region of the leaflets.

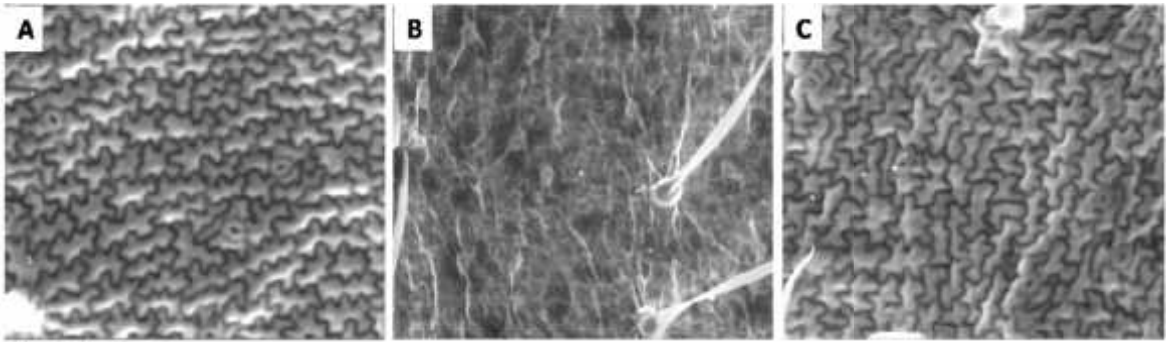


Figure 3.4 Morphological analysis of chickpea leaf surface after application of WIB BCME at different times [A] Control [B] 5 min [C] 24 h. Images were captured at magnification of 1000X and 100 µm scale.

3.2.6 Leaf Retention of CanPI-7 Incorporated in WIB-BCME

CanPI-7 incorporated in WIB BCME was also tested for ex vivo leaf stability and activity. In this experiment, CanPI-7 lodged on the leaf surface using water or WIB BCME was later recovered through water wash and quantified using the trypsin inhibition assay. Remarkably, inhibition activities measured from wash concentrate containing CanPI-7 recovered from WIB BCME spotted leaflets were three times less than the inhibition activity of the corresponding wash concentrate for the CanPI-7 recovered from water spotted leaflets (**Fig. 3.5A**). The activities determined at 3 h and 24 h time points did not have a significant difference indicating that the activity, and hence the CanPI-7 retention on leaf surface, was not influenced by the duration of contact of the CanPI-7 with the leaf surface in either cases. These experiments clearly indicate that when CanPI-7 incorporated in WIB BCME is placed on the leaf surface, permeation of CanPI-7 onto the leaf is taking place almost instantaneously and is further maintained (at least up to 24 h). Whereas with CanPI-7 aqueous solution, there was no detectable leaf permeation of CanPI-7 as the activity was almost completely recovered from the corresponding wash concentrate. CanPI-7 delivered using WIB BCME cannot be recovered from the leaflets even with extensive water wash unlike the aqueous counterpart. There is indeed a quantitative difference in the PI activities

recovered in both cases. The significant decrease in inhibition activity corresponding to lower amount of CanPI-7 recovered from the leaf could be attributed to two possible scenarios. Firstly, CanPI-7 is adhering strongly to the leaf surface/cuticle following the efficient delivery using WIB BCME in comparison with water. Secondly, it possibly traverses through the cuticle and epidermis and is irreversibly incorporated in the mesophyll cells or intercellular spaces. The wettability and spreadability of WIB BCME enable the distribution of CanPI-7 more evenly over a large leaf surface possibly leading to greater retention and permeation. The E-SEM images indicating that the WIB BCME interacts with the cuticular waxy layer on the epidermis of chickpea leaf also strengthen the hypothesis that it might be facilitating intralaminar PI delivery. This type of foliar uptake is also important as it minimizes the toxic effects of a pesticide on off-target organisms and further increases the efficacy of the pesticide formulation. To validate protein retention on leaf, similar study was conducted using trypsin. 5µg trypsin was incorporated in 10 µl WIB and water and spread on chickpea leaflets in replicate of 10 and allowed to dry. After 3 h, leaflets were washed extensively and protein amount was estimated by Bradford assay (**Bradford 1976**). Trypsin units obtained in the water wash of WIB and water spread leaf showed significant variation accounting to 0.02 units in WIB-trypsin leaf wash against 0.18 units in water-trypsin leaf wash (**Fig. 3.5B**). This confirms that trypsin with molecular weight of 23.3 kDa also exhibited significant retention on leaf surface.

In gel visualization of protease inhibitory activity was carried out in the leaf wash of WIB-PI and water-PI painted leaves using gel X-ray contact print technique. Equal amount of leaf wash were loaded on native-PAGE after which gels were treated with trypsin and exposed to gelatin coated x-ray film for various time points. Localized inhibition of trypsin digested gelatin was visualized due to the presence of PI. It was observed that the intensity of the bands were high in water-PI leaf wash as compared to WIB-PI leaf wash (**Fig. 3.5C**) due to the presence of more amount of PI in water-PI wash.

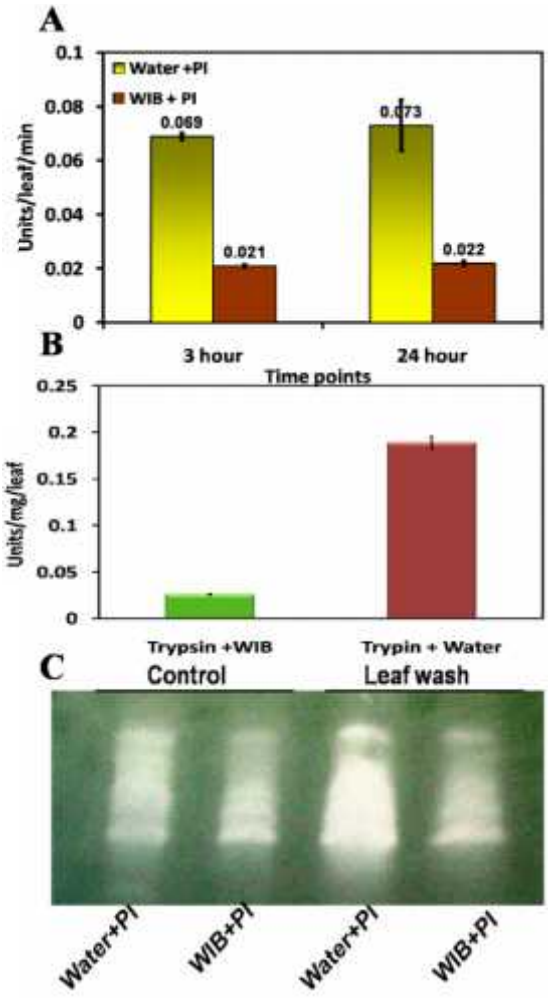


Figure 3.5: Protein stability and retention on leaf surfaces after incorporating in WIB was assessed [A] leaves spotted with WIB-PI and water-PI were washed after 3 and 24 h to determine retained trypsin inhibitory activity. PI activity was recovered expressed in units/min/leaf and 0.07 units of PI were recovered from water-PI leaf wash as against 0.02 from WIB-PI leaf wash [B] Typsin incorporated in WIB and water was spotted on leaf and its leaf wash was further examined to estimate protein amount in terms of units/mg/leaf. 0.02 units in WIB-tyrpsin leaf wash were measured as compared to 0.18 units in water –trypsin leaf wash. Standard error bars are shown at appropriate places for n=3.

3.2.7 *In vivo* Evaluation of WIB-PI Formulation on *H. armigera*

To investigate the *in vivo* effect of WIB-PI formulation on the growth and development of larvae, feeding studies were carried out. Chickpea plants were treated with WIB-PI with a concentration of 0.8HGPI units per leaf and allowed to dry for 6

h. 5 neonates were left on each leaf and 30 insects were used per replicate. Appropriate controls were used which includes WIB treated and control (without treatment) leaves. Assay was carried out for 6-8 days until neonates have metamorphosed to 2nd instar larvae in control leaves (**Fig. 3.6A**). Larval mass of the insects fed on different diets were measured and observed to be significantly reduced in CanPI-7 containing diet exhibiting 20-25 mg weight as compared to WIB painted (P value≤0.05) and control plant (P value≤0.01) showing an average of 35 and 59 mg of weight respectively (**Fig. 3.6B**). Interestingly, significant variation in the larval mass of insects fed with WIB and control leaves were observed. Although microscopy analysis reveals no alteration on the leaf surfaces, it reveals that insects are sensitive to the spray of WIB even after 6 h and hence their feeding on WIB leaves is limited. Further investigation to understand this differential behavior of insect towards WIB and control leaves is required. Survival rate of insects were also observed to be affected significantly in WIB-PI leaf fed insects revealing 30% insect survival. Whereas, control plants displayed highest survival rate accounting to approx. 90% and 60% in WIB leaf fed insects (**Fig. 3.6C**). As number of survived insects were found to be less in WIB treatment, it is likely that spread of WIB on leaf have deterred insects for further eating which has affected insect survival rate. To validate this, detailed analysis of physiological status of insects upon WIB feeding needs to be assessed. A representative image of insects fed on control and WIB-PI fed diet is shown (**Fig. 3.6D**).

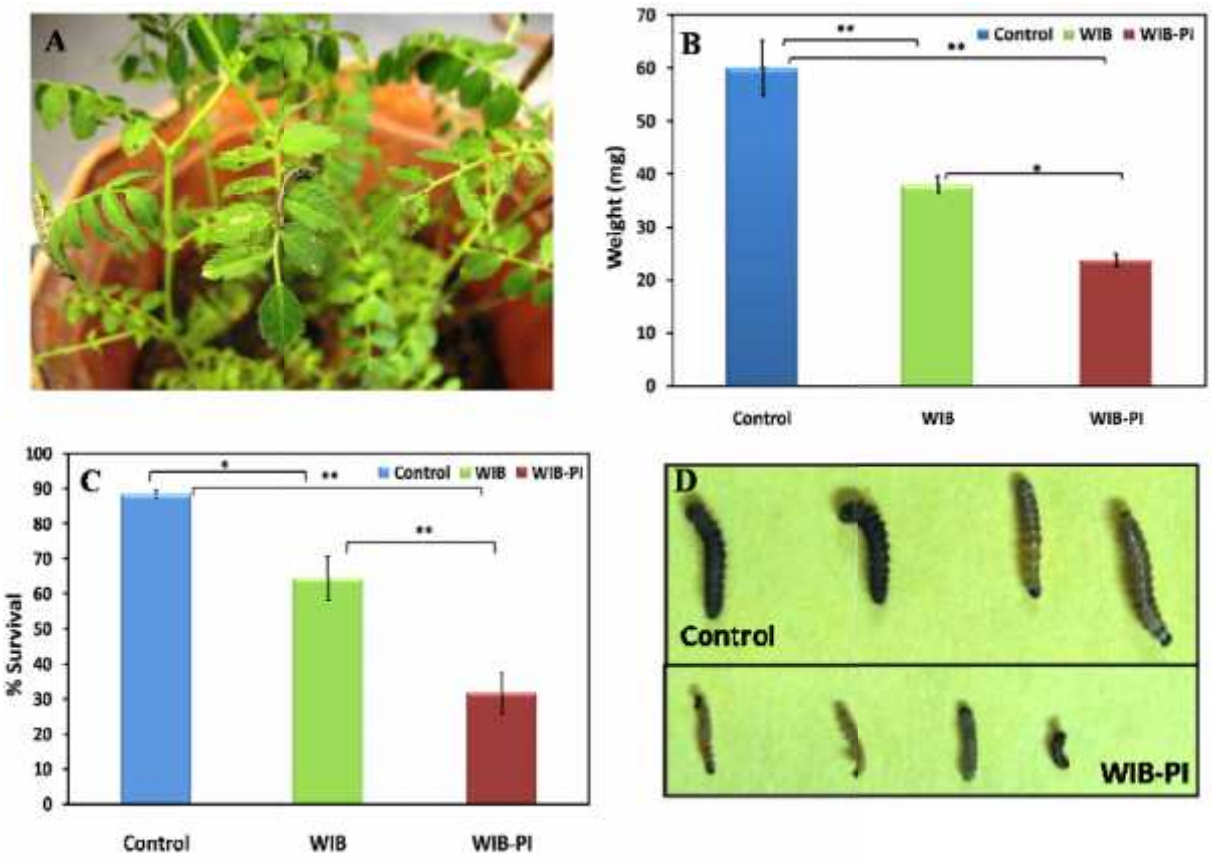


Figure 3.6: [A] A representative image of insect feeding on chickpea plant is shown, [B] Neonates were subjected to three different treatments viz. WIB-PI treated chickpea leaves, WIB treated leaves and control leaves without any treatment and their larval mass was measured. Experiment was conducted in three replicates each with 30 insects. Significant variation among the treatments are measured by their P values and shown in the figure using relevant star sign [C] Number of survived insects were shown in percentage by counting the survived number of insects with initial number of insects that were used for each treatment. Significance is shown. [D] Representative image of insect fed on control and WIB-PI diet is given. (P>0.05=ns; P≤ 0.05=*; P≤ 0.01= **; P≤0.001***)

3.2.8 Evaluation of Gut Proteases and HGP Inhibition

Gut proteases extracted from larvae fed on different treatments were used to resolve in activity polyacrylamide gel (PAGE). Protease activity was visualized by exposing gel to gelatin coated X-ray film for time points ranging from 20 min to 2 h. Time point showing best image were selected and analysed for the protease activity bands. Gut proteases of the insect fed with control diet showed largely 6 protease band which

can be distinctly seen to be diminished in CanPI-7 fed larvae gut (**Fig. 3.7A**). Previous studies have also shown that insect gut proteases were inhibited when fed with CanPI-7 and other isoforms of CanPIs (**Tamhane *et al.*, 2007, Mishra *et al.*, 2010**). Interestingly, inspite of decreased weight and survival of insects in WIB control, protease bands in WIB fed larvae did not show much variation as compared to control. Hence, it is again validated that WIB does not interfere with the gut protease activity. This was also confirmed by estimating BAPNase activity in terms of HGP unit which did not reveal any variation among control and WIB, whereas protease activity is reduced significantly in WIB-PI fed larvae (**Fig. 3.7B**). Induction of insensitive proteases by insects was also reported as a defense mechanism but such observation was not found in this study. Newly hatched neonates were fed on WIB-PI and WIB painted chickpea leaves to determine the patterns of protease inhibition. Protease activity gel displayed intense band in control fed larvae as compared to WIB-PI fed insect gut proteases. As studies showed the overexpression of proteases or production of insensitive proteases upon encountering with PIs, targeting insect at neonatal stage could be useful in order to delay the resistance. It is possible to extend feeding studies till 4^{th} instar larval stage or one generation to understand the prolonged effect of WIB-CanPI-7.

It is evident that *H. armigera* changes its gut complement while feeding on diets differ in their nutritional content (**Sarate *et al.*, 2012**). Thus, *in vitro* analyses were conducted on gut proteases of insects fed on artificial and natural diets. In general, gut proteases were extracted from 4^{th} instar larvae fed on chickpea based artificial diet, chickpea, okra, maize, marigold and sorghum. Activity of CanPI-7 against artificial diet and okra showed highest inhibition of gut proteases followed by marygold and so on. Insects upon exposure to different nutritional components diet exhibit physiological diversity and complexity as previously stated (**Sarate *et al.*, 2012**). These changes enable insect to adjust with the plant type and obtain more nutrition. In addition, to deal with plant defense system, insect pursue modifications in its feeding habits as well as physiological changes.

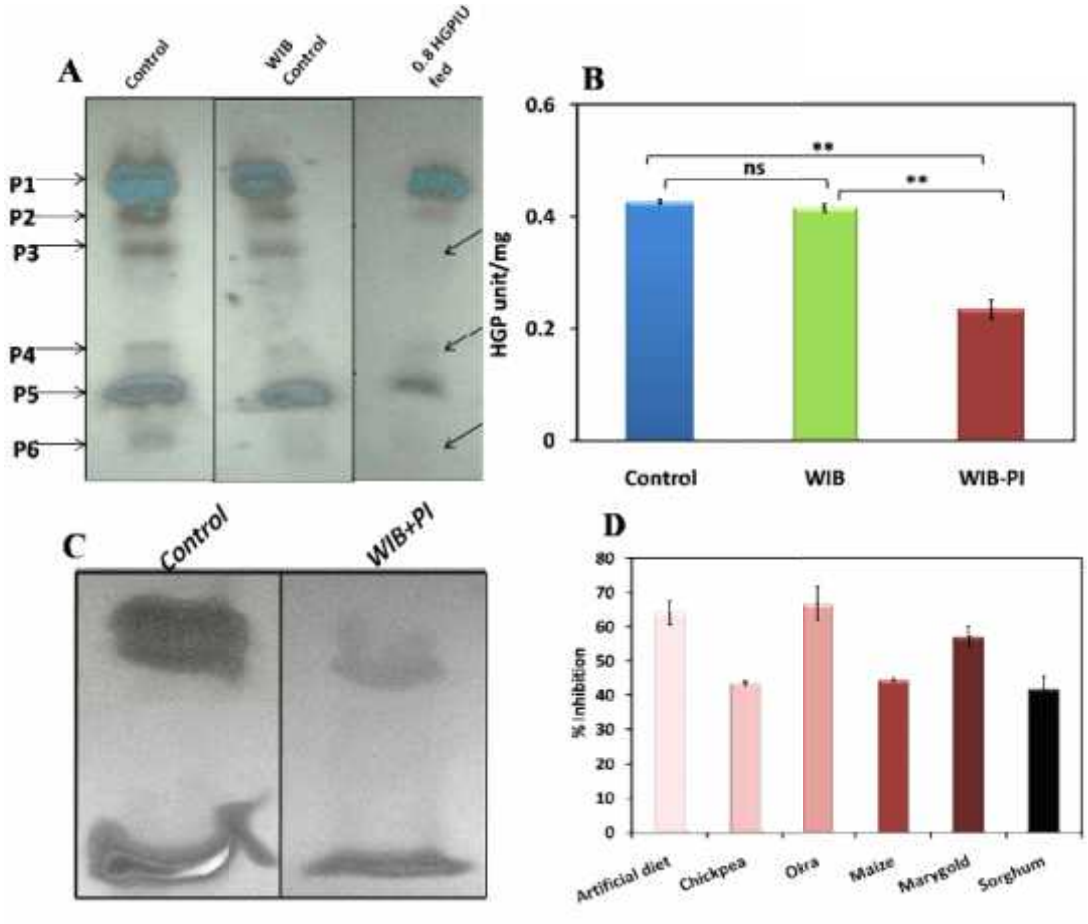


Figure 3.7: [A] gut proteases from insects fed on control and WIB-PI containing 0.8 HGPI unit diets were extracted and loaded on activity gel for protease activity visualization by gel x-ray film contact print technique, arrows shows the protease bands diminished in PI fed insect, [B] HGP BAPNase activity was estimated in CanPI-7 fed insects and their respective controls, [C] neonates subjected to control and WIB-PI diet were used for protease activity determination, [D] HGP extracted from 4th instar larvae fed on various diets were treated *in vitro* with CanPI-7 to determine its activity against complex and variable get proteases.

3.3 Conclusion

In conclusion, we have demonstrated the optimization and translation of a surface chemistry approach toward protein delivery in a biopesticidal application. The delivery of CanPI-7 incorporated in WIB BCME enabled its efficient leaf permeation and enhanced retention for crop protection against insect pests. The bicontinuous aqueous domains in the microemulsion preserve the inhibition activity of PIs on

leaves as well as upon storage for several months. Wettability and spreadability of BCME enable greater surface retention of the PI on the hydrophobic leaf surfaces. The microemulsion seems to reversibly alter the leaf surface through self repair to enable deeper penetration of the CanPI-7. The WIB BCME itself has no adverse effects on the growth of the chickpea plant. BCME-enabled encapsulation and delivery opens newer avenues for protein and peptide delivery, previously limited to reverse micellar systems (**Karpe and Ruckenstein 1991; Bauduin *et al.*, 2005; Rodakiewicz-Nowak and Ito 2005; Dasgupta *et al.*, 2005; Majumder *et al.*, 2005; Uda *et al.*, 2011**). The bicontinuous domains have been previously reported to stabilize proteins and enzymes and have been envisaged as media for enzymatic reactions. This study establishes that the excellent interfacial properties of the BCME enable them as delivery vehicles for encapsulations of biocolloids stabilized by the water-rich domains. Encapsulation and subsequent release of proteins and peptides via a microemulsion delivery vehicle have rarely been explored for biopesticidal applications although the PIs have been well characterized previously (**Tamhane *et al.*, 2005, 2007; Mishra *et al.*, 2010; Joshi *et al.*, 2014**). Use of PI encapsulated BCME as biopesticides presents a viable, 'greener,' and acceptable alternative to chemical pesticides and transgenic approaches. The versatility and generic nature of the BCME allow encapsulation of various PIs to target a variety of pests on different plants. Trans-laminar/leaf absorption of the protein incorporated in BCME is a novel approach for plant protection and immunization that overcomes the adherence limitations generally associated with topical applications (**Whetstone and Hammock 2007**). The microemulsion–protein–leaf surface interactions present an opportunity for further investigations and may lead to novel applications. Currently, insect studies involving *H. armigera* at various stages of growth and maturity are underway, and preliminary results indicate feasibility of this approach.

3.4 Materials and Methods

3.4.1 Materials

1-Butanol (HPLC grade), 2-propanol (LC MS grade), 1-pentanol (>99%), tetradecane (>99.5%), cetyltrimethylammonium bromide (CTAB), limonene, Tween 80, trypsin (type II: crude from porcine pancreas), and N -benzoyl-L-arginine-4-nitroanilide hydrochloride (BApNA) were from Sigma–Aldrich. Deionized water (18.2 MX) was

used for all purposes. Soyabean lecithin was obtained from Oil Tech Inc. GmBH, Germany. For the cloning and expression of CanPI-7, EasyComp Transformation Kit™ and Pichia Expression Kit, Invitrogen Carlsbad, CA, USA was used. Purification of recombinant protein was carried out by hydrophobic interaction chromatography, Amersham Pharmacia Biotech Piscataway, NJ. For the inhibition assays, Konica X-ray films were used.

3.4.2 Preparation and Characterization of Microemulsion

The compositions used for preparing the BCME were as follows and were adapted from previously published literature [11,29-31]: CTAB BCME: CTAB:1-pentanol:water:tetradecane in 17.5:35:35: 12.5 w/w%; Soyabean lecithin and limonene BCME: lecithin: propanol: water: limonene in 19:19:5:57 w/w %; Tween 80 BCME: Tween 80: 1-butanol: water in 35:10:55 w/w%; WIB BCME:water:2-propanol:1-butanol in 55:35:10 w/w%. For preparing CanPI-7 incorporated WIB BCME, 550 µL of 2.1 µg μL^{-1}. Aqueous solution of CanPI-7 was mixed with 450 mg of 2-propanol and 1-butanol (35:10, respectively) mixture to give overall concentration of 105µg of PI μL^{-1}. For the control experiments, 105 µg μL^{-1} of CanPI-7 aqueous solution was prepared. The microemulsions were tested on chickpea (*Cicer arietinium*) and tomato (*Solanum lycopersicum*) leaflets.

3.4.3 Conductivity and Contact Angle Measurements

Conductivity was measured using the Mettler Toledo InLab™ 730 instrument equipped with a stainless steel electrode operational in the range 0.01–1000 mS/cm, having a cell constant 0.1050 at 25^{o}C. The change in the WIB microemulsion system from ‘water-in-oil’ to ‘oil-in-water’ through a bicontinuous region with the increase in water content was monitored using several points in each of the regions (at least three points each) in the phase diagram previously reported (**Wang *et al*., 2008**). The exact ratios of the components in the system were deduced and conductivities measured. The conductivities of individual points were plotted as a function of increase in water content. NaCl (100 µM) solution was used for preparing the respective microemulsions for conductivity studies.

Contact angle measurements were performed on a GBX Digidrop (France) instrument. 4 µL of the WIB BCME and CanPI-7 incorporated in WIB BCME were placed on the central portion of the adaxial surface of the leaflet, and the contact angles were measured. CanPI-7 aqueous solution and water were spotted similarly on chickpea leaflets, and contact angle measurements were taken.

3.4.4 Environmental Scanning Electron (E-SEM) Microscopy

Chickpea plants var Digvijay grown for one month in the greenhouse (maintained at 25 _C with 60% relative humidity, 16 h light, and 8 h dark environments) were used for the E-SEM studies. The adaxial leaflet surface was completely covered with the WIB BCME and allowed to dry at room temperature. These leaflets were plucked immediately and fixed on a stainless steel sample holder using a conductive carbon tape so that the adaxial leaflet surface was facing upwards. The analysis was done in the environmental mode on a Quanta 200 3D, FEI, Netherlands, SEM instrument equipped with a tungsten filament and operated at an accelerating voltage of 20 kV. Images were collected at 1000X and 200X magnification for 100 µm and 500 µm scales, respectively.

3.4.5 Expression and Purification of CanPI-7

Mature peptide region of CanPI-7 cloned in pPIC9 vector was used for transformation in Pichia pastoris GS115 by using Easy Comp Kit. Transformants were selected on histidine-deficient regeneration dextrose base (RDB) media as per the manufacturers' protocol (Invitrogen, Carlsbad, CA, USA). Transformants were screened for the presence of transgene, pilot scale protein expression and further for the large scale expression of CanPI-7 as previously described (**Tamhane et al., 2005**). The expressed protein was purified by using phenyl Sepharose column (hydrophobic interaction chromatography) by applying a reverse NaCl gradient (2.0–0.0 M). Aliquots of eluted fractions were analyzed by carrying out PI activity assays and running them in 12% SDS–PAGE gel followed by activity visualization and staining by CBB-R250. Fermentation was repeated to obtain sufficient quantity of protein; proteins were checked for purity by SDS–PAGE and stored at 4^{o}C for subsequent use in assays.

3.4.6 HGP Preparation

Laboratory culture of *H. armigera* was established by initially collecting larvae from chickpea fields at Agriculture College, Pune or MPKV, Rahuri, Maharashtra, India. Field collected larvae were reared in the laboratory on chickpea flour based artificial diet. Guts of the fourth/fifth instar larvae were dissected out and stored at -20 ^{o}C until further use. Proteases from the gut tissue of *H. armigera* larvae (HGPs) were extracted by homogenizing the tissue in equal volume of glycine–NaOH buffer of pH 10.0 and allowed to stand for 2 h at 4 ^{o}C. The suspension was then centrifuged at 10,000 rpm for 10 min at 4 ^{o}C. The resulting supernatant was collected, frozen in aliquots, and used for further protease inhibitor activity gels and assays.

3.4.7 Proteinase and Proteinase Inhibitor Assays

Protease activity of Trypsin and HGP were estimated using chromogenic synthetic substrate BApNA (**Erlanger, 1961**). 150 µL of diluted enzyme for BApNA assay was added to 1 mL of 1 mM substrate solution and incubated at 37 ^{o}C for 10 min. The reaction was terminated by the addition of 200 µL of 30% acetic acid and absorbance was checked at 410 nm. One protease unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions. For enzyme inhibitor assay, the inhibitor was mixed with the enzyme and the premix was incubated at 25-27 ^{o}C for 30 min. The residual enzyme activity was then estimated as above. A PI unit is defined as the amount of inhibitor required for inhibiting one protease activity unit. For protease inhibitor activity assays in WIB, the above method was modified accordingly. To 1 mL of WIB BCME, 12 µg of trypsin or 0.6 µL of HGP with or without CanPI-7 (1.2 µg) was added in WIB BCME. For activity comparison, the above assay was performed using buffer instead of the WIB BCME. To these, 0.44 mg of BApNA was added and mixed. Optical density at 410 nm was measured after allowing a reaction time of 10 min at 25–27 ^{o}C. Protease inhibitor activity was measured using their respective controls (Buffer and WIB BCME) where no CanPI-7 was added in the above mixture. Protease inhibitor activity from the leaf wash was calculated in terms of PI unit present per leaf.

3.4.8 In Gel Visualization Of Proteases and Protease Inhibitor

0.4 units of HGP were resolved on 8% native-PAGE for activity visualization by gel X-ray film contact print technique (GXCT) (**Pichare and Kachole, 1994**). After run, gel was overlaid on x-ray film and allowed to incubate for different time points in order to capture clear bands. Films were washed in luke warm distill water and air dried. Activity bands were visualized as digested gelatin over x-ray film due to the presence of trypsin. To detect PI activity, gel was washed in tris-cl buffer pH 7.8 for 10 min followed by wash in 0.03 % trypsin solution with another brief wash in buffer solution. Trypsin facilitates gels were overlaid on gelatin coated x-ray film for various time points and washed films were analyzed for trypsin inhibitory activity

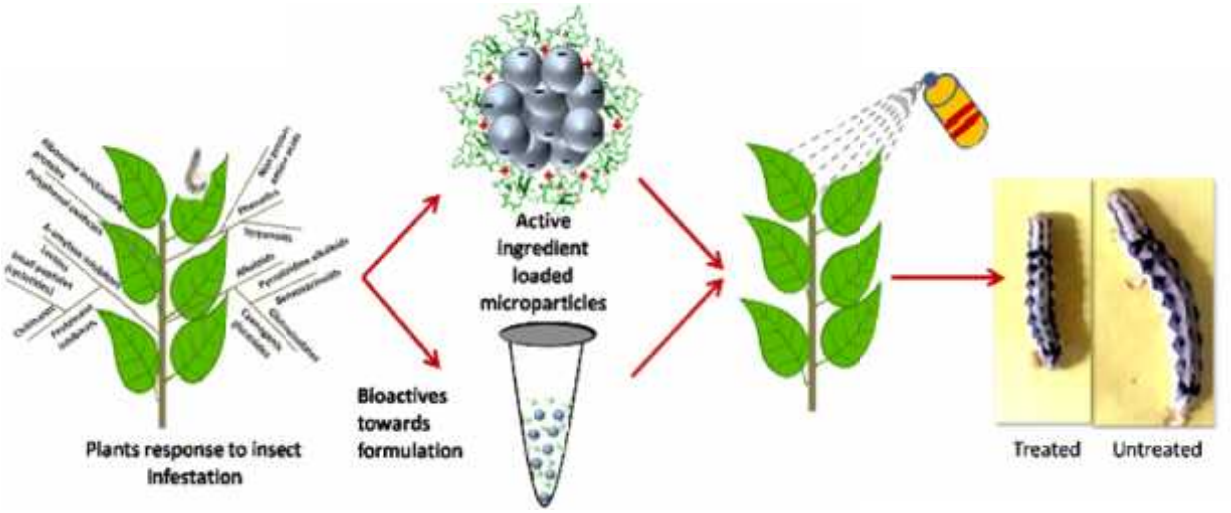
3.4.9 Ex- vivo Stability and Activity Studies

These experiments were performed on green house grown (25 ^{o}C with 60% relative humidity, 16 h light, and 8 h dark environments) chickpea plants. 4 µL of 1.1 mg mL CanPI-7 incorporatedin water or WIB BCME, respectively, was individually spotted on adaxial surface of chickpea leaflets. Water (4µL) and WIB BCME (4µL) without CanPI-7 were also placed on separate chickpea leafletsto be used as negative controls. The experiment was performed by spotting 30 leaflets of chickpea with 4µL solution, respectively, for the four treatments. The spot of either water or WIB BCME onthe leaflet was allowed to evaporate completely, which took amaximum 5 min in the green house conditions. After 5 min, 3 h, and 24 h, respectively, ten leaflets from each treatment were pooled and treated as one replicate. The pooled leaflets were washed with water twice with gentle swirling, (a) with 3 mL of initial wash followed by (b) another 5 mL wash. The washings were pooled (8 mL total) and further concentrated in a cooling vacuum evaporator (Labconco, Speedvac) to 200 µL (wash concentrate). This process was designed essentially to ensure the recovery of CanPI-7 lodged on the leaf either through water or WIB spotting. The wash concentrate was used for determining the PI activity expressed in terms of units/leaflet/min by using BAPNA assay described above.

3.4.10 Feeding Assays

H. armigera larvae were collected from chickpea fields of Mahatma Phule Krishi Vidyapeeth (MPKV) and reared on chickpea based artificial diet in laboratory conditions. For feeding assays, 0.8 HGPI units were used to feed insects at neonatal stage for 6-8 days. 2-3rd instar larvae were dissected to remove gut for protease extraction and stored for further analysis. For protease band visualization at neonate stage, whole insect were snap freezed and stored at -80^{o}C for protease and inhibitor assays.

Chapter 4



Bio-physical evaluation and *in vivo* delivery of plant proteinase inhibitor immobilized on silica nanospheres

Chapter 4: Bio-physical evaluation and *in vivo* delivery of plant proteinase inhibitor immobilized on silica nanospheres

4.1 Introduction

Delivery of bio-molecules often entails both biological and synthetic milieu for their release at the necessary site of action (**Tang *et al.*, 2013; Chou *et al.*, 2014; Jiang *et al.*, 2013**). Bio-delivery of protein/peptide(s) owing to their magnificently diverse and remarkable structural and functional properties is of primary interest. These features are governed by their structural conformation which in turn is tailored by surrounding environment such as molecular crowding, protonation, temperature, pH, etc. (**Koide *et al.*, 2002; Minton *et al.*, 2000; Di Russo *et al.*, 2012**). Small peptides, owing to their multi-level function in biological system are produced recombinantly or chemically synthesized for their broad spectrum application in the form of biomedical drugs (**Craik *et al.*, 2013**), signalling, (**Murphy *et al.*, 2012**) inhibitors of specific enzyme targets (**Dunse *et al.*, 2010**) and many more. Thus, efficient application of protein/peptide(s) in the non-native environment depends on the designing of delivery carrier without altering the function in achieving competent targeted emancipation. To achieve this, nanoparticles (NPs) have been considered as one of the potential system for targeted delivery in the desired environment (**Mackowiak *et al.*, 2013**). Choice of NPs of varied sizes and structures depends on its specific application. Specifically, NPs have long been known for their drug delivery applications. Several studies have investigated the interaction of NPs with complex set of proteins such as serum proteins. In addition, due to their physico-chemical features, such as chemical composition, surface functionalization, shape, angle of curvature, surface charges, etc. (**Nel *et al.*, 2009; Cedervall *et al.*, 2007**). NPs adapts to the physiological environment by forming protein 'corona' through adsorption thereby attaining biological identity in the system (**Walkey *et al.*, 2012; Walkey and Chan, 2012**). Engineered particles can be utilized as a potential platform to develop specific protein 'corona' in vitro. It can thus deliver target molecule to desired site in order to enhance their applicability in specific environment (**Cifuentes-rius *et al.*, 2013**). Towards this, biocompatible silica based nanomaterials which possess unprecedented advantages as drug delivery nanocarriers have been structurally and chemically modified for their successful explorations (**Tang *et al.*, 2012; Zhao *et al.*, 2013; Liu *et al.*, 2007**). In this study, we explored silica nanospheres (SiO_2N) and Santa Barbara Amorphous (SBA-15) for the maximum loading of proteinase inhibitor (PI) peptide and its stability.

Application of recombinantly expressed PIs which was sourced from insect nonhost plant, *Capsicum annuum*, may prove to be a potential system for providing protection from Lepidopteran insects to limit the crop damage (**Joshi *et al.*, 2014**). In the initial screening we noted SiO_2N to be the promising material. Hence, we endeavoured to use silica-based nanocarrier with PI peptide for their potential use in agriculture implications. Towards this, silica nanospheres (SiO_2N) offer physical and chemical tunability with resourceful usage as a prudent bioactive nanocarrier (**Tang *et al.*, 2012**). Also, large pore diameter and high surface area of self assembled silica particles provides platform for immobilization of many bioactive molecules imparting additional advantage of sturdiness and durability. Furthermore, it is prerequisite to protect the stability of protein/peptide in the outer environment from chemical and physical parameters such as temperature, moisture, plant's chemical secretions, soil pH, composition, etc. (**Arakawa *et al.*, 2001**). Thus, we aimed to use biocompatible SiO_2N–PI duo complex, firstly to avoid such constraints (**Joshi *et al.*, 2014**) and secondly to allow inhibitor molecule to reach and maintain function in the gut of insect. In addition, the comprehensive mapping of the nano-bio interface following immobilization is anticipated along with its effect in vivo. Exceptionally, in vivo delivery of SiO_2N–PI is unconventional over to the traditional acidic milieu delivery below 5.5 pH. To our knowledge, this is the first report to establish alkaline pH mediated PI-peptide release from charged silica solid surface to develop an effective biopesticide for crop protection.

4.2 Results and discussion

4.2.1 Characterization of CanPI-13

PIs are natural defensive proteins found in the plants and induced upon insect attack/damage in local and systemic tissues (**Green *et al.*, 1972**). C. annuum PI (CanPI-13) is a small peptide of around 5.9 kDa and is one of the variant amongst series of inhibitors, belonging to wound inducible Pin-II PIs confined to Solanaceae family (**Green *et al.*, 1972; Harsulkar *et al.*, 1999**). PI was expressed in yeast (*Pichia pastoris*) system to obtain recombinant inhibitor protein. Multiple sequence alignment of PI and its variants revealed that it consists of single functional domain which determines its specificity against trypsin-like serine proteases as evident by the appearance of conserved Lysine (K) or Arginine (R) in the sequence at reactive P1 residue. Furthermore, the presence of eight cysteine residues in the sequence will lead to the formation of four disulphide bonds imparting structural stability to PI molecule

(**Fig. 4.1A**). Activity of recombinant CanPI-13 was validated against commercially available bovine trypsin and gut extracted trypsin(s) of *Helicoverpa armigera* larvae exhibiting maximum 100% and 91% inhibition, respectively (**Fig. 4.1B**). PI inhibitory potency towards insect trypsin is supported by its interference with the protein digestion leading to arresting the growth, development and hamper fecundity and fertility of pest *H. armigera* (**Joshi *et al.*, 2014**). Importantly, by virtue of this inhibitor peptide, which exhibits more activity towards cocktail of insect (*H. armigera*) trypsins (IC_{50} 0.01 M) than bovine trypsin (IC_{50} 0.09 M), is potentially advantageous for their insecticide use.

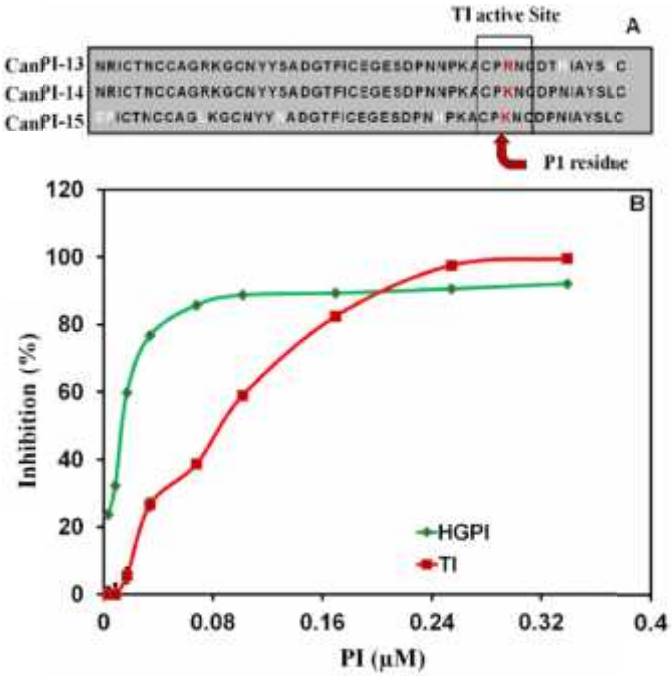


Figure 4.1: [A] Sequence comparison of CanPI-13 with CanPI-14 and 15, highlighting conserved trypsin inhibitory active site along with presence of lysine (K) and Arginine (R) residue; other sequence variations are shown in white colour and [B] proteinase inhibitory activity against bovine trypsin and *H. armigera* gut protease mixture (HGP) was measured at increasing concentration. Standard error bars are shown for $n = 3$.

4.2.2 Immobilization of CanPI-13 and Biophysical Evaluation

In initial study, SiO_2N and SBA-15 were chosen to compare the loading capacity of recombinant CanPI-13. SBA-15 was synthesized as per reported procedure (**Zhao *et al.*, 1998**). It was found that the Brunauer, Emmett and Teller (BET) N2 adsorption

surface area 902 m2/g with Barret–Joyner–Halenda (BJH) adsorption cumulative volume of pores 0.98 cm3/g and BJH adsorption average pore having width (4V/A) = 90.2A. ˚ Whereas, Stöber method was followed to synthesize SiO_2N by hydrolyzing tetraethyl orthosilicate (TEOS) in highly basic media (**Biradar *et al.*, 2011; Yokoi *et al.*, 2006**). The surface area and hydroxyl group density of obtained SiO_2N was increased by mild etching in aqueous KOH solution. Furthermore, BET surface area was found to be 13 and 25 m2/g with BJH adsorption cumulative volume of pores 0.066757, 0.212843 cm3/g and BJH adsorption average pore having width (4V/A) = 411.892 and 569.993A. ˚ The 29Si NMR (nuclear magnetic resonance) analysis showed increase in Q3 species (−100 ppm) than the parent SiO_2N, which indicated increase in hydroxyl density on the SiO_2N surface (**Fig. 4.2A, B**).

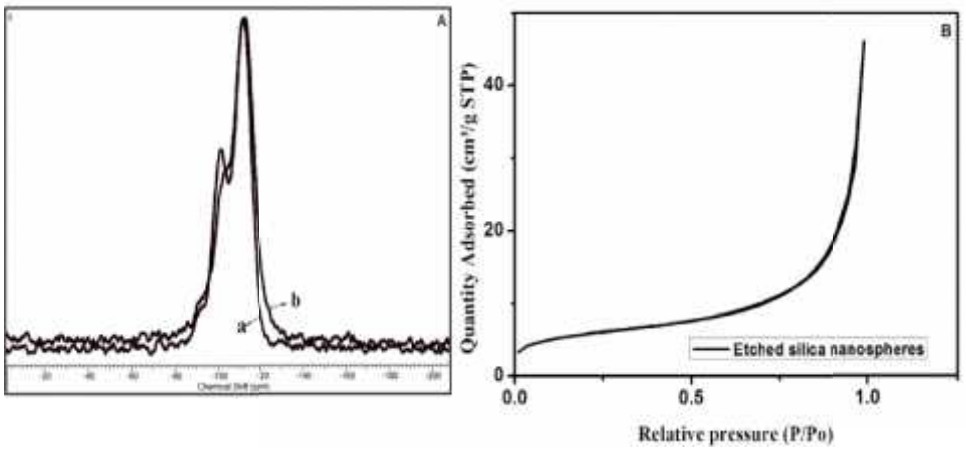


Figure 4.2: [A] ^{29}Si NMR of silica nanospheres (a) and 4 h etched SiO_2N (b); (B) Nitrogen adsorption/desorption isotherms of 4 h etched SiO_2N.

At first, 1 mg of PI was incubated with 10 mg SBA-15 and SiO_2N at pH 4.0. CanPI-13 immobilization on silica was carried out by considering peptide's isoelectric point (pI = 6.2), which upon varying the solution pH below and above pI endowed net positive/negative charge on rCanPI-13 due to gain or loss of protons (H+). However, equal amount of rCanPI-13 was estimated to be adsorbed on SiO_2N despite of the presence of large surface area of SBA-15 as compared to SiO_2N (**Fig. 4.3A**). Zeta potential measurements revealed the surface charges on silica nanostructures and native protein as observed at different pH. SBA-15 carried almost neutral surface charge whereas, SiO_2N displayed negative charge (**Table 4.1**).

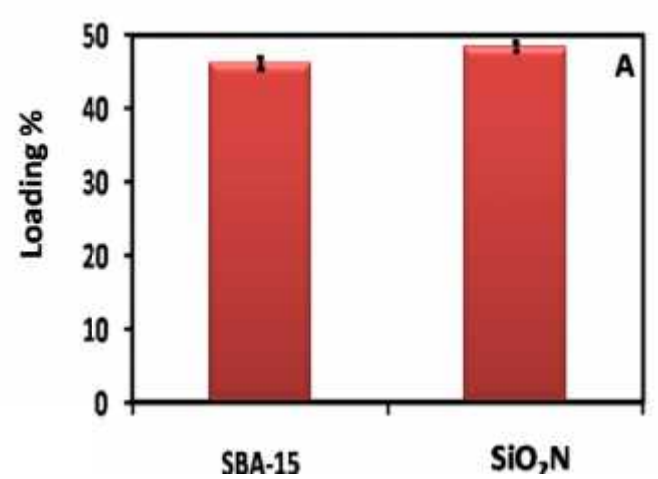


Figure 4.3: [A] Amount of PI bound to SBA-15 and SiO_2N from 1mg PI is calculated as % loading

Table 4.1: Zeta potential measurements of different samples at different pH. Values are expressed in the units mV.

Table 4.1

Samples	pH2	pH 4	pH 7	pH 10
SiO_2N	11.18	-5.46	-14.78	-38.68
SBA-15	9.05	-1.22	-16.68	-29.08
PI	12.61	2.16	-10.36	-16.27
SiO_2N-PI	19.04	16.82	-29.32	-44.03

The study revealed that protein adsorption on the particle surfaces rely majorly on electrostatic interaction and protein flexibility to access nano-surface and is further regulated by specific amino acid residues (**Mathè *et al.*, 2013**). Thus, rCanPI-13 was incubated with negatively charged SiO_2N and immobilization was found to be occurring at pH 4 with negligible adsorption at alkaline pH 7 (**Fig. 4.4A**). This is because of the positive mean zeta potential (2.16 mV) of CanPI-13 at pH 4 as compared to negative zeta potential (−10.36 mV) displayed at higher pH. Nevertheless, zeta potential of SiO_2N–PI showed positive charge showing 16.82 mV at pH 4 (**Fig. 4.4B**) which clearly stated the dominance of positively charged PI peptide on silica surface.

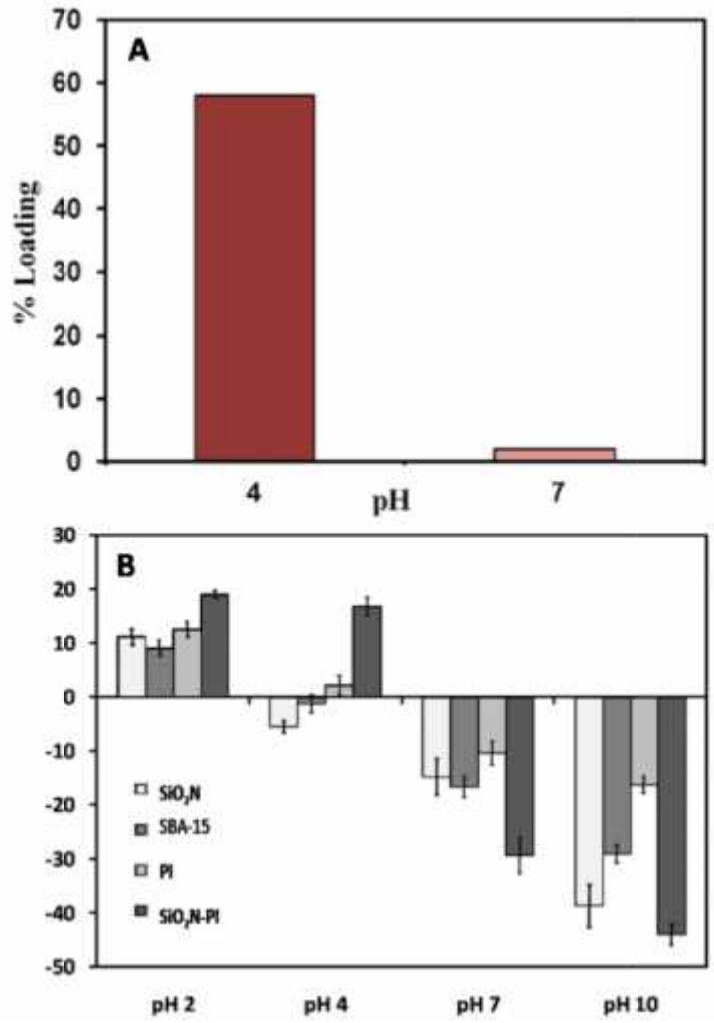


Figure 4.4: [A] Loading of 2.0 mg PI on 10 mg SiO_2N at two different buffers viz. 0.1 M acetate pH 4.0 and 0.1 M phosphate pH 7.0 and [B] (A) Surface charge measurements of SiO_2N, SBA-15, PI and SiO_2N–PI at different pH, each reading was carried in the replicate of 10, standard error bars are shown for n = 2

Also, the formation of PI mono or multi layers on SiO_2N surface substantiates the SiO_2N–PI protonation occurring at pH 4 as corroborated in previous studies (**Bain *et al.*, 1989; Röcker *et al.*, 2009)**. These SiO_2N were observed to be mono dispersed (**Fig. 4.5A and B**) with size of ~240 nm demonstrated by scanning electron microscopy (SEM). SBA- 15 was also visualized for their morphology with and without PI adsorption (**Fig. 4.5C and D**). SBA-15, due to its non-distinct advantages over SiO_2N together with its tedious synthesis methods, was not characterized further. Hence, further studies are involved in understanding SiO_2N–PI interaction and application. PI immobilization was clearly observed as a layer formation on silica spheres.

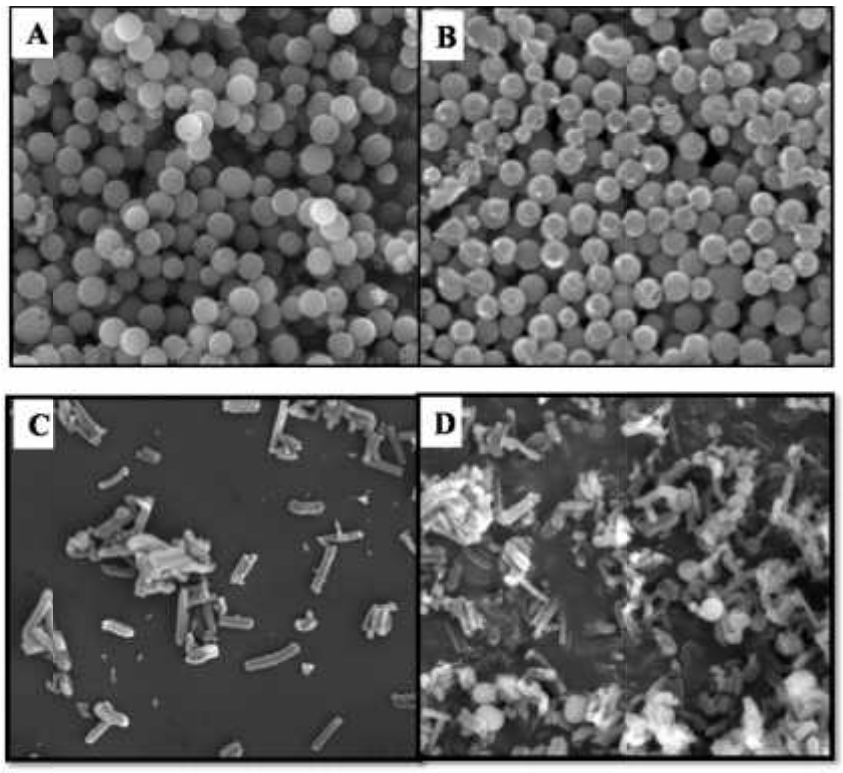


Figure 4.5: SEM images were captured for (A) SiO_2N (30000×, 1 µm scale); (B) SiO_2N–PI (30000×, 1 µm scale); (C) SBA-15 (25000×, 1 µm scale) and (D) SBA-15-PI (25000×, 2 µm scale).

Transmission electron microscopy (TEM) technique was employed to reveal more fine structural details of native and corrugated silica surfaces (**Fig. 4.6A and B**). However, TEM was not found to be a suitable technique to visualize presence of peptide on silica particles (**Fig. 4.6C**). To establish a stable conjugation between SiO_2N and PI, it was characterized for structural stability and PI activity. Any possible change in the peptide conformation at SiO_2N–PI interface was studied by virtue of sensitivity of secondary structure of peptide by Fourier transform infrared spectroscopy (FTIR) and optical chirality. Peptide binding with silica spheres was confirmed by the appearance of stretching frequencies of amide I (1650 cm^{-1}) corresponding to C-O and amide II (1540 cm^{-1}) bands for N-H and C-N stretching in bound and unbound peptide (**Fig. 4.6D**) (**Mandal and Kraatz 2007; Biradar *et al.*, 2009**). This indicated successful immobilization of peptide on nanospheres. PI interaction with SiO_2N was further investigated for any conformational change by circular dichroism (CD) to establish secondary structural features. CD spectra at far UV range demonstrated characteristic feature dominating in PI peptide by the

emergence of negative ellipticity around 215 nm, indicative of sheet conformation (**Slocik *et al.*, 2011**) which was also observed in SiO_2N–PI (**Fig. 4.6E**). This displayed no significant alteration in the secondary structure of the protein owing to the stable tertiary structure of PI.

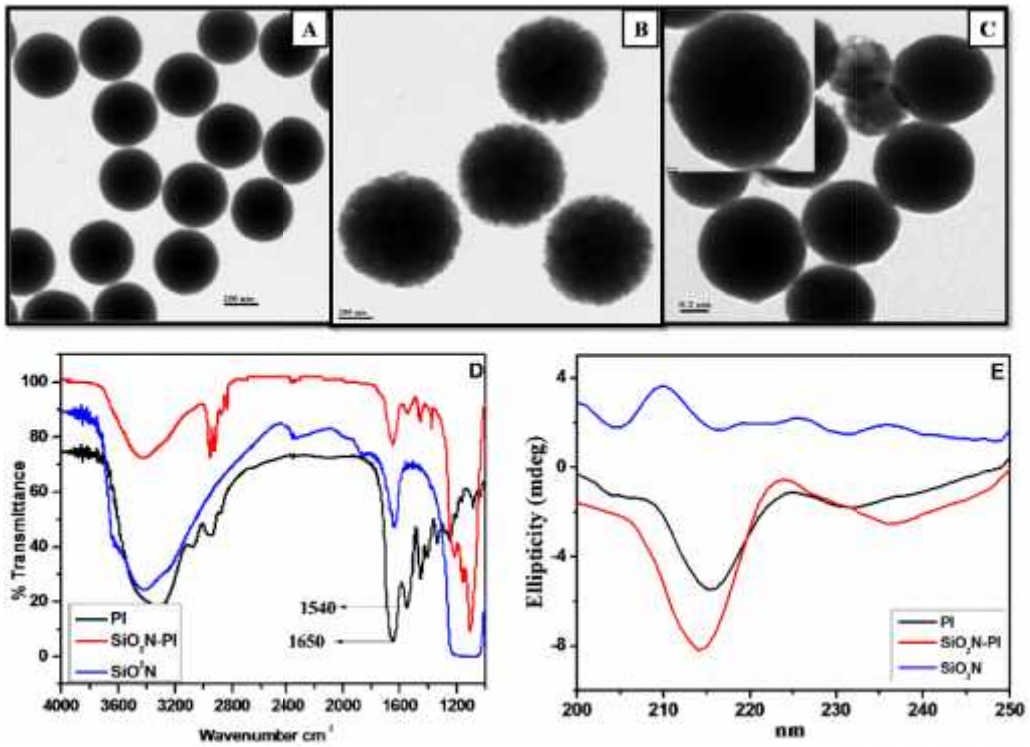


Figure 4.6: TEM images of (A) SiO_2N; (B) etched SiO_2N; (C) SiO_2N–PI (inset is the high resolution image of PI immobilized on SiO_2N); (D) FTIR and (E) CD measurement of SiO_2N, PI and SiO_2N–PI conjugated complex

Measuring binding affinity of peptide with silica nanospheres is of significance to understand the stability of the dual nanosystem. PI exhibited characteristic emission spectrum for tyrosine, displaying highest peak at 370 nm which was quenched with increasing concentration of SiO_2N (**Fig. 4.7A**). Excitation of PI was recorded at 280 nm with selected emission range from 300 to 550 nm. With increase in SiO_2N amount, decrease in intensity of PI was observed which suggested stable dynamic interactions at nano-bio interface which was also measured using classical Stern–Volmer equation (**Stern *et al.*, 1919**).

$F0/F = 1 + kq \cdot t_0 \cdot [Q] = 1 + Ksv \cdot [Q]$

Where F0 and F are the intensities of PI before and after interaction with SiO_2N, Ksv is the Stern–Volmer quenching constant, Q is the concentration of SiO_2N and 0 is the life-time of the fluorophorein the absence of quencher. A plot of [F0/F] versus [Q]

from the quenching data, yielded a straight line (R2 = 0.97) explaining collisional quenching (**Eftink *et al.*, 1991**). Ksv which is equal to the slope calculated by following linear fitting of the model presenting value of 4.2 × 105 G−1 (**Fig. 4.7B**). The slope reveals the accessibility of the quencher to the fluorophore and a linear plot defines the presence of single class of fluorophores evenly accessible to the quencher (Khandare *et al.*, 2010) [34]. The dissociation constant (Kd) has value of 3.9 × 10−5 shows high affinity at SiO_2N–PI interface.

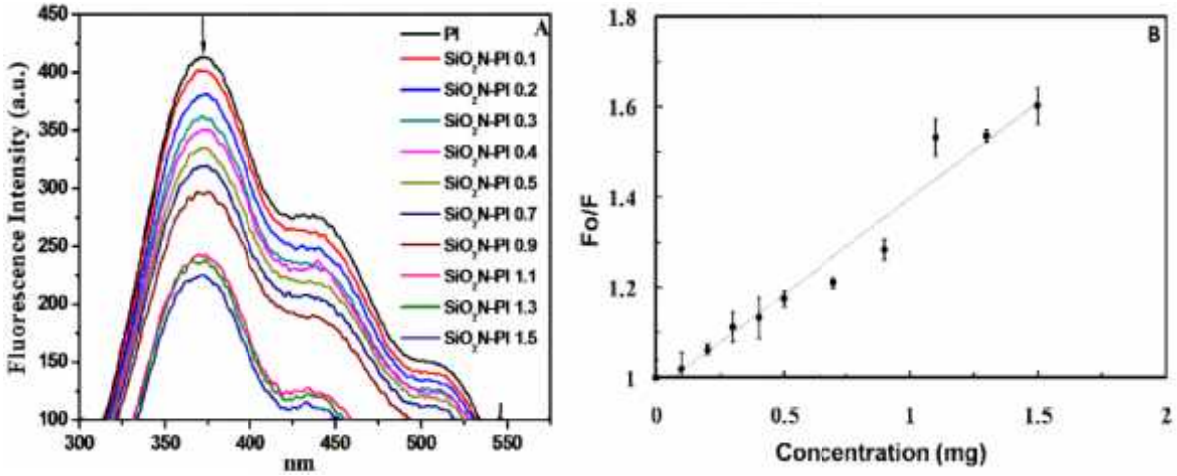


Figure 4.7: (A) Emission spectra of free PI (Ex-280 nm) monitored after the addition of SiO2N with increasing concentration. Arrow reflects the maximum fluorescence intensity of PI (0.5 M) and (B) Stern–Volmer plot for tyrosine quenched by silica NPs. Standard error bars are shown (n = 3).

4.2.3 CanPI-13 Loading and Release

To understand binding dynamics occurring at the protein nanomaterial interface, quantitative methods along with protein exchange kinetics have been suggested (**Lynch *et al.*, 2009**). Loading capacity of PI with SiO_2N was established, by incubating increasing amount of PI (0.25–5.0 mg) with 10 mg silica nanospheres for three hours at 4 ◦C in 0.1 M acetate buffer of pH 4. Immobilized amount of PI on SiO_2N was then quantified by Bradford protein estimation method (**Bradford 1976**). It was observed that the protein amount bound to SiO_2N increased subsequently with increasing concentration. However, its overall loading efficiency showed a steady decline after reaching a peak at a concentration of 2.0 mg (**Fig. 4.8A**). As reported earlier, change in the protein identity and concentration influences the composition of ‘corona’. This phenomenon has been well explained with fibrinogen which initiates

imparting its dissociation from NPs surface due to increase in the competition with other plasma proteins (**Monopoli *et al.*, 2011; Mahmoudi *et al.*, 2013**). Thus, it can be assumed that with increasing concentration of PI, it is subjected to the greater competition with other PI molecules along with less exposure to surface available on SiO_2N thus leaving PI with reduced probability to get adsorb in a stated time. In addition, the PI-peptide adsorption continues to occur until it reaches the level of surface saturation. Further increase in the PI-peptide concentration may contribute in the formation of densely packed mono or multi layers (**Mathè *et al.*, 2013**). As expected, PI predominantly maintained its activity in bound state as compared with free form of protein which was studied by gel-Xray film contact print technique (**Fig. 4.8B**) (**Pichare and Kachole 1994**). SiO_2N–PI interaction was screened at two different temperature viz., 4 and 25 ◦C to study the change in the adsorption pattern. Significant variation was observed with high adsorption taking place at 4 ◦C (**Fig. 4.8C**). The finding is in contrast with the study on Human serum albumin (HSA) and apo-TF protein adsorption on NPs by (**Mahmoudi *et al.*, 2013**), illustrating high affinity with increasing temperature. However, the major determining factor in guiding the interaction depends on the peptide conformation and its interaction with surrounding environment. Also,the physico-chemical properties ofthe particle which is under the influence of given conditions such as temperature and atmospheric pressure plays an important role. We infer from our data that at increasing temperature peptides tend to dissociate from each other to increase translational entropy which does not corroborate with earlier reports (**Mahmoudi *et al.*, 2013**). With an assumption of charge mediated interaction of SiO_2N and PI, peptide release from the silica surface was evaluated by changing the PI net charge by dispersing SiO_2N–PI conjugated system in buffer of pH 7 and 10. Estimated release of PI at alkaline pH was 62% at pH 7 and 56% at pH 10 which simulates Lepidopteran insect gut environment i.e. pH > 10 (**Fig. 4.8D**). We noted that the amount released from SiO_2N is primarily due to the repulsive force interacting at nano-PI interface which came into play at alkaline pH by imparting negative charge on PI. On the other hand, remaining PI was retained on the silica nanospheres suggesting the possible involvement of other forces at play such as particle curvature, particle size and aggregation (**Nel *et al.*, 2009**) at the nano-PI interface. Additionally, retention of PI can be explained by the fact that payload release from protein ‘corona’ in physiological system is affected by the presence of mixture of ‘hard’ or ‘soft’ protein

corona in the environment (**Cifuentes-rius *et al.*, 2013**). Thus exchange is initiated between the proteins in the corona due to their differential binding affinities encountered in the surrounding environment. In spite of its higher concentration, replacement of lower affinity protein occurs with proteins bearing high affinity having lower concentration. This could be a supporting fact to justify why retention of PI on SiO_2N remained high. As SiO_2N is now surrounded maximally by PI peptide after partial release, the exchange phenomena between the peptides are supposed to be halted and hence existence of similar charge and same binding affinity in the SiO_2N vicinity could not lead to further release. Surprisingly, significant difference was obtained in PI release in water as compared to alkaline pH. This is a desirable observation in terms of application of PI in the outer environment with varying moisture content in different seasons. Specific ionic strength becomes one of the major concerning factors for the dissociation of the peptide from the particle in aqueous medium. Furthermore, as the activity of PI was retained in the bound state, absolute release of PI in the alkaline pH cannot be considered as any kind of apprehension for its use as a biopesticide.

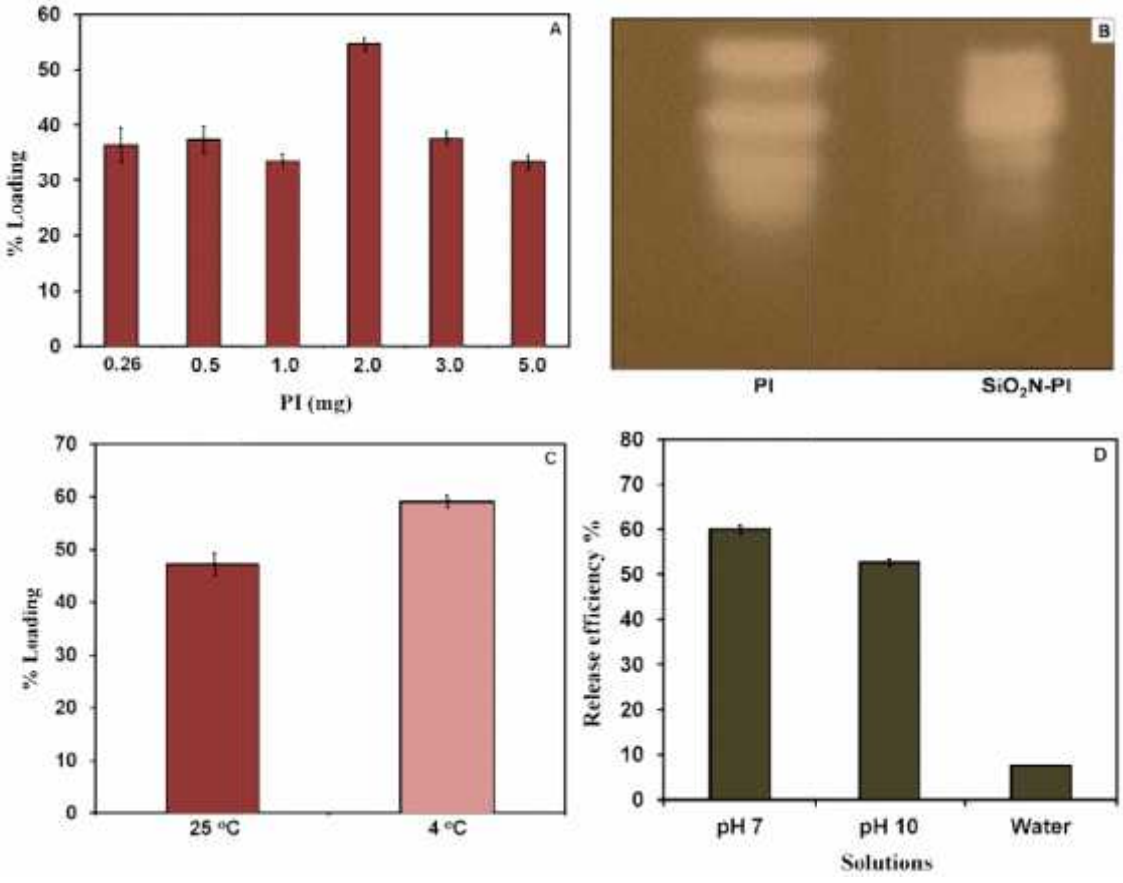


Figure 4.8: (A) Loading efficiency of PI on SiO_2N was measured after loading PI at different concentration in the replicate of 6, standard error bars are shown n = 6; (B) activity visualization of PI (0.2 TIUs) in bound state to SiO_2N; (C) loading of PI on SiO_2N at different temperatures (SE, n = 3) and (D) release of bound PI from SiO_2N was estimated in buffer pH 7, 10 and in aqueous (SE, n = 3).

4.2.4 *In vivo* Evaluation of SiO_2N–PI Complex

In vitro confirmation of PI peptide adsorption was extended for their in vivo studies in insect gut by performing feeding bioassays. Thus, 2 days old insect neonates were allowed to feed on SiO_2N immobilized with recombinant PI peptide based artificial diet (SiO_2N–PI–AD). Along with this, control diets i.e. AD, SiO_2N–AD and PI–AD were also used to monitor insect's growth till they metamorphosed into 4th instar larvae. Insect metamorphosis was found to be delayed in SiO_2N–PI–AD diet as compared to control SiO_2N–AD diets. All the insects turned to 4th instar in case of control SiO_2N–AD diets. Whereas, those feeding on PI–AD and SiO_2N–PI–AD diet showed significant reduction in their growth as measured in terms of body mass (**Fig. 4.9A**). Insect's weight was measured and compared with their respective controls, such as SiO_2N–PI–AD was compared with SiO_2N–AD ($p < 0.0001$) and PI–AD with AD ($p < 0.0001$). In addition, to understand the effect of only SiO_2N on insect growth, insect surviving on this diet were also compared with AD showing $p = 0.023$. As shown in Fig. 6B, almost 50% insects showed mortality to PI treatment. Small variation in the SiO_2N–PI treatment was observed corresponding to 60% survival of the total insects (**Fig. 4.9B**). Therefore, we inferred from these results that in addition to SiO_2N–PI complex being effective on insect system, silica might create an extra burden on the insects due to indigestion (**Fig. 4.9C and D**). Hence, insects resisted to consume SiO_2N–PI–AD diet, which was also tested by comparing insect feeding on control silica and artificial diet. Although, the variation in insect body mass and percent survival in AD and SiO_2N–AD treatments is not highly significant, the effect might be enhanced in the presence of PI. Presence of SiO_2N were also observed in the frass of SiO_2N–PI–AD fed insects and compared with PI-fed insects (**Fig. 4.9E and F**).

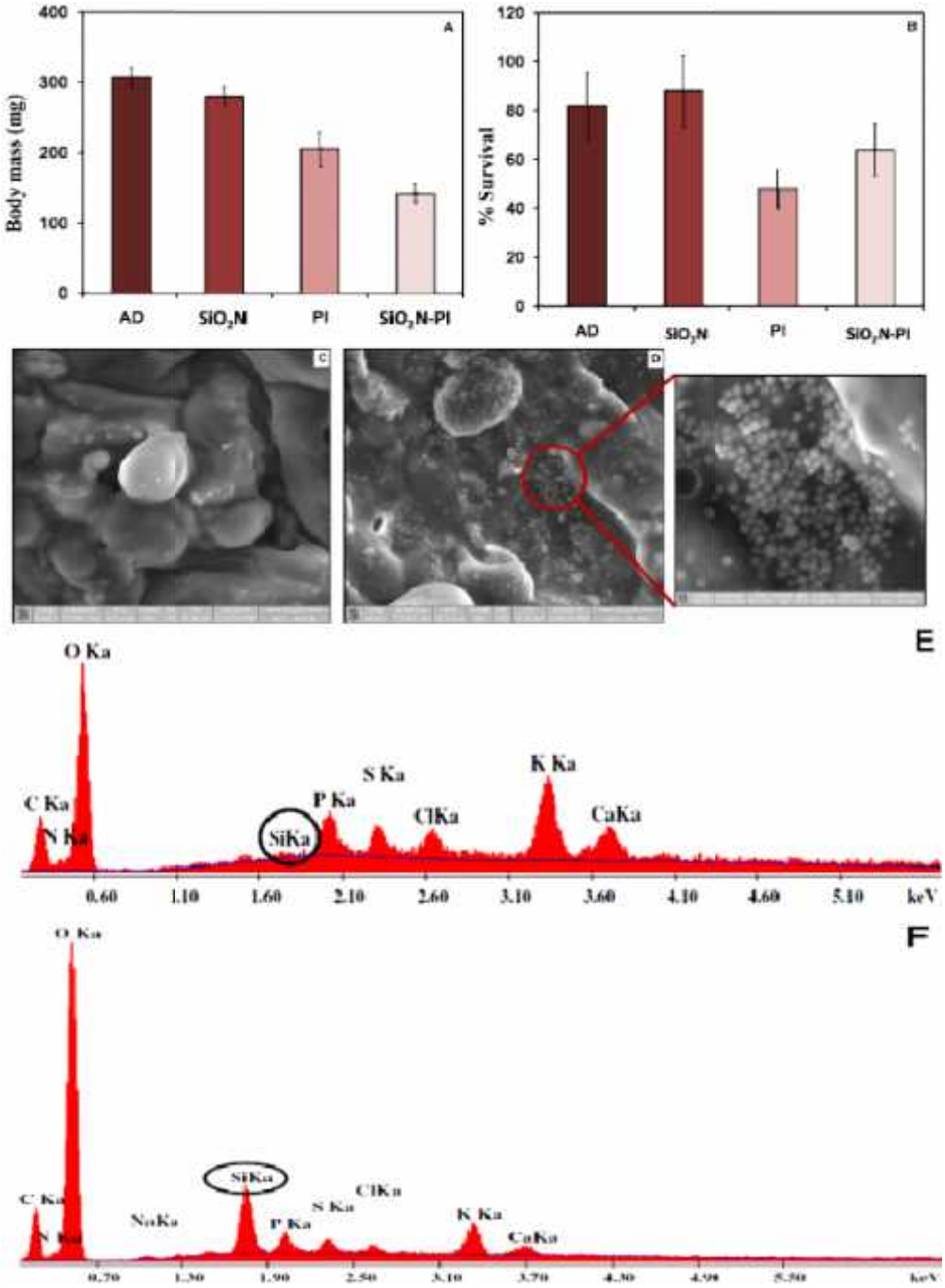


Figure 4.9: Insects were fed on four diets viz. AD, SiO_2N, PI, and SiO_2N–PI (approx. 8 activity units) each treatment was carried out in the replicate of three with 30 insects per replicate; standard error bars are shown (n = 3); (A) insects average mean weight was calculated after feeding on different diets; (B) % survival of the insect feeding on diets were calculated. SEM measurements of insect frass; (C and E) frass of PI fed insect with no detectable silica peak; (D and F) frass of SiO_2N–PI–AD fed insect, inset showing high magnification of silica present in frass and showing presence of SiO_2N

To confirm the activity of PI in the gut of the insect, frass extracted PI activity gel visualization was performed revealing PI stability in the gut with and without loading on SiO_2N (**Fig. 4.10A**). Variation in the insect size was also easily distinguishable among PI containing and control diets (**Fig. 4.10B**). Our study represents PI-peptide immobilized on to silica based delivery platform for higher pH gradients milieu in insects and in establishing the activity of PI peptide.

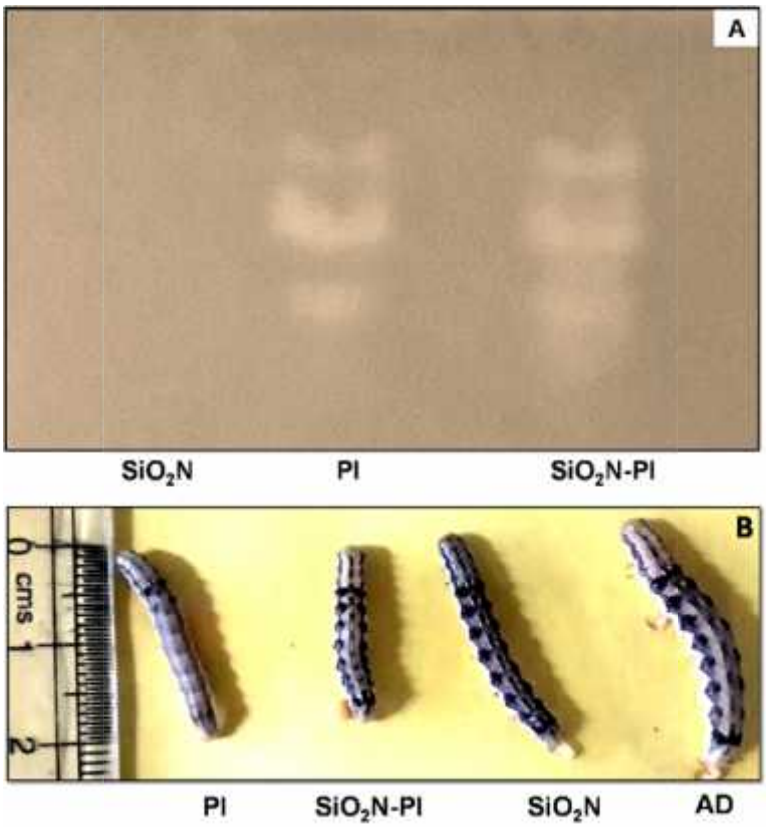


Figure 4.10: (A) Activity visualization of PI in the heat treated frass of insect feeded on SiO_2N, PI and SiO_2N-PI and (B) Insects fed on different diets showing size variation.

4.3 Conclusion

Delivery of bioactive PI-peptide via silica nanocarrier was achieved for their augmented accessibility in the outer environment having potential defensive property against insect pest. For the successful delivery, it was prudent to realize the dynamic interactions, which might exist at the interface of SiO_2N and PI-peptide leading to a stable peptide immobilization. Variation in peptide loading at different pH and temperature is the direct evidence of specific forces at the interface such as charge and ionic strength. In vivo studies clearly demonstrated the feasibility and potency of the cargo delivery at large scale and open new avenues to design bio active-multi

functionalized nanocarrier for their broad spectrum application in the nature for the protection of the plants from insect damage. This study provides a further opportunity to understand the various aspects of physical and chemical interaction such as size, shape, surface roughness/smoothness, chemical composition, charge, etc. to establish bioactive uptake, efficiency, enhanced retention time in the targeted system for their successful implications in developing sustainable agricultural practices for crop protection.

4.4 Experimental

4.4.1 Synthesis of Silica Nanospheres and SBA-15 Nanorods

Silica nanospheres were synthesized by following the reported procedure (**Zhao *et al.*, 1998; Biradar *et al.*, 2011**). In brief, a 1000 mL plastic bottle was charged with 400 mL of ethanol, 14.4 mL distilled water and 40 mL of ammonium hydroxide and stirred vigorously at 1000 rpm for 2 minutes. Then 23.39 g of tetraethyl orthosilicate (TEOS) was added slowly into the reaction mixture and allowed to mix at moderate stirring (600 rpm) for 3 h. Then obtained white solid was collected by the centrifugation and washed thrice with ethanol and allowed to dry at room temperature (∼24 ◦C). The weight of final product was 7 g (yield = 97%). To generate etched surface of SiO_2N, 4 g of synthesized silica nanosphere was dispersed in 50 mL distilled deionized water by sonication. Then 1500 mL of (0.01 M) KOH was added into it and the reaction mixture was stirred at slow speed (250 rpm) for 4 h. Finally, etched silica was collected by centrifugation and washed with copious amount of water. This step was repeated at least three times. For the synthesis of SBA-15 nanorods solution containing 6 g of poly (ethylene oxide)-block-poly(butylene oxide)-blockpoly(ethyleneoxide) [(PEO)20(PPO)70(PEO)20] Pluronics 123, 161 g deionized water, and 36 g HCl (36 wt%) was prepared and stirred vigorously at 40 ◦C until the Pluronics template dissolved. Then tetraethyl orthosilicate (TEOS), (12.8 g) was added, and stirring of the solution was continued at 45 ◦C for 24 h. The solution was kept static in an oven at 80 ◦C for 24 h; solid particles were then separated by filtration on filter paper. The particles were washed with deionized water and ethanol (3 mL × 80 mL) and dried at ambient conditions to produce as-synthesized SBA-15 particles. The Pluronics template from the as-synthesized SBA-15 was removed by calcination, and the final product was named as SBA15-cal.

4.4.2 Expression and Purification of CanPI-13 in *P. pastoris*

GS115 strain of *Pichia pastoris* were transformed with recombinant pPIC9 plasmids containing insert CanPI-13. Recombinant expression was carried out by following Easy Comp Kit (Invitrogen, Carlsbad, CA, USA). Transformants were selected on histidine deficient RDB (Regeneration dextrose base) media (Invitrogen; medium contained 1.86% sorbitol, 2% dextrose, 1.34% yeast nitrogen base (YNB) with ammonium sulphate and without amino acid, biotin, l-glutamic acid, l-methionine, l-lysine, l-leucine, lisoleucine each at 0.005% and 2% agar). Colonies were further screened for positive transformants which were selected for protein expression. Recombinant P. pastoris was initially grown at 28 ◦C in 'minimal glycerol (MGY) medium' (Invitrogen; 1.34% YNB without amino acids, 4 × 10−5% biotin, and 1% glycerol) for 24 h till the growth reached ∼1 OD at 600 nm. The cell pellet was collected by centrifugation at 5000 rpm, for 10 min at 25 ◦C. Cells were dispersed in 100 mL 'buffered methanol-complex (BMMY) medium' (Invitrogen; each litre of medium contained 10 g yeast extract, 20 g peptone, pH 6.0 100 mM potassium phosphate buffer, 13.4 g YNB, 0.4 mg biotin, 10 mL methanol) dispensed in one litre baffled flasks. Methanol (1%) was supplemented every 24 h and the culture was allowed to grow for ∼96 h. Culture was passed through 0.2 m membrane column to remove cells from the solution. Partial purification of the protein solution was achieved by passing through 30 kDa cut off membrane, followed by ammonium sulphate precipitation (70%) and dialysis with 1 kDa membrane. Protein was further passed through Sephadex G-25 medium using PD SpinTrap G-25 kit (GE Healthcare) for sample cleanup.

4.4.3 PI Immobilization and *in vitro* Release

For PI immobilization, parameters such as pH, and temperature were standardized to achieve maximum loading capacity. At first, 2 mg PI was immobilized on SiO_2N at two different pH, acetate buffer pH 4.0 (100 mM) and phosphate buffer pH 7.0 (100 mM). For this, 1 mL of each buffer was added to 10 mg silica nanospheres and was kept for sonication for 30–40 min with intermittent shaking upside down to ensure uniform dispersion via sonication. The total volume was then adjusted to 3 mL by the addition of respective pH buffers after adding desired concentration of PI. This mixture was incubated at temperature 4 ◦C for 3 h with mild shaking. For temperature

standardization, SiO_2N–PI mixture in pH 4 buffer was incubated at both 4 and 25 ◦C. Above mixtures was centrifuged at 5000 rpm for 5 min at their respective temperatures. Supernatant was carefully removed by inverting tubes upside down and examined for protein estimation present in free form, subtracting which withthe initial amount gives the amount bound to the silica. Protein estimation was carried out by Bradford assay (**Bradford, 1976**). To determine protein loading capacity, different concentration of PI (0.5, 1.0, 2.0, 3.0 and 5.0 mg) was incubated with 10 mg SiO_2N and proceeded to calculate bound amount of PI as described above. To determine the PI release, the above obtained SiO_2N–PI was kept for drying at 25 ◦C and then it was dispersed in four different solvents viz. acetate buffer pH 4.0 (100 mM), phosphate buffer pH 7.0 (100 mM), glycine-NaOH pH 10.0 (100 mM) and water. 1 mL of each solvent was used for dispersing SiO_2N–PI dry powder and kept for vortexing for 3 h at 25 ◦C. Samples were centrifuged for 6000 rpm for 6 min and supernatant was removed for further estimation for the released amount by Bradford assay.

4.4.4 Estimation of PI activity

Bovine trypsin and trypsin-like activity of HGPs were estimated using enzyme-specific chromogenic substrate, BApNA. 150 µL of diluted enzyme for BApNA assay were added to 1 mL of 1 mM substrate solution and incubated at 37 ◦C for 10 min. The reaction was terminated by the addition of 200 L of 30% acetic acid and absorbance was recorded at 410 nm. One proteinase unit was defined as the amount of enzyme required to increase the absorbance by 1.0 OD under the given assay conditions. For enzyme inhibitor assay, increasing amount of proteinase inhibitor was mixed with trypsin and HGP in desired amount (µM or µL) and the premix was incubated at 25 ◦C for 10 min. The residual enzyme activity was then estimated as explained for proteases. One PI unit is defined as the amount of inhibitor required for inhibiting one proteinase activity unit.

4.4.5 Visualization of PI Activity

PI activity was visualized by following X-ray gel contact print technique as reported (**Bradford, 1976**). Silica bound and unbound PI was loaded on native PAGE to check trypsin inhibitory activity. After electrophoresis, gel was equilibrated in 0.1 M Tris–

HCl buffer (pH 7.2) and then incubated in 0.04% bovine trypsin for 15 min. Gel was washed again with Tris–HCl buffer to wash off excess trypsin and overlaid on gelatin coated X-ray film for different time points to obtain best activity image. As trypsin degrades gelatine present on X-ray film, inhibitory activity bands appear (protected gelatin) at specific sites due to the presence of PI and inhibits trypsin from degrading gelatin. To determine PI activity in frass, it was suspended in 0.1 M Glycine-NaOH buffer pH 10 in the ratio of 1:2 and was heated at 70 ◦C for 15–20 min to inactivate other contaminant proteins. X-ray gel contact print technique was performed with 0.2 units of PI to visualize activity as explained.

4.4.6 Bio-physical Evaluation of Individual and Conjugated SiO_2N and PI

Surface charge measurements of SiO_2N, PI and SiO_2N–PI was carried out and represented by mean zeta potential (mV). Readings were measured by PALS (Phase Analysis Light Scattering) Zeta Potential Analyzer version 3.54 (Brookhaven Instruments corp.). For aqueous samples disposable acrylic cells and Plexiglas based electrodes were used. Readings were measured at pH 2 (0.1 M Glycine-HCl), 4, 7 and 10 (buffer composition explained above). Diluted samples were used for the measurements with 10 cycles per run. For FTIR, 1 mg of SiO_2N, PI (dried powder) or SiO_2N–PI powder was added in 100 mg KBr powder to make pellet. The FTIR spectrum was recorded on Thermo-Nicolet 670 spectrometer in the range of 400–4000 cm−1. To predict secondary structure variation, circular dichroism spectrum was monitored. To record spectra, JASCO J810 spectrometer equipped with peltier water circulation, thermostated six position automatic cell changer and variable slit system was used. Initial purging of the system was carried out with nitrogen gas. For sample preparation, 0.5 M PI bound to 1.2 mg of SiO_2N was used along with SiO_2N and PI as controls. Spectra were recorded at 25 ◦C in far UV region with scan speed 100 nm per minute, slit width of 100 m and the range was selected from 180 to 300 nm. Data analysis was done using origin Lab program 8.0 version. Fluorescence quenching experiment was carried out using monochromator based PerkinElmer LS-55; fluorescence spectrometer with FLWinLab as operating software was used which employs high energy pulsed Xenon source for excitation. It is equipped with peltier controlled fluid circulation, 4 position automatic cell changer accessories and variable slit system. To measure binding affinity quenching was carried out with 0.4 µM

protein quenched with increasing concentration of SiO_2N (0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 0.9, 1.1, 1.3 and 1.5 mg) and the total volume was make up to 2 mL. This SiO_2N–PI mixture was incubated for 30-45 min and used for spectra capturing. Samples were excited at 280 nm and scanned at emission range from 300 to 550 nm. Readings were recorded at 25 ◦C with scan speed of 100 nm per min, and slit width 9 nm. Data analysis was carried out by using Origin Lab programme.

4.4.7 Extraction of *H. armigera* Gut Protease (HGP) Activity

Midgut tissue was dissected from the fourth instar larvae of H. armigera and immediately frozen in liquid nitrogen and stored at −80 ◦C. For the extraction of HGPs, midgut tissue was homogenized in 0.2 M Glycine-NaOH buffer, pH 10.0 in 1:1 ratio and kept at 4 ◦C for 2 h. The suspension was centrifuged at 4 ◦C for 10 min at 13,000 × g and the resulting supernatant was used as a source of HGPs.

4.4.8 Insect Bioassays

Newly hatched larvae were transferred to chickpea flour based artificial diet (AD). For feeding bioassay, 2 days old neonates were then transferred to SiO_2N–PI containing artificial diet, along with control diets namely, Silica, PI, AD. For insect feeding, 90 Trypsin inhibitory units (TIUs) of PI were loaded on 10 mg SiO_2N which was further estimated by BApNA assay to calculate bound units of PI on SiO_2N which was found to be approx. 45 TIUs. Each insect was treated with 2 mg SiO_2N–PI containing 8–10 TIUs per gram of diet and assay was carried out for 8 days until insect turned to 3rd instar larvae. Similarly, control diets were prepared by adding same amount of SiO_2Nor PI as above. Following this, gut was extracted from the insects and stored at -80 ˚C for further biochemical assays.

Chapter 5

General Discussion and Future Outlook

Chapter 5: General Discussion and Future Outlook

It is astonishing to witness natures' bequest to its players with the capability to adapt and strive in a challenging niche. One of the important examples exhibiting this feature is evident in the plant and insect interplay displaying chemically mediated communication. Interaction of plants with insects occurs at a multi-trophic level involving pollinators, herbivores and predators. In particular, the dependency of herbivores on plants to obtain food and shelter in their natural ecosystem has led them to develop strategies to deal with the diverse array of metabolites produced by plants. At the same time, plants evolved to adjust their phenotypical status to limit herbivore attack. Crops, over a longer duration were domesticated to produce genotypes with improved yields, which have compromised their defense mechanism to some extent. Various strategies were developed to limit pest population *viz.* introducing desired traits to tolerate or resist pest attack, using chemical pesticides and developing transgenics. While the conventional system provides robustness; contemporary sustainable methodologies are required to accelerate the process of development of crops with desired traits. Thus, realizing the need of the hour for sustainable practice of pest management, plant defense compounds were identified from time to time.

Plant defense system is comprised of constitutive and inducible defense responses, which together are composed of plants morphological barriers and chemical compounds exerting toxic, repellent and antinutritive effects. It is pre-requisite to study the specificity of chemical diversities between particular plant-herbivore pair to devise appropriate controlling tactics. Wild relatives and non-preferred host plants were identified as important sources of resistance that can be explored for identification of defense compounds enabling their strategic utilization in limiting pest population. . Application of active ingredients *via* delivery vehicles provides several benefits, i) eco-friendly and cost effective, ii) flexibility to use in contrasting environments iii) targeted and controlled release and iv) plasticity to incorporate active ingredients with different mode of action for efficient efficacy by delaying pest resistance.

In this study, we have attempted to analyze the specific responses induced in wild relatives of *C. cajan* and cultivated pigeonpea to *H. armigera* and associated elicitation treatments. In a more targeted approach, well-documented plant defense

molecules called serine PIs were characterized from a non-host plant *C. annuum*. PIs efficacy and applicability in the external environment against *H. armigera* was evaluated.

5.1 Induced Proteome Changes in Pigeonpea and Wild Relatives of *C. cajan*

It is of primary importance to investigate the plant metabolic responses induced upon insect attack. These responses enable the plant to deal with pest damage either by tolerance or by producing defense molecules. On the contrary, insect-mediated changes in plant physiology may allow them to avoid plant defense mechanism while obtaining nutrition.

In the present study, an important legume, pigeonpea was studied to investigate changes in proteome upon elicitation and herbivory using tandem mass spectrometric analyses. Due to lack of extensive research at the molecular level and limited scope of crop improvements programs for insect resistance, it appeared apparent to conduct detailed proteome analyses.Wild relatives of pigeonpea were previously demonstrated to have potential against *H. armigera* (**Mallikarjuna *et al*., 2007**). Hence three wild relatives were selected for this study along with two cultivated varieties. Wild relatives selected for the study included *F. stricta*, *C. platycarpus* and *C. scarabaeoides* which belong to the quaternary, tertiary and secondary genepool, respectively. They were subjected to mechanical wounding with insects oral secretion treatment. Similar treatments were given to cultivated pigeonpea species namely: Vipula (tolerant) and ICPL 87 (susceptible), grown under similar conditions. Treatment with oral secretion functions by mimicking insect attack due to the presence of herbivore associated molecular patterns (HAMPs) in the form of specific chemical elicitors that can be readily distinguished by the plants (**Felton and Tumlinson, 2008; Mithofer and Boland, 2008**). Analysis revealed that most proteins showedsignificant up-regulation in WOS treated plants as compared to mechanical wounding. These findings ascertain plants ability to specifically perceive herbivore attack. Analysis of differentially expressed proteins revealed their role in photosynthesis, photorespiration, ROS signaling, energy and amino acid metabolism. For instance, proteins involved in limiting ROS damage such as carbonic anhydrases, malate dehydrogenases and glyceraldehyde 3-phosphate dehydrogenases were shown to be upregulated in various studies upon herbivory (**Fan *et al*. 2012; Giri *et al*. 2006;**

Collins *et al.* 2010). In addition, regulation of α- and β- isoforms of RCA was suggested to support in optimizing plant performance under attack (**Giri *et al.*, 2006**). Herbivory induced changes in primary metabolism have observed effects of elevated or suppressed photosynthesis together with remobilization of carbohydrate and amino acids (**Reymond *et al.*, 2004; Giri *et al.*, 2006; Wei *et al.*, 2009; Bilgin *et al.*, 2010; Coppola *et al.*, 2013; Appel *et al.* 2014; Botha *et al.*, 2006**). Reassignment of components from primary metabolism apparatus can have two consequences, one is to support the production of defensive metabolites and the other explanation is in regard with endowed ability of the plant to attain tolerance against herbivory. However, it is also challenging to decipher the function of metabolic reconfiguration whether it is induced by an insect for their own benefit (**Schwachtje and Baldwin, 2008**). Interestingly, Vipula, a tolerant variety of pigeonpea exhibited relatively greater fold changes in defense proteins against its respective control upon elicitation. This was further validated by employing 2D DIGE technique followed by protein identification by MALDI-TOF-MS.

It is clear that advancement in proteomics has contributed to ascertain important information in plant-insect interaction. Together with this, a high throughput combinatorial profiling methods, alongside bioinformatics tools may grant better insights into plants' overall responses to determine these changes. Proteins significantly upregulated in this study can be investigated for potential role in plant defense by gene silencing and/or overexpression experiments, (**Halitschke *et al.* 2003; Zavala *et al.* 2004; Kessler *et al.* 2004; Mitra *et al.* 2008**). Earlier studies on wild pigeonpea relatives suggested the presence of phagostimulants or antifeedants in the pod surface secretion exerting a differential effect on *H. armigera* growth/survival (**Sujana *et al.* 2012**). Thus, metabolite analysis of wild relatives could be performed to identify candidate molecules. This study can further be progressed by carrying out detailed molecular analyses at different time points to get a comprehensive view of plant physiological status and discover novel compounds for utilization against herbivores.

5.2 Plant Defensive Molecule: Microemulsion- based PI delivery and Applicability

The function of PIs has been documented in several studies demonstrating its antagonistic effect on *H. armigera* insect/larvae. In particular, *C. annuum* PIs were explored extensively for their structural and functional variation exhibiting their differential inhibitory activity against insect gut proteases (**Harsulkar *et al*., 1999; Tamhane *et al*. 2005; 2007; Mishra *et al*., 2010; Mahajan *et al*., 2013**). Its efficacy can be translated from lab- related studies to field level trials by developing stable transgenics (**Dunse *et al*., 2010; Gatehouse, 2011**). However, considering stringent regulatory issues and possibility of developing resistance by pest, it is imperative to look for an alternative application system. In this study, we have identified water: isopropanol: butanol (WIB) microemulsions (ME) as an efficient delivery vehicle for rCanPI-7 on crop plant(s) including chickpea and tomato. Microemulsions with bicontinuous domains form a thermodynamically stable water rich system for high protein loading. It further exhibits properties of easy synthesis, improved solubilization capacity, dose uniformity and longer shelf life. WIB bicontinuous ME (WIB-BCME) proved to be a promising blend of compounds displaying no adverse effect on chickpea and tomato leaf as monitored macroscopically or microscopically. Incorporation of CanPI-7 in BCME system maintained its activity upto several months and exhibited property of enhanced protein retention on the leaf. Interestingly, penetrating properties of the microemulsion in subcutaneous applications substantiated our result of BCME mediated locking of CanPI-7 on chickpea leaf. Hence, it would be appealing to explicate the fate of recombinant protein over time by determining its stability and overall efficacy.. Moreover, enhanced wettability and spreadability of WIB BCME were also recognized when applied on leaves offering an advantageous property to be used in application purposes.

Microemulsion system provides flexibility to readily mix defensive molecules with a different mode of action in order to delay resistance or to achieve broad-spectrum efficacy. However, the concentration range of protein to be incorporated is largely determined by the water percentage in addition to heat and cold storage stability considering environmental temperature variation. Presence of oil in microemulsion may serve to improve protein adherence on leaves avoiding the runoff

in moist conditions (**Paul and Moulik, 2001**). In addition, presence of surfactants in microemulsion not only stabilizes the system but also improve pesticide effectiveness by facilitating evenly spreading of pesticidal foliar sprays with prolonged adherence (**Ytl *et al.*, 2007**). These features altogether endowed microemulsion to become an attractive media for loading of active ingredient with wider range of solubility and increased bioavailability.

5.3 Evaluation of Nanoparticles as an Efficient PI delivery Vehicle

Research in nanoparticles has witnessed unprecedented growth in various branches of sciences. They are well- recognized for their drug delivery applications in terms of targeted delivery and controlled release. In this study we have used silica nanoparticles as a carrier system displaying unique features such as small size, large surface area and most importantly their surface functionalization enable them to be able to bind proteins, peptides or probes for their high loading. Mesoporous silica nanoparticles are attractive delivery vehicles due to its tunable pore size, high surface to mass ratio and biocompatibility (**Zhou *et al.*, 2015**). In this study, negatively charged monodisperse silica nanospheres in the size range of 200 to 250 nm were used and interacted with CanPI-13. CanPI-13 (6 kDa; pI 6.2) is a single IRD protein exhibiting specificity to trypsin whose activity against *H. armigera* gut proteases was determined. Ionic interactions mediated immobilization of CanPI-13 on SiO_2N at pH 4 facilitated by a slightly positive charge on the protein. Results obtained by incubating low to high range of CanPI-13 protein concentrations on nanospheres determined its high loading efficiency. Loading of protein on nanoscpheres is largely dependent on protein concentration which implies that as saturation is reached, repulsive forces among similarly charged PIs leads to its low rate of adsorption While investigating PI activity in bound state and its stability by determining secondary structure conformation, retention of its activity accompanied with no significant alteration in the secondary structure was obtained. This could be attributed to the presence of eight cysteine residues in the CanPI-13 sequence combine together in the form of four di-sulphide bridges rendering structural stability to the protein. In an attempt for the targeted release of CanPI-13 in the insect gut, pH-gated release of protein was performed with a range of pH and demonstrated effective release (56%) at alkaline pH, simulating insect gut environment (pH 10). It was noted that this

amount of CanPI-13 released was due to repulsive action as alkaline media imparts a negative charge on its surface. However, retention of remaining protein on silica surface suggests the involvement of other forces at the interface such as particle curvature, particle size and aggregation enabling tight binding for no further release.

We demonstrated the efficacy of silica nanosphere for stable loading of active ingredient and their targeted delivery in external environment. However, this study provides further opportunity to optimize particle characteristics such as surface functionalization, size, shape, surface smoothness/ roughness for augmented uptake and delivery purposes. Exploration of particles for high and controlled payload release is feasible *via* mechanisms such as adherence, adsorption or covalent binding as per the application. Plasticity to synthesize particles with chemical diversities and surface modifications enable loading of proteinaceous or non-proteinaceous molecules for their exploitation in plant protection.

5.4 Establishing *in vivo* Efficacy of PI on *H. armigera*

In this study we have used two structurally and functionally different isoforms of CanPIs having four (CanPI-7) and one (CanPI-13) functional inhibitory repeat domains, IRD. Previous studies have established their efficacy against *H. armigera* gut proteases (**Mahajan *et al*. 2013, Joshi *et al*. 2014**), whereas herein, we investigate its applicability while maintaining the efficacy of these proteins for their exploration at field level. BCME incorporated CanPI-7 sprayed on chickpea plants along with their respective controls were fed to newly hatched larvae till they metamorphosed to second instar larval stage. Larvae showed significant reduction in weight and mortality when fed on WIB-CanPI-7- treated plants. Analysis of insect gut protease by protease activity gel revealed inhibition of protease activity bands suggesting specific inhibition of proteases isoforms by the presence of CanPI-7. This observation is in consenses with previous study wherein diminishing of protease bands were observed along with induction of insensitive protease as a defense mechanism (**Mishra *et al*., 2010**). It is likely that targeting insects at neonatal stage may delay the ability of insect to undertake protective measures for its survival. For this, feeding assays for subsequent generation can be carried out to determine their adaptation to PI molecule and thus designing more efficient strategy.

In another approach, we have utilized silica-based nanoparticles for CanPI-13 targeted delivery in the larval alkaline gut milieu. *In vivo* evaluation of feeding CanPI-13 immobilized SiO_2N to *H. armigera* second instar larvae by incorporating in artificial diet significantly altered its physiology in terms of growth and development. Insect frass was analyzed to determine the fate of silica particles in the gut of insect by microscopic analysis. Intact silica particles were visualized whose presence was also validated by elemental analysis in CanPI-13- SiO_2N fed diet. Though, *in vivo* results, showed the small difference in the survival rate between CanPI-13 and CanPI-13- SiO_2N fed insects with high mortality in the former treatment. This can be explained by the presence of silica particle in the frass after several hours of feeding, which indicates insect inability to digest it, eventually creating an extra burden on the insect gut and restricting it from subsequent feeding.

In conclusion, it is prudent to understand the specific responses of plants upon herbivory to identify important proteins and/or metabolites having a role in defense. Advancement of high throughput technologies together with molecular biology and bioinformatic tools has made it feasible to carry out profiling experiments on large-scale while contributing significant information to the existing literature. Information obtained can be translated in a more applied way by employing interdisciplinary approaches. This requires identifying a potential compound or a strategy to limit pest invasion and their further characterization for enhanced applicability and efficacy at the field level.

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Neha Khandelwal

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Academic Profile:

2011-till date	National Chemical Laboratory, Pune, India Pursuing doctoral studies in Biotechnology (thesis submission in process)
2005-2007 (Distinction)	Rajiv Gandhi College (A.P.S. University), Satna, India Masters in Biotechnology with aggregate 77%
2002-2005	Indira Gandhi College (A.P.S. University), Satna, India Bachelors in Biology with 63%
2001	Higher Secondary Examination with 73%, Satna, India

Publications:

- Khandelwal N, Barbole RS, Banerjee SS, Chate GP, Biradar AV, Khandare JJ, Giri AP,Trends in integrated pest management using advanced micro- and nano-based formulations: challenges and perspectives. *J Integr Plant Biol*, 2015 (Under Review)

- Khandelwal N, Doke DS, Khandare JJ, Jawale PV, Biradar AV, Giri AP, Bio-physical evaluation and *in vivo* delivery of plant proteinase inhibitor immobilized on silica nanospheres. *Colloids Surf B: Biointerfaces*, 2015, ***130***: 84-92

- Tamhane VA, Dhaware DG, Khandelwal N, Giri AP, Panchagnula V, Enhanced permeation, Leaf retention and plant protease inhibitor activity with bicontinuous microemulsions. *J Colloid Interface Sci*, 2012, ***383***:177-83 (Equal contribution)

- Mishra M, Tamhane VA, Khandelwal N, Kulkarni MJ, Gupta VS, Giri AP, Interaction of recombinant CanPIs with *Helicoverpa armigera* gut proteinases reveals their processing patterns, stability and efficiency. *Proteomics*, 2010, ***10***: 2845-2857

Book Chapter:

Khandelwal N, Joshi RS, Gupta VS, Giri AP (2012) Protease inhibitors as bio-pesticides: Potential and constraints. Biopesticides in Environment and Food

Security, issues and strategies, Editors: Koul O, Dhaliwal GS, Khokhar S, Singh R, Scientific Publishers (India), Chapter #9, pp 146-181

Present Research Profile:

1. Legume, *Cajanus cajan* was studied for their molecular responses upon Lepidopteran insect attack. Differentially regulated proteins were identified in domesticated varieties and wild relatives of *C. cajan*. This was further validated with candidate gene expression profiling using semi quantitative expression techniques.

2. A non preferred host plant *Capsicum annuum* was explored for their potential to express defensive molecule, proteinase inhibitor against Lepidopteran insect. Existence of diverse types of proteinase inhibitor molecule was established with varied structure and function.

3. Cloning and recombinant expression of proteinase inhibitor was carried out to establish their activity in reducing growth and development of the insect *in vitro* and *in vivo.*

4. Efficiency of proteinase inhibitor was assessed by utilizing it in the form of bio-formulation using different delivery vehicles. Nanoparticles and micro emulsions delivery systems were utilized for the augmented activity and accessibility of proteinase inhibitor in the outer environment. These carriers demonstrated the feasibility and potency of the cargo delivery at large scale and open new avenues to design bio actives-multi functionalized carriers for their broad spectrum application for the protection of the plants against insect pests.

Techniques Learned

- Protein extraction and separation using 2-D DIGE (Difference Gel Electrophoresis)
- Identify differentially expressed protein spots using Progenesis SameSpots software
- Protein identification by MALDI-TOF and shot gun proteomic approach
- Transcript profiling of candidate genes by semi quantitative expression techniques
- Gene cloning, protein expression in *Pichia pastoris*, chromatographic separation of proteins, biochemical activity assays
- Techniques like Electron microscopy (SEM & TEM), Contact angle, and Conductivity measurements, Circular dichroism, Fourier transform infrared spectroscopy, Fluorescence intensity measurements

Teaching Abilities:

Attended workshop on "Introduction to teaching and learning in higher education-Part-A" held at Newcastle University, Newcastle upon Tyne, UK

Carried out demonstrations for one year in practical classes for students pursuing Bachelors in Science in Newcastle University, Newcastle upon Tyne, UK

Academic Awards:

- 2012-2013, awarded commonwealth split-site doctoral scholarship, UK to carry out part of PhD work at Newcastle University upon Tyne
- 2011-2014, awarded with council of scientific and industrial research – senior research fellowship (CSIR-SRF), India
- 2010, qualified graduate aptitude test of engineering (GATE) exam from biotechnology subject with 92 percentile
- 2010, won 2nd prize in poster presentation on science day held at National Chemical Laboratory - title "Development of recombinant proteinase inhibitor incorporated microemulsion for plant defence against pest"

Conferences and Workshops Attended:

Jan 15- Jan 17, 2015
Workshop on "Basic R for Life Sciences" organized by BioSakshat, Venture Centre, NCL Innovation Park, Pune, Maharashtra, India

Jan 20- Jan 22, 2015
Symposium on "Accelerating Biology 2015" held at Centre for Development of Advanced Computing (C-DAC), Pune, Maharashtra, India

Jan 16- Jan 18
8th Maharshtra State Inter-University Research Convention on "Sustainable Growth through Innovation" held at North Maharashtra University, Jalgaon, Maharashtra, India

Feb 24- Mar 01, 2013
Gordon research conference on plant –herbivore interactions held at Ventura, California, U.S

Oct 03- Oct 08, 2012
VI International Conference on Legumes Genetics and Genomics (VI ICLGG) held at Hyderabad, A.P, India

Nov 14- Nov 16, 2010
Volunteered for International Symposium on “Food Safety Issues- with Specific Emphasis on GM crops” held at National Chemical Laboratory, Pune, Maharashtra, India

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Additional interests and skills:

Reading- novels, science fiction, anecdotes
Writing- poems, circumstances based events, scientific articles,
Photography and badminton

IT skills: MS office (word, excel, PowerPoint and endnote)
