STUDIES ON THE CLONING AND EXPRESSION OF A SPIDER NEUROTOXIN, MU-DIGUETOXIN-DC1A, IN ENTOMOPATHOGENIC FUNGI FOR ENHANCED BIOACTIVITY

A thesis submitted for the award of the degree of

Doctor of Philosophy

Under the Supervision of

Prof. Gurvinder Kaur Saini

By

Balwant Singh

Roll No. 126106032

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Department of Biosciences and Bioengineering

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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Every challenging work needs self-efforts as well as the guidance of elders, especially who are very close to our heart. My humble efforts are

<u>*Dedicated to*</u>

My sweet and loving grandparents

whose affection, love, encouragements and prays of day and night make me able to get such success

My respected supervisor and teachers

whose guidance and constant support make me able to get such an honour

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
Department of Biosciences and Bioengineering
Guwahati – 781 039 (India)

DECLARATION

I do hereby declare that the content embodied in this thesis entitled "**Studies on the cloning and expression of a spider neurotoxin, Mu-diguetoxin-Dc1a, in entomopathogenic fungi for enhanced bioactivity**" is the result of investigations carried out by me in the **Fungal Biotechnology Research Laboratory**, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of **Prof. Gurvinder Kaur Saini**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

Date: 30/06/2020
Place: Jawali

Balwant
Balwant Singh
(Roll No. 126106032)

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
Department of Biosciences and Bioengineering
Guwahati – 781 039 (India)

CERTIFICATE

This is to certify that the work described in the thesis entitled "**Studies on the cloning and expression of a spider neurotoxin, Mu-diguetoxin-Dc1a, in entomopathogenic fungi for enhanced bioactivity**", submitted by **Mr. Balwant Singh** (Roll no: 126106032) for the award of the degree of **Doctor of Philosophy** is an authentic record of the research work carried out under my supervision in the **Fungal Biotechnology Research Laboratory**, Department of Biosciences and Bioengineering, Indian Institute of Technology, Guwahati, India.

This thesis or any part thereof has not been submitted elsewhere for the award of any other degree or diploma.

Date: Prof. Gurvinder Kaur Saini

Place: (Thesis Supervisor)

Tel. (Off.): 91–361–258 2207; Fax: 91–361–258 2249
Email: gurvinder@iitg.ac.in

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List of Abbreviations

Nucleic acid abbreviations

A	Adenine	G	Guanine
T	Thymine	C	Cytosine

Amino acid abbreviations

Amino acid	*Single-letter code*	*Amino acid*	*Single-letter code*
M	Methionine	R	Arginine
E	Glutamic acid	L	Leucine
S	Serine	V	Valine
A	Alanine	G	Glycine
K	Lysine	D	Aspartic acid
Y	Tyrosine	Q	Glutamine
P	Proline	C	Cysteine
W	Tryptophan	F	Phenylalanine

Other abbreviations

AaIT	*Androctonus australis* insect toxin
AcMNPV	*Autographa californica* nuclear polyhedrosis virus
BkCa	Calcium-activated big potassium channel
bp	Base pair
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
CAI	Codon Adaptation Index
Ca_v	Voltage gated calcium channel
CD	Circular dichroism
CDA	Czapek-dox agar
cDNA	Complementary DNA
CHCA	α-cyano-4 hydroxycinnamic acid
CNS	Central nervous system
Cry	Crystal δ-endotoxins
CTAB	Cetyltrimethylammonium bromide

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Cyt	Cytotoxins
Dc1a/DTX9.2	Mu-diguetoxin-Dc1a
DDT	Dichlorodiphenyltrichloroethane
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUM	Dorsal unpaired medican
EMBL-EBI	European Bioinformatics Institute of the European Molecular Biological Laboratory
EPA	Environmental Protection Agency
FACS	Fluorescence-activated cell sorter
FAO	Food and Agricultural Organization
FE-SEM	Field emission scanning electron microscopy
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
GABA	Glutamate- and γ-aminobutyric acid
GC	Guanine-cytosine
gcua	Graphical Codon Usage Analyzer
GM	Genetically modified
IC_{50}	Inhibitory concentration, i.e. concentration required for 50 % inhibition *in vitro*
IPTG	Isopropyl β- d-1-thiogalactopyranoside
ISVPs	Insecticidal spider venom peptides
JC-1	5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine Iodide
LB	Luria-Bertani broth
LC_{50}	The median lethal time
LD_{50}	The median lethal dose
LT50	Median lethal time i.e., the time taken to kill 50 % of the test population
MALDI-ToF	Matrix-assisted laser desorption/ionization-time of flight

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MBP	Maltose Binding Protein
MMP	Mitochondrial membrane potential
mre	Molar residual ellipticity
MTCC	Microbial Type Culture Collection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
nAChRs	Nicotinic acetylcholine receptor
Na_V	Voltage-gated sodium channels
NMWL	Nominal molecular weight limit
OD	Optical density
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PD_{50}	The dose of antiserum or vaccine that protects 50 % of the animals challenged
PDA	Potato dextrose agar
PDB	Protein Data Bank
pH	The decimal logarithm of the reciprocal of the hydrogen ion activity in a solution
PI	Propidium iodide
PMCL1	*Metarhizium* collagen-like protein promoter
PMSF	Phenylmethylsulfonyl fluoride
rDc1a	Recombinant Dc1a
rHPLC	Reverse-phase high-performance liquid chromatography
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SDA	Sabouraud dextrose agar
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SMGs	Selectable marker genes

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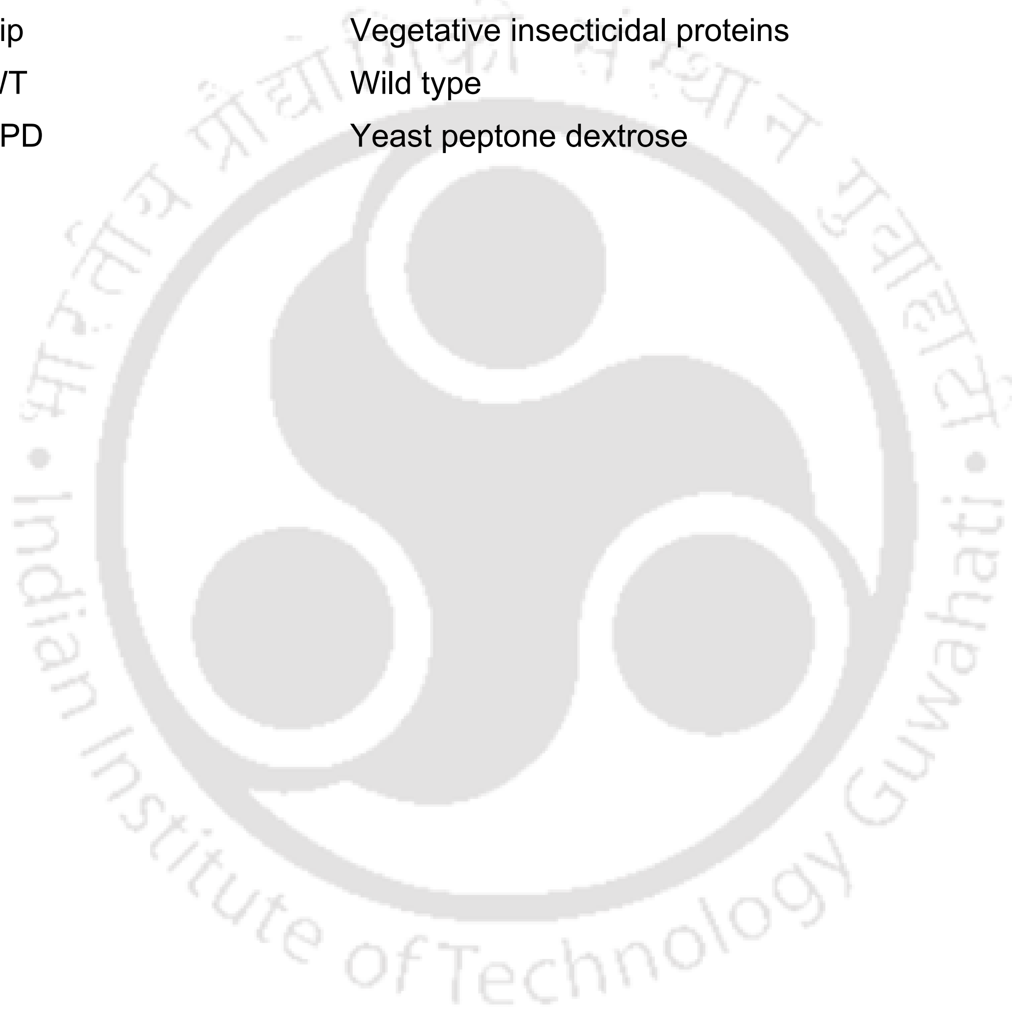
SVP	Spider venom peptide
TB	Terrific broth
TTX	Tetrodotoxin
UN	United Nations
UV radiation	Ultra violet radiations
VGSCs	Voltage-gated sodium channels
Vip	Vegetative insecticidal proteins
WT	Wild type
YPD	Yeast peptone dextrose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Units and symbols

A	Ampere
cm	centimeter
i.c.v.	Intracerebroventricular injection
i.p.	Intraperitoneal injection
kDa	kilo Dalton
L	Litre
M	Molar
mg	Milligram
min	Minutes
mL	Millilitre
mM	Millimolar
mM	Millimolar
ng	Nanogram
nm	Nanometer
nM	Nanomolar
rcf	Relative centrifugal force
rpm	Revolutions per minute
s	Second
v/v	Volume by volume
w/v	weight/volume
w/w	weight/weight
%	Per cent
°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometre
µM	Micromolar
′	Prime

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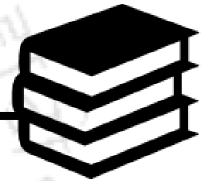
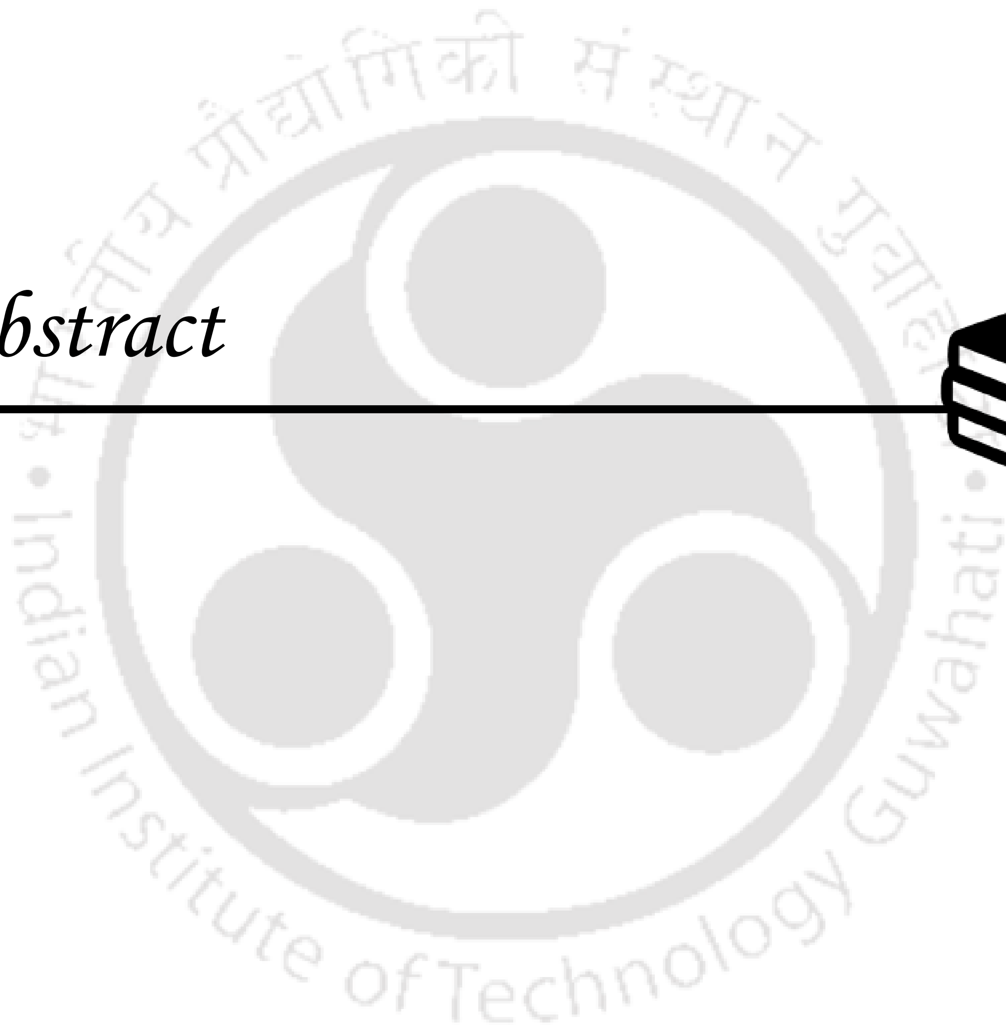
Abstract





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Insect pests are global problems that cause severe damage to crops and spread a multitude of insect-borne diseases. Their control is commonly based on chemical insecticides. The need for alternatives has heightened after the development of resistance in insect pests to almost all classes of chemical pesticides because of their persistent use from decades. Insect pathogenic fungi are considered as a promising alternative to chemical insecticides for efficient as well as environmentally benign insect pest control. However, the slow killing speed and requirement of high conidial concentrations are limiting this approach to become viable. Genetic engineering of fungal entomopathogens to express insecticidal transgenes encoding toxins have been shown to increase the efficacy and decrease the required conidial dose.

The insecticidal spider venom-peptide (ISVP) Mu-diguetoxin-Dc1a (Dc1a) from the desert bush spider (*Diguetia canities*) is one of the most potent insect-specific neurotoxins isolated to date. The toxin binds to the S1-S2 and S3-S4 loops in the domain II voltage-sensor of insect voltage-gated sodium (Na_V) channels (i.e. receptor site 4) in the insect central nervous system, a mechanism quite distinct from existing chemical insecticides. It induces paralysis that precedes death in a taxonomically wide range of insects. Dc1a's broad spectrum of target insects, a novel mode of action, and absence of toxicity to vertebrates make the Dc1a gene an attractive tool for engineering fungal entomopathogens. Thus, the primary aim of this thesis was to engineer transgene encoding highly potent ISVP Dc1a into *Metarhizium* and *Beauveria* in order to enhance its efficacy in controlling insect pests of different orders.

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To facilitate this, an efficient *Escherichia coli* periplasmic expression system was developed to produce sufficient amounts of rDc1a for characterisation and structural studies. Injection bioassays revelled that the intrinsic insecticidal activity of rDc1a was maintained and caused a spastic paralysis with median lethal doses (LD_{50} i.e., the dose required to kill 50 % of the test population) for tobacco cutworm (*Spodoptera litura*) was 0.416 nmol/g and for Cotton bollworm (*Helicoverpa armigera*) was 0.397 nmol/g, conclude that rDc1a has a similar lethality in lepidopteran larvae when compared with native toxin. Moreover, feeding bioassays showed a significant level of oral toxicity in Housefly (*Musca domestica*) (>75 % mortality at 48 h post-feeding) with LD_{50} of 85.39 nmol/g against adults of the dipteran insect vector. In contrast, rDc1a showed ~1000-fold less cytotoxicity effects towards cultured insect cells *in situ* compared to lepidopteran pests *in vivo*.

To achieve the primary aim of this thesis, the potent ISVP Dc1a was successfully expressed in *M. anisopliae* strains 892 & 3210 and *B. bassiana* 984. Using the promoter of MCL1 for genetically engineering *Metarhizium* and *Beauveria*, the expression of the target genes was limited to the hemocoel of the target insects, ensuring the targeted expression of the ISVPs in genetically engineered fungal strain. As a result, it will restrict the release of ISVPs into the environment, thereby engineered fungus would not interfere with non-target insects beyond their host range. Hemolymph induced expression of transgene Dc1a started within 20 min of induction and moderated until 12 h. Insect bioassays of genetically engineered strains against lepidopteran and dipteran insect orders revealed a significant improvement in virulence compare to wild type (WT) strain, with the engineered

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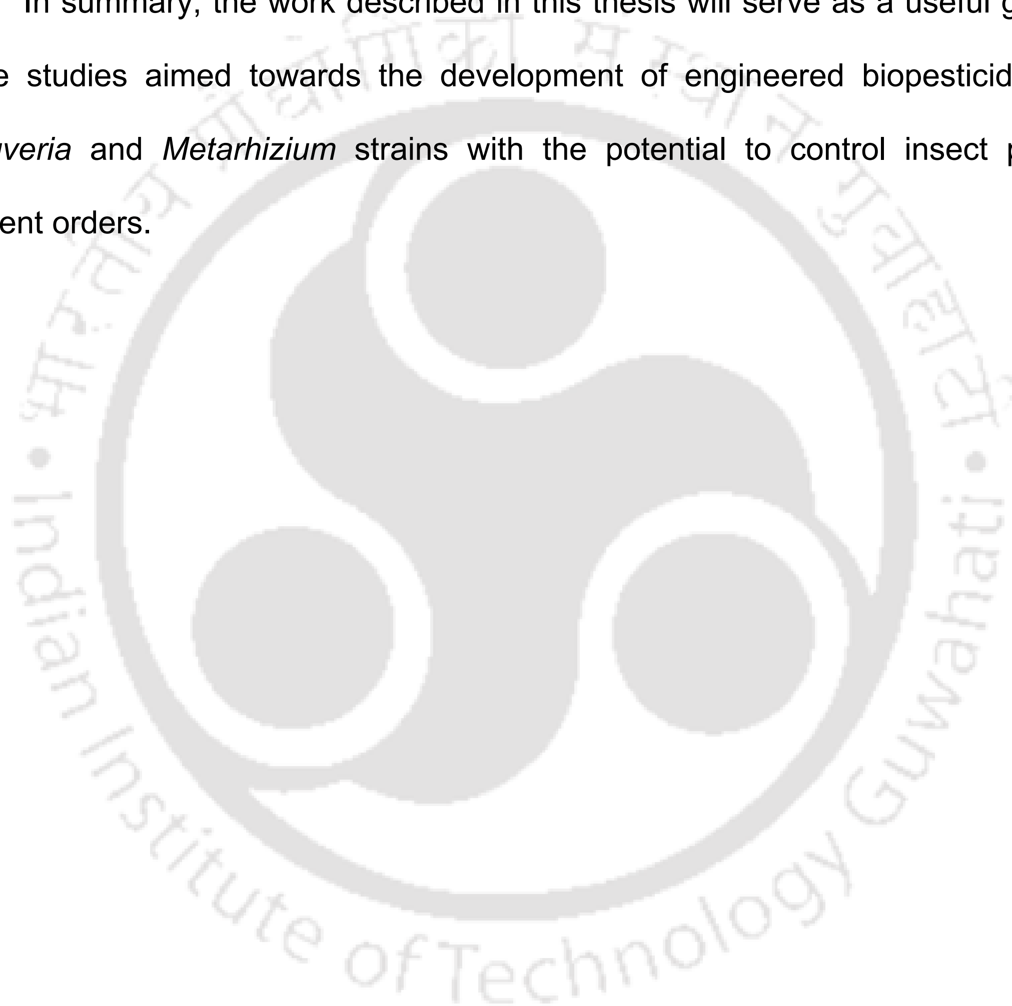
strains requiring less time to kill insect pests. The LT_{50} (i.e., the time taken to kill 50 % of the test population) values for genetically modified (GM) *Beauveria* and *Metarhizium* strains expressing ISVP Dc1a were 20–35 fold lower than wild-type strains.

In summary, the work described in this thesis will serve as a useful guide for future studies aimed towards the development of engineered biopesticides from *Beauveria* and *Metarhizium* strains with the potential to control insect pests of different orders.



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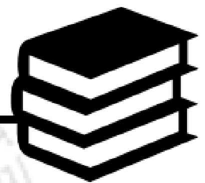
Chapter One



Introduction, Literature review and Aims

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This thesis illustrates studies aimed at developing genetically engineered fungal entomopathogen as an alternative mean of controlling insect pests. In brief, the bio-insecticidal potential of recombinant **i**nsecticidal **s**pider **v**enom **p**eptide (ISVP), Mu-diguetoxin-Dc1a, against cultured insect cells and **g**enetically **m**odified (GM) fungal entomopathogen expressing ISVP against lepidopteran-dipteran insect pests has been explored. Before addressing the scope of the work and the specific aims, an overview of the biological control agents and their prospects are highlighted. The currently used control measures with their potential limitations are explained. Besides this, significant importance of the bio-control agents, particularly, insect-pathogenic fungi expressing insecticidal transgenes, have been pointed out. However, social acceptance of these GM fungal entomopathogen fungi would be ensured through rigorous risk-benefit analysis considering short- and long-term aftereffects on non-target organisms and the environment.

1.1 General Introduction

Three billion, i.e. world's half of the population live in rural areas and approximately 2.5 billion of them are dependent on agriculture for their livelihood (FAO 2013). It has been projected by the **U**nited **N**ational (UN) that the global demand for food will rise to 70 % by 2050 (FOA 2009). Therefore, intensification and expansion of agriculture are required to substantially meet the growing demand for food (Choudhary *et al.* 2014). However, changes in climatic conditions, the resurgence of insect pests and disease pose threats to agricultural production (Sundström *et al.* 2014). More than 10,000 insect species have been found to damage food crops, with an estimated annual loss of 14 % globally (Oerke and

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Dehne 2004; Pimentel 2009) which costs approximately USD 100 billion. Besides this, three million people are killed annually by mosquito-borne disease, and over 130 human pathogens were carried by housefly (Enserink *et al.* 2013; Ansari, Moraiet and Ahmad 2014; Khamesipour *et al.* 2018).

In the present scenario, chemical pesticides are dominantly used for controlling insects from damaging crops, thus helping to feed the overgrowing populations and playing a significant role in protecting millions of lives from malaria and other insect-borne diseases. Approx. 4.6 million metric tons of pesticide are being applied to the crops globally to increase the productivity and control the vector-borne diseases. However, it leads to potential threats to human health, development of insecticide resistance, emergences of the secondary pest as a primary pest, and effects on non-target insects (Enserink *et al.* 2013; Ansari, Moraiet and Ahmad 2014). These issues with chemical pesticides are triggering the need to find alternatives (Vontas *et al.* 2011; Zimmer *et al.* 2014).

One approach is to use biocontrol agents such as bacteria, viruses, and fungi, which are naturally parasitic to insects. Among these, insect-pathogenic fungi, *Beauveria bassiana and Metarhizium anisopliae*, are now being extensively utilised as an alternative to pesticides. However, fungal pathogens have not satisfied the expectations as biocontrol agents because of relatively slow kill compared to chemical pesticides, the requirement for high amounts of inocula and inconsistent results compared to the chemicals with which they compete (Gressel 2001). Unlike bacteria and viruses that are ingested in order to cause disease, pathogenic fungi infect insects by breaching the host cuticle. Proteases, chitinases, and lipases

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secreted by these insect pathogens degrade the major constituents of the cuticle (i.e. protein, chitin, and lipids) and allow hyphal penetration (Cho *et al.* 2006; Wang and St. Leger 2006). In order to enhance the potency of *M. anisopliae*, St. Leger et al., (1996), engineered a strain that constitutively expressed an endogenous subtilisin-like protease gene (pr1A) and killed caterpillars (*Manduca sexta*) 25 % faster than the **w**ild-**t**ype (WT) strain (St Leger *et al.* 1996). A similar report on the over-expression of a chitinase gene (Bbchit1) enhanced the virulence of *B. bassiana* to aphids (*Myzus persicae*) (Fang *et al.* 2005). Recent advancement in molecular techniques has provided insight to increase the fungal virulence and specificity by incorporation of heterogenous genes such as scorpion, spider, and ant neurotoxins (Lu *et al.* 2008; Fan *et al.* 2012). These GM strains demonstrated the potential of insect toxic genes to increase fungal efficacy.

1.2 Food Security and Global scenario

Food security is a major global issue, as the rising human population has drastically increased the demand for food production, particularly in the developing countries (Boulter *et al.* 1990; Sharma, Sharma and Crouch 2004). It has been projected that global demand for food will significantly increase to 70 % by 2050 (FAO 2013) and food production has to be doubled to sustainably and equitably feed the projected 9 billion people. Along with this, rapid urbanisation and diminishing availability of arable land, it is important to increase crop yields to support the growing population (Tilman *et al.* 2011). Unfortunately, the effects of global warming have an immense negative influence on crop yields (Peng *et al.* 2004), causing an increased occurrence of crop failures and global movement of insects from warm to

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what were previously cooler regions (Porter, Parry and Carter 1991; Khasnis and Nettleman 2005). It has been estimated that one-fifth of the world's total crop production is destroyed by herbivorous insects annually (Oerke 2006). Hence, the **F**ood and **A**gricultural **O**rganization of the United Nations (FAO) has rightly stated: "To feed a growing world population, we have no option but to intensify crop production. But farmers face unprecedented constraints. In order to grow, agriculture must learn to save" (FAO 2009).

1.3 Crop losses and Human health risks

Arthropod insect pests cause extensive damage to the crops, which accounts for substantial economic loss to farmers annually (Oliveira *et al.* 2014). Consequently, their control is critical for achieving optimal crop yields. Around 20-50 % of the crop losses are caused by 10,00 species of arthropod pests globally (Scholthof 2003). Despite the annual investment of USD 40 billion on chemical pesticides use of biological control strategies and applications of chemical insecticides, current insect pest control measures are not able to reduce crop losses to a significant level. The major crop losses are caused by Phytophagous (plant-eating) insects which include insect pests like locusts and grasshoppers (*Orthoptera*), moths and butterflies (*Lepidoptera*), and beetles (*Coleoptera*) (Novotny *et al.* 2002). The most destructive of them is lepidopteran larvae (McCaffery 1998). Moreover, the insect species from the orders *Hemiptera* (plant sap-sucking bugs), Diptera (flies), Acarina (mites), and Thysanoptera (thrips) are also considered as major crop pests (McCaffery 1998; Nicholson 2007).

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Besides threatening food security, insect pests carry a variety of human and animal pathogens and pose a significant risk to human health (Tabachnick 2010; Mehlhorn *et al.* 2012). Mosquitoes cause more suffering than any other organisms as they transmit a variety of disease-causing parasites and viruses (Benelli 2015). Tropical fevers such as dengue fever, Chikungunya and Zika fever caused by flaviviruses are transmitted by *Aedes* sp. mosquitoes (Benelli 2015). Besides this, infectious disease caused by housefly, and vector-borne diseases like dengue fever, malaria will result in more than one million human deaths globally every year (Grillet *et al.* 2019).

1.4 Problems with chemical insecticides

Despite the increasing use of biological control and transgenic crops, chemical insecticides remain the dominant way to control arthropod pest (Whetstone and Hammock 2007; Day 2016). Although there is vast arsenal of available chemical insecticides but most of these insecticides act by altering the function of one of the six molecular targets in insect synapse: glutamate receptor: avermectins; **g**lutamate- and γ-**a**mino**b**utyric **a**cid (GABA)-gated chloride channels: fipronil and cyclodienes; voltage-gated sodium (Na_V) channels: pyrethroids, dihydropyrazoles and **d**ichloro**d**iphenyl**t**richloroethane (DDT); **n**icotinic **a**cetyl**ch**oline **r**eceptor (nAChRs): imidacloprid and spinosad; ryanodine receptors: cyazypyr and rynaxypyr; and acetylcholinesterase: carbamates and organophosphates (Tedford *et al.* 2004b; Nicholson 2007; Sattelle, Cordova and Cheek 2008). However, over 600 arthropod species have now developed resistance to one or more chemical insecticides due to

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the prolonged and widespread use of these insecticides (Bass and Field 2011; King and Hardy 2013) (Fig. 1.1).

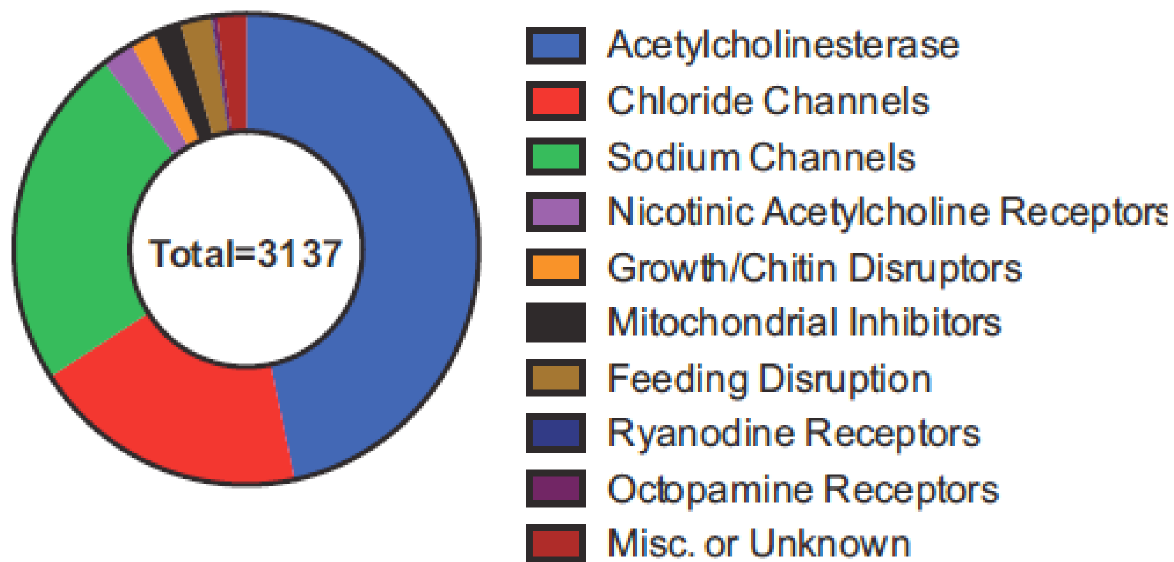

Fig 1.1: Total number of insecticide resistance cases grouped according to molecular target (adapted from Hardy 2014)**.**

For instance, there is 10 to 20-fold decrease in sensitivity to DDT or pyrethroid in Housefly (*Musca domestica*) due to a point mutation of leucine to phenylalanine (L1014F) in Na_V channels. It is further enhanced to >500-fold by a methionine to threonine substitution (M918T) in Na_V channels (Williamson *et al.* 1996). Similarly, fruit fly (*Drosophila melanogaster*) has developed resistance to cyclodienes and acetylcholinesterase inhibitors due to an alanine to serine substitution (A302S) in the GABA receptor (Ffrench-Constant *et al.* 1993) and four-point mutations in acetylcholinesterase (G265A, F330Y, G368A, and I1161V), respectively (Menozzi *et al.* 2004).

Besides the issues related to the development of insecticide resistance, there are biodegradability and poor selectivity concerns regarding the use of certain

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chemical insecticides. High toxicity and poor species selectivity of organophosphates pose ecological and health risks. In addition, exposure to organophosphates has been shown to have adverse effects on reproduction, development and cancer onset (Weisenburger 1993; Eskenazi, Bradman and Castorina 1999; Greenop *et al.* 2013). Some insecticides such as organochlorines are persistent organic pollutants that resist degradation leading to detrimental environmental effects and bioaccumulation (Haynes and Johnson 2000; Vives *et al.* 2005). The United States **E**nvironmental **P**rotection **A**gency (EPA) has deregistered and cancelled 169 chemical insecticides between the years 2006 to 2009 due to health, safety and environmental concerns, while only nine new insecticides were approved during this period (King and Hardy 2013; Smith *et al.* 2013). Thus, there is an urgent need to discover new and safe alternative methods for controlling insect pests so that crop productivity can be enhanced sustainably with reduced dependence on chemical insecticides.

1.5 Microbial control agents as bioinsecticides

Due to the problems of chemical insecticides mentioned above, there is an urgent need to develop new, eco-friendly insecticides. Crop protection strategies using biological control offers a promising area and an attractive alternative for insect pest management. Microbial control agents comprised of any molecules from the biological origin, whether it may be of the whole organism(s) or products derived from them (Villaverde *et al.* 2014). Their complex mode of action makes them less prone to resistance and hence considered to be less harmful to the environment. Concerning biopesticides usage, North America is the largest user (44 %), followed

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by Europe and Latin America and then Asia using 6 % of global biopesticide (Bailey, Boyetchko and Längle 2010). The use of microorganisms such as viruses, bacteria, fungi, and nematodes as bio-control agents has some notable successes.

1.5.1 Bacteria as bio-control agents

***B**acillus **t**huringiensis* (Bt), a soil-borne insect pathogen, secretes **cry**stal δ-**e**ndotoxins (Cry), **cyt**otoxins (Cyt) and **v**egetative **i**nsecticidal **p**roteins (Vip). It kills its host by effectively binding to insect midgut receptors such as cadherin, aminopeptidase-N and alkaline phosphatase, leading to the formation of pores in the epithelial cells residing on midgut (Bravo *et al.* 2011). *B. thurigiensis* based formulations have been used to control *Ostrinia nubilalis* Hubner (Lepidoptera: *carmbidae*), a major pest of corn in temperate regions (Crava *et al.* 2014), Beet armyworm (*Spodoptera exigua*) (Naimov *et al.* 2014) and combination of toxins also used against *S. littoralis* and *Ephestia kuehniella* (Elleuch *et al.* 2014).

1.5.2 Viruses as bio-control

Baculovirus is an insect pathogenic virus, having circular double-stranded DNA molecule. It has the potential to be used as a bioinsecticide because of its high specificity towards insects, which is considered safe to vertebrates and plants. Over 50 baculovirus products have been developed against a variety of insects (Bonning and Hammock 1996). The fusion of *Bt* toxins to the baculovirus expression system improves virulence to diamondback moth *Plutella xylostella* (Chang *et al.* 2003). In addition, expression of insect-specific neurotoxin AaIT along with Cry toxin in baculovirus showed reduced lethal time against *S. exigua* and increased insecticidal activity against *P. xylostella* larvae (Shim *et al.* 2009).

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1.5.3 Entomopathogenic fungi as bio-control

Entomopathogenic fungi or insect pathogenic fungi are those fungi that are pathogenic to arthropods such as insects, mites, and ticks, and responsible for their premature death (Glare and Milner 1991). There are almost 100,000 species of fungi that exist in different global ecosystems. Out of these about 750 have been identified as insect pathogens. They can survive for a long time in the soil as saprophytes (Keller and Zimmermann 1989). However, under favourable environmental conditions, these fungal pathogens cause natural epizootics in their susceptible insect hosts. Compared to other biocontrol agents, fungal entomopathogens infect a broader range of insects which includes *Diptera*, *Lepidoptera*, *Coleoptera*, *Hymenoptera* and *Homoptera* (Vilcinskas, Matha and Götz 1997). However, some entomopathogenic fungi infects a specific host, eg. *M. acridum and M. flavoviride*. Therefore, a wide variety of insects can be infected by conidia or blastospores of entomopathogenic fungi (Ferron 1978). Fungal conidia are robust and can withstand a range of environmental conditions. In addition, they can be applied in a different type of formulation (oil-based, water-based, granules or powder-based) (Prior, Jollands and Le Patourel 1988) and simple artificial media can be used for their mass-production (Jenkins and Goettel 1997). Minimum loss of viability occurs for conidial formulations stored at low temperatures (Stathers, Moore and Prior 1993; Hedgecock *et al.* 1995).

Over the past three decades, there has been much research on developing fungal bioinsecticides to control arthropod pests, mostly insect pests of crops and vector of human diseases. Most research has been directed to the genera

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Metarhizium and *Beauveria* whose distribution is diverse in nature. *M. anisopliae* is the most widely used *Metarhizium* species, *and B. bassiana* is the most widely used and studied *Beauveria* species throughout the world.

1.5.3.1 *Beauveria bassiana*

Beauveria bassiana is a facultative pathogen, named after *Agosatinio Bassi,* its discoverer which caused white muscardine fungal disease in silkworm.

The species *B. bassiana* can be taxonomically classified as:

Kingdom	:	Fungi
Division	:	Ascomycota
Class	:	Sordariomycetes
Order	:	Hypocreales
Family	:	Cordycipitaceae
Genus	:	*Beauveria*
Species	:	*B. bassiana*

It has been used to control pests of a wide variety of crops (Ferron 1981). The fungus has proven to be virulent against larvae of *Anopheles albimanus, Cx. tarsalis, Culex pipiens*, and Cx. *tritaeniorhynchus* (Clark *et al.* 1968; Sandhu, Rajak and Sherma 1993; Geetha and Balaraman 1999). Additionally, it has been shown to successfully infect and kill adult anopheline mosquitoes (Blanford *et al.* 2005; Farenhorst *et al.* 2008; Mnyone *et al* .2009), immature and mature stages of various species of ticks (Mwangi, Kaaya and Essuman 1995; Frazzon *et al.* 2000; Onofre *et al.* 2001; Samish, Ginsberg and Glazer 2004), *Triatominae* (Luz and Fargues 1997; Lecuona *et al.* 2001; Luz and Batagin 2005), and pupa and adults of the Mediterranean fruit fly *Ceratitis capitata* (Quesada-Moraga, Ruiz-Garci´a and Santiago-A´LvarezLVAREZ 2006).

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1.5.3.2 *Metarhizium anisopliae*

The fungus *Entomopthora anisopliae*, later renamed *Metarhizium anisopliae* Sorokin, was used for the first time in the 1880s to infect and kill healthy larvae of the scarab beetle *Anisoplia austriaca* (Steinhaus 1956).

The species *M. anisopliae* can be taxonomically classified as:

Kingdom	:	Fungi
Division	:	Ascomycota
Class	:	Sordariomycetes
Order	:	Hypocreales
Family	:	Clavicipitaceae
Genus	:	*Metarhizium*
Species	:	*M. anisopliae*

Studies using insects as bait for entomopathogenic fungi suggest that *Metarhizium* is a soil-borne fungal pathogen and predominantly infects the soil-dwelling insects (Rath, Koen and Yip 1992; Zimmermann 2007). It has a broad host range and has been used extensively as a biological control agent against arachnids, and insects of different orders (Boucias and Pendland 1998; Frazzon *et al.* 2000; Dutra *et al.* 2004).

1.5.3.3 Mode of action

Unlike, bacteria and viruses, which infect their host through the gut, most of the fungal entomopathogens cause infection by direct penetration through the insect cuticle (Fig. 1.2). Under favourable conditions, fungal conidia adhere to the insect cuticle, conidial bodies produce germ tubes and breach the cuticle layer to differentiate into swollen “holdfasts” called appressoria which will help in penetration.

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Roles attributed to the appressorium include anchorage for penetration, softening and degradation of the cuticle through enzymes (lipases, proteases, and chitinases), and concentration of components involved in penetration (Rath, Guy and Webb 1996).

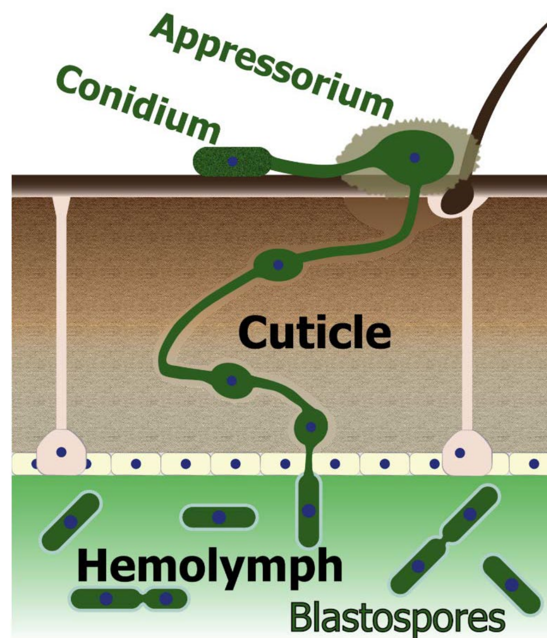

Fig. 1.2: Diagrammatic representation of cuticle penetration by entomopathogenic fungi using an appressorium followed by budding off as blastospores in the hemolymph (adapted from Lovett and St Leger 2017)**.**

There is a change in the morphology of the fungus from filamentous to proliferate to yeast-like hyphal bodies which will lead to the death of the insect because of a combination of actions, including physical obstruction, depletion of nutrients, or toxicosis or invasion of organs (Acevedo *et al.* 2007). Infection process takes several days, with the overall time to death depending mostly on fungal dose and virulence of the fungal isolate. These hyphal bodies will subsequently re-emerge from the cadaver to produce conidia. Since fungal pathogens do not require

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ingestion by the host, unlike bacteria and viruses, they can be used against sucking insects, such as aphids and mosquitoes.

1.5.3.4 Commercial development of fungal entomopathogens as bio-control agents

US EPA has approved more than 100 fungus-based biocontrol agents since 1995 (Shah and Pell 2003; de Faria and Wraight 2007). Mycotrol is a *Beauveria*-based formulation which is commercially produced in the USA and used as mycoinsecticide for control of aphids, grasshoppers, thrips and whiteflies (de Faria and Wraight 2007). GreenMuscle is a conidial formulation of *M. anisopliae* var. *acridum* that is commercially produced in Africa; which is selectively pathogenic to locusts and grasshoppers. These conidia formulations can remain infective for >12 months when stored at 25-30 °C (Jenkins and Goettel 1997; Lomer *et al.* 2001; de Faria and Wraight 2007) and showed no detrimental effects on non-target organisms (Lomer *et al.* 2001). Above mentioned studies demonstrate that fungal biopesticides are relatively safe for the environment, especially when compared to chemical pesticides.

1.5.3.5 Problems with entomopathogenic fungi as bio-control

Compared to the chemical insecticides, fungal pathogens infect insects at a slow rate and requires high inoculum volume, which is ultimately accounted for their small market share. Moreover, inconsistent performance in the field mitigates their potential as biological control agents (Fang, Azimzadeh and Leger 2012). The sensitivity of fungal pathogens to environmental stress conditions is responsible for their inconsistencies in performance in the field (Lovett and Leger 2015). Even use

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of more virulent strains takes 2-5 days to kill the host. It would allow the insects to make major crop damage even after the infection. Therefore, effective strategies are required to increase the virulence, by doing so, the pathogen could reduce the median **l**ethal **d**ose (LD_{50} i.e. the dose required to kill 50 % of the test population) and the median **l**ethal **t**ime (LT_{50} i.e. the time taken to kill 50 % of the test population) required in achieving high mortality.

1.6 Strategies used to improve the fungal virulence

The efficacy and virulence of mycoinsecticides can be improved by genetic engineering to tolerate environmental stresses. The main concern in improving the virulence of fungal pathogens is to reduce time to kill and conidial dosage. Major strategies currently being exploited to improve the virulence of entomopathogenic fungi are summarised in Table 1.1.

The most promising methodology for this approach includes overexpression of endogenous genes like proteases, chitinases, lipases, esterase, metalloproteases which are already present and expressed in entomopathogenic fungi during host infection. St. Leger *et al.* (1996) developed a method to improve the virulence by overexpressing a toxic protease Pr1 in *M. anisolpliae,* which exhibited a decrease in median lethal time to 25 %. Expression of endochitinase gene Bbchit in *B. bassiana* significantly reduced the level of lethal time to 50 % and dose to 50 % compared to that of WT strain (Fang *et al.* 2005). The fusion of chitin-binding domain BmChBD from *Bombyx mori* and chitinase from *B. bassiana* Bbchit1 can specifically bind to the chitin polymer targets in insects (Fan *et al.* 2007, 2011). It has been observed that transformant overexpressing *B. bassiana* Pr1A homolog (CDEP1) and Bbchit1

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Table 1.1: Genes and metabolic pathways that have been used to improve fungal virulence and tolerance to abiotic stresses (adapted from Zhao, Lovett and Fang 2016).

Aim	Type	Source
	Improve virulence	
Genes from entomopathogenic fungi		
Pr1A	Subtilisin-like protease	*Metarhizium robertsii*
CDEP1	Subtilisin-like protease	*Beauveria bassiana*
Bbchit1	Chitinase	*Beauveria bassiana*
Mr-Npc2a	Sterol carrier	*Metarhizium robertsii*
ATM1	Trehalase	*Metarhizium acridum*
Mr-Ste1	Esterase	*Metarhizium robertsii*
BbBqrA	Benzoquinone oxidoreductase	*Beauveria bassiana*
Genes from insect predators		
AaIT1	Na_V blocker	*Androctonus australis*
BmKit	Na_V blocker	*Buthus martensi*
LqhIT2	Na_V blocker	*Leiurus quinquestriatus hebraeus*
BjaIT	Na_V blocker	*Buthotus judaicus*
u-HXTX-Hv1a	Ca_V blocker	*Atrax robustus*
k-HXTX-Hv1c	BKCa blocker	*Hadronyche versuta*
Hybrid-toxin	Ca_V and KCa channel blocker	*Hadronyche versuta*
Genes from other insect pathogens		
Vip3A	Vegetative insecticidal protein	*Bacillus thuringiensis*
	Improve tolerance to abiotic stresses	
Try	Tryosinase	*Aspergillus fumigatus*
BbSOD1	Superoxide dismutase	*Beauveria bassiana*
DHN-melanin synthesis pathway	Three genes	*Alternaria alternate*
MrPhr1	CPD photolyase	*Metarhizium robertsii*
HsPHR2	CPD photolyase	*Halobacterium salinarum*
trxA	Thioredoxin	*Escherichia coli*
HSP25	Heat shock protein 25	*Metarhizium robertsii*

Na_V: Voltage gated sodium channel; Ca_V: Voltage gated calcium channel; BKCa: Calcium-activated big potassium channel

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exhibited a 24.9 % reduction in LT_{50} and 60.5 % reduction in LC_{50} (median **l**ethal **c**oncentration i.e., the concentration required to kill 50 % of the test population) (Fang *et al.* 2009).

Although endogenous genes have been exploited, an approach considered to have an enormous potential is to engineer the entomopathogenic fungi with genes from the heterogenous origin. It was first attempted in baculovirus using AaIT1 neurotoxin from *Antroctonus australis,* which acts explicitly on insect Na_v channel (Stewart *et al.* 1991). The same gene was also used by Wang and Leger (2007) to express specifically in insect hemolymph using ***M**etarhizium* **c**ollagen-**l**ike protein **p**romoter (PMCl1), which increased fungal toxicity 22-fold against adult yellow fever mosquitoes and tobacco hornworm caterpillars. Expression of ***A**ndroctonus **a**ustralis* **i**nsect **t**oxin (AaIT) gene along with protease gene Pr1A from *M. anisolpliae* in *B. bassiana* reduced the LT_{50} value to 40 % against the larvae of *Dendrolimus punctatus* (Lu *et al.* 2008). *M. acridum* has been genetically-modified to express BjαIT, an insect selective neurotoxin from *Buthotus judaicus* which resulted in increased virulence against locusts (Peng and Xia 2015). Cloning and expression of a vegetative insecticidal protein (Vip3Aa1) from *B. thuringiensis* increased 23-35 % virulence against *S. litura* (Qin *et al.* 2010) and *P. xylostella* (Liu *et al.* 2013).

1.7 Arthropod Venom peptides as bioinsecticides

Over the course of evolution (Fig. 1.3), arthropod predators such as centipedes, scorpions and spiders, have developed venoms for predation, defence and competitor deterrence (Brodie III and Brodie Jr 1999; Schwartz *et al.* 2012; King and Hardy 2013; Smith *et al.* 2013). Since early civilisation, humans have harnessed

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the biotechnological potential of arthropod venoms for varied and seemingly contradictory purposes.

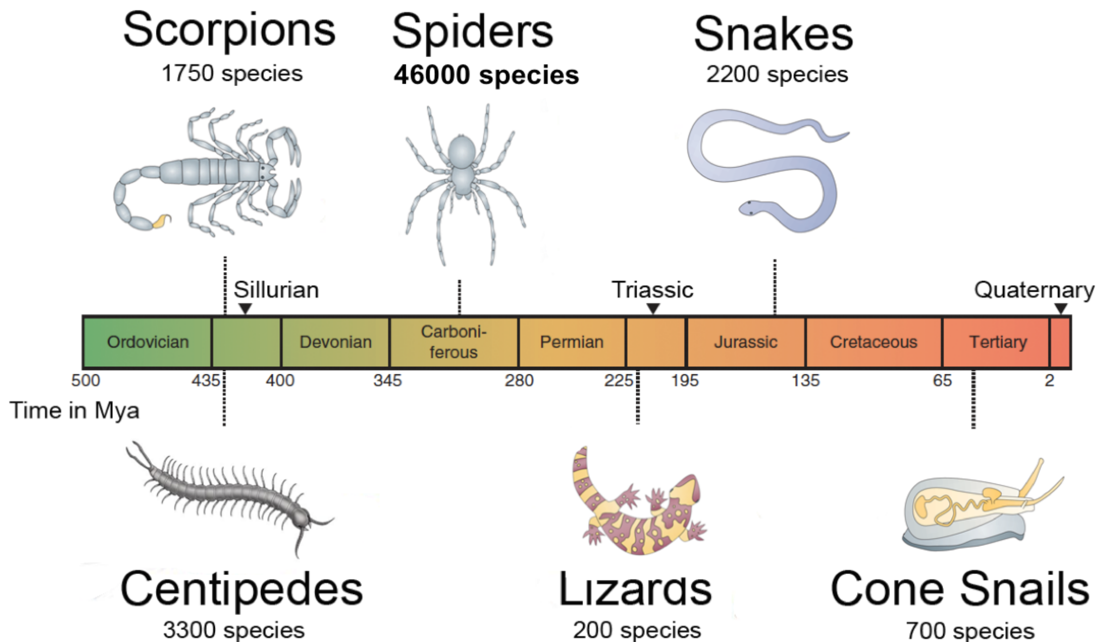

Fig. 1.3: Evolutionary timeline of selected venomous animals (adapted from King 2011)**.**

The incredible pharmacological diversity, complexity and potency of arthropod venoms have led to an explosion of venomics-based research and discovery programs for biotechnological applications such as the development of bioinsecticides (Lewis and Garcia 2003; Vetter *et al.* 2011; King and Hardy 2013). Arthropod venoms mostly consist of biologically active peptides (3−10 kDa) and proteins (> 10 kDa), and small organic molecules (< 1 kDa) (Escoubas, Sollod and King 2006). These peptides typically target the nervous system causing rapid incapacitation or paralysis, and they can be highly insect-selective. This remarkable selectivity and potency for their molecular targets have been developed in arthropods due to the selection pressure from millions of years (Smith *et al.* 2013).

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1.7.1 Spider venoms as a source for novel insecticidal peptides

Spiders are one of the most abundant terrestrial predators (Windley *et al.* 2012a). It is estimated that the number of existing spider species are nearly ~45,000 http://research.amnh.org/iz/spiders/catalog/counts.html). Based on a conservative estimate from ArachnoServer (www.arachnoserver.org), approximately 70 % of SVPs are predicted to have insecticidal activity (Herzig *et al.* 2010). Most spider venoms consist of disulphide rich peptides as the dominant compound, and they are responsible for the venom's insecticidal activity. These insecticidal peptides target pre or postsynaptic ion channels receptors either at synapses in the insect **c**entral **n**ervous **s**ystem (CNS) or at peripheral neuromuscular junctions (Fig. 1.4) (King and Hardy 2013; Smith *et al.* 2013).

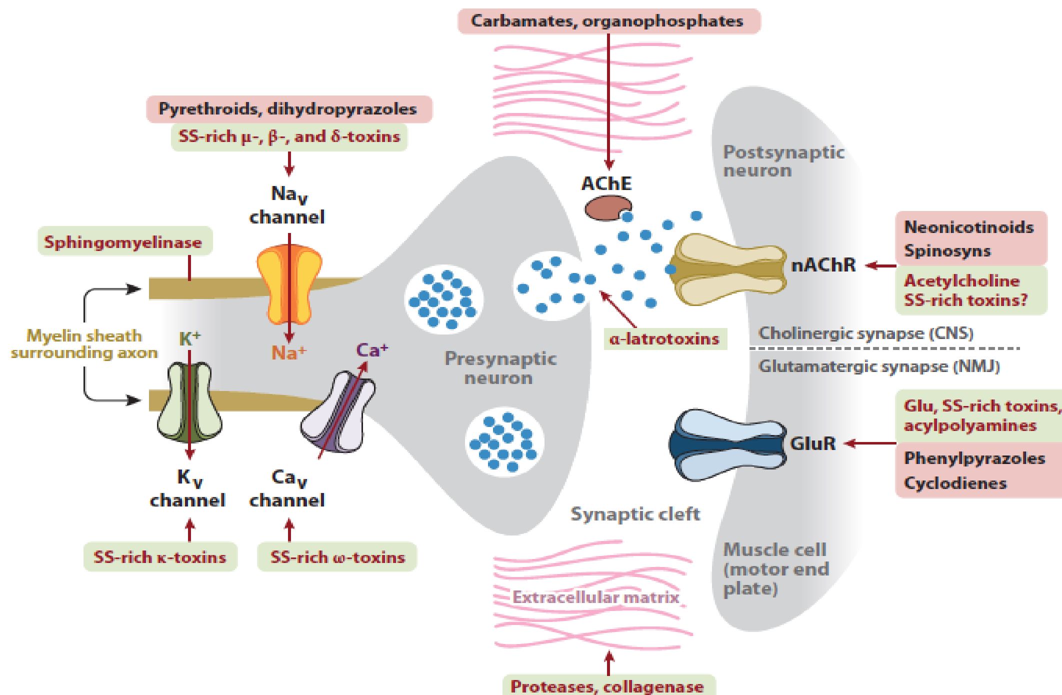

Fig. 1.4: Schematic of an insect synapse showing the molecular targets of spider-venom components (green boxes) and chemical insecticides (red boxes) (King and Hardy 2013)**.**

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Spiders mostly prey on insects, although other arachnids such as opilionids and mites, often contribute to their diet (Kuhn-Nentwig, Stöcklin and Nentwig 2011). Hence, the primary rationale for investigating spider venoms peptides as a potential source of bioinsecticides is that these insecticidal peptides are expected to have little or no vertebrate activity. To date, more than 200 disulphide-rich SVPs have been sequenced (Maggio *et al.* 2005; Windley *et al.* 2012a) having a molecular weight from 3.3 to 9.0 kDa (King and Hardy 2013). Table 1.2 provides a summary of characterised SVPs.

Some peptides isolated from Australian funnel-web spiders are lethal to multiple insect genera (*Coleoptera*, *Orthopetera*, *Lepidoptera* and *Diptera*) but harmless when injected into mice (Atkinson *et al.* 1998; Wang *et al.* 2000; King, Tedford and Maggio 2002). Recently, an ISVP was approved by the EPA for sale as a bioinsecticide in the United States (www.vestaron.com/epa-approval/). Although venoms are typically injected and not thought to be orally-active, several SVPs isolated from an Australian tarantula were found to be insecticidal when fed to termites (*Coptotermes acinaciformis*), mealworm beetle (*Tenebrio molitor*) larvae and ticks (*Amblyomma americanu*), further highlighting the potential of SVPs as bioinsecticides (Mukherjee *et al.* 2006; Hardy *et al.* 2013).

1.7.2 Insecticidal spider venom peptides to increase the bio-efficacy of entomopathogenic fungi

Entomopathogenic fungi are the natural enemies of insects-pests and can transport spider toxins to haemolymph by acting as a vector (Bonning and Nusawardani 2007; Whetstone and Hammock 2007). The genes encoding toxin

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Table 1.2: Insecticidal peptides from spider venoms together with their molecular target and effective dose.

Venom peptide	Source	Size* (in aa)	Molecular Target	Acute toxicity test species	Effective Dose	Reference
δ-ctenitoxin-Pn1a	*Phoneutria nigriventer*	48	Na_V channel	*Musca domestica*	LD_{50} 0.418 pmol	(Figueiredo *et al.* 1995)
κ-hexatoxin-Hv1c	*Hadronyche versuta*	37	BKCa channel	*A. domestica*	LD_{50} 167 ± 10 pmol/g	(Wang *et al.* 2000)
				M. domestica	LD_{50} 320 ± 20 pmol/g	(Maggio and King 2002a)
				M. domestica	LD_{50} 91 ± 5 pmol/g	(Maggio and King 2002b)
μ-agatoxin-Aa1d	*Agelenopsis aperta*	37	Na_V channel	*M. sexta*	LD_{50} 9,510 pmol/g	(Skinner *et al.* 1989)
μ-diguetoxin-Dc1a	*Diguetia canities*	56	Na_V channel	*Heliothis virescens*	PD_{50} 3,800 pmol/g	(Krapcho *et al.* 1995)
ω-hexatoxin-Hv1a	*Hadronyche versuta*	37	Ca_V channel	*A. domestica*	LD_{50} 89.0 pmol/g	(Wang *et al.* 1999; Mukherjee *et al.* 2006)
				M. domestica	LD_{50} 77.0 pmol/g	(Mukherjee *et al.* 2006)
					IC_{50} 279,000 pmol (M-LVA)	(Chong *et al.* 2007)
					LD_{50} 1080,000 pmol (HVA)	(Chong *et al.* 2007)
U1-AGTX-Ta1a	*Tegenaria agrestis*	50	N.D.	Tobacco budworm	PD_{50} 890 pmol/g	(Johnson *et al.* 1998)
				Cabbage looper	PD_{50} 780 pmol/g	(Johnson *et al.* 1998)
				Beet armyworm	PD_{50} 900 pmol/g	(Johnson *et al.* 1998)
				Southern corn rootworm	PD_{50} 2,000 pmol/g	(Johnson *et al.* 1998)
γ-ctenitoxin-Pn1a	*Phoneutria nigriventer*	47	NMDA receptor	*M. domestica*	LD_{50} 1.03 pmol	(de Figueiredo *et al.* 2001)
δ-ctenitoxin-Pn1b	*Phoneutria nigriventer*	48	Glutamate uptake	*M. domestica*	LD_{50} 3.84 pmol	(Oliveira *et al.* 2003)
U1-CUTX-As1c	*Apomastus schlingeri*	76	N.D.	Subcutaneous injection	LD_{50} 2.4 pmol/g	(Skinner *et al.* 1992)
μ-cyrtautoxin-As1a	*Apomastus schlingeri*	37	Na_V channel	*M. sexta*	LD_{50} 133 pmol/g	(Skinner *et al.* 1992)
				S. exigua	LD_{50} > 2.66 pmol/g	(Skinner *et al.* 1992)
				Lucilia cuprina	PD_{50} 700 ± 35 pmol/g	(Bende *et al.* 2013)
ω-hexatoxin-Hv2a	*Hadronyche versuta*	45	Ca_V channel	*A. domestica*	PD_{50} 160 ± 9 pmol/g	(Wang *et al.* 2001)
				Bee brain neurons	EC_{50}~139 pmol	(Wang *et al.* 2001)
U1-NETX-Csp1a	*Calisoga sp.*	39	N.D.	*Heliothis virescens*	PD_{50} 0.265 pmol/g	(Johnson, Kral Jr and Krapcho 1997)

*: Mature toxin; HVA: High voltage activated; LVA: Low voltage activated; IC_{50}: The half maximal inhibitory concentration; PD_{50}: The dose of antiserum or vaccine that protects 50 % of the animals challenged; LD_{50}: The median lethal dose; EC_{50}: The half maximal effective concentration, N.D.: Not determined; Na_V: Voltage gated sodium channel; Ca_V: Voltage gated calcium channel; BKCa: Calcium-activated big potassium channel; NMDA receptor: N-methyl-D-aspartate receptor

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peptides could be engineered into entomopathogens and be produced in the insect host after pathogen infection, which was proven to be a beneficial method to avoid spider toxins being degraded by enzymes in the insect gut (Ikonomopoulou and King 2013). For example, some fungi, which have already been used for insect pest control, whilst not ingested by the hosts, could penetrate the cuticle directly (Leger and Wang 2010). Engineering *M. anisopliae* fungus to overexpress its cuticle degrading protease, Pr1, increased the efficacy of this fungus (St Leger *et al.* 1996). This is because a large amount of Pr1 can cause more degradation of insect cuticle, which facilitates *M. anisopliae* to get into the haemolymph to infect the target insects-pests easily. Furthermore, *M. anisopliae* was also engineered to express AaIT. The toxicity of AaIT in the engineered recombinant fungal pathogen was highly improved against the mosquitoes, tobacco hornworm and coffee berry borer beetle (Pava-Ripoll *et al.* 2008). Therefore, if *M. anisopliae* fungus was engineered to express spider toxins, these fusion proteins should be more insecticidal as the fungus could mediate transport of spider toxins to hemolymph by acting as the vector.

1.7.3 Improving the efficacy of mycoinsecticides to control vector-borne diseases

Arthropod insects (*Anopheles* or *Aedes* mosquitoes, biting midges, and xylem feeding leafhoppers) act as vectors and cause malaria, dengue fever, bluetongue, and Pierce’s disease in human, animal, and plants. Most of these vectors are prone to entomopathogenic fungi infection (Fang, Azimzadeh and Leger 2012) such as *M. anisopliae* and *B. bassiana*, can naturally kill adult mosquitoes. However, the

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efficacy of fungal pathogen towards these vectors is very less, and it takes about 14 days for *Plasmodium* to develop from ingested gametocytes to infectious sporozoites. However, malaria transmission by mosquitoes could be blocked by fungal strains with enhanced killing speed even at a late-stage of *Plasmodium* infection. *Metarhizium*-based insecticides have been developed and applied to sheep to manage vector *Psoroptes* mites (Brooks *et al.* 2004; Abolins *et al.* 2007) to improve their health simultaneously. The virulence of *M. anisopliae* and *B. bassiana* can be increased to a remarkable extent by expressing spider venom peptides, which can reduce the LC_{50} many-fold without developing resistance.

1.7.4 Minimizing effects on beneficial insects via Tissue-specific expression of insecticidal transgenes

Pollinators such as bees and natural predators such as beetles and wasps that prey on pest insects are considered as beneficial insects. However, due to the extensive use of generic chemical pesticides, these beneficial insects are targeted. Besides this, transgenics expressing insecticidal toxins unintentionally subjected these beneficial insects. However, via the use of tissue-specific promoters, the expression of these insecticidal toxins can be limited to a specific tissue. This is one of the attractive strategies to avoid detrimental effects on beneficial insects. Additionally, targeted expression of transgenes using development stage-specific promoters can be used to facilitate gene expression at certain stages of fungal development. An important strategy is to employ promoters that are tightly regulated by insect-related signals to ensure targeted deployment of insect-specific toxins. MCL1 gene is required for defence against phagocytosis and it is one of the

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pathogen's most highly up-regulated genes in the insect hemolymph (Wang and St. Leger 2006). Using the promoter of MCL1 (PMCl1) to drive transgene expression ensures that the transgene is expressed in the insect body cavity, but not outside the insect. PMCl1 is the only development stage-specific promoter used in the genetic engineering of entomopathogenic fungi till date (Wang and St. Leger 2006). With the genome-wide characterisation of fungal development in insects, more development stage-specific promoters can be identified.

1.7.5 Approaches to reduce the safety concerns associated with transgenic entomopathogenic fungi

Safety and environmental concerns are associated with transgenic fungal entomopathogens similar to genetically-modified organisms. Primarily, GM pathogens should not reproduce and persist in the agricultural fields. It can be achieved by providing selective conditions to reduce the ecological fitness for recombinant fungal pathogen so that it will lose competitiveness with native strains and possibly go extinct from the environment (Council). It was found that removal of *M. robertsii* photoreactivation system resulted in a strain that was highly sensitive to UV radiation (Fang, Azimzadeh and Leger 2012). UV hypersensitive strains with hypervirulence from expression of anti-insect or antimalarial proteins will be especially useful in controlling mosquitoes and malaria transmission because the genetically engineered strains cannot spread into the environment from the application site (e.g., mosquito traps or houses). Moreover, the ability of *Metarhizium* to survive over winter in a temperate climate could be greatly reduced by disrupting the RNA binding proteins that allow it to adapt to the cold (Fang and St. Leger 2010).

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M. robertsii is a potent insect pathogen that is also ubiquitous in the soil community, where it establishes mutualistic interactions with plants as a rhizospheric fungus (Behie, Zelisko and Bidochka 2012). It was found that an oligosaccharide transporter (Mrt) is essential to the rhizosphere competency of *M. robertsii* (Fang and Leger 2010). Disrupting Mrt significantly reduced rhizosphere competence, but not its pathogenicity to insects, demonstrating that Mrt is exclusively involved in *M. robertsii*'s interactions with plants. The reduction in rhizosphere competence could significantly reduce the persistence of fungus in the environment (Wang *et al.* 2011).

Selection agents and **s**electable **m**arker **g**enes (SMGs) are useful tools in the selection of transformants of genetically engineered fungi from untransformed cells. Genes that confer herbicide or antibiotic resistance traits are expressed by most of these SMGs. In many countries, the existence of SMGs in transgenics is a matter of concern as they are subjected to special government regulations. Hence, it is desirable to remove genes encoding SMGs in the final genetically engineered strains or to use alternatives (Luo *et al.* 2007). Auxotrophic mutants are considered as an obvious alternative to SMGs. Moreover, Luo et al., (Luo *et al.* 2007) proposed an SMG-removal method in transgenic plants with increased precision of genome modification. Similar strategies like Cre-loxP recombination can be applied to genetically engineered entomopathogenic fungi. The resulting strains are free of SMG and have only a short DNA fragment of the LoxP recognition sites.

1.8 The insecticidal spider-venom peptide Mu-diguetoxin-Dc1a

Mu-diguetoxin-Dc1a (alternate name DTX9.2 or Dc1a) is one of the most potent insect-specific SVP isolated from the desert bush spider (*Diguetia canities*).

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The species *D. canities* can be taxonomically classified as:

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Arachnida
Order	:	Araneae
Family	:	Diguetidae
Genus	:	*Diguetia*
Species	:	*D. canities*

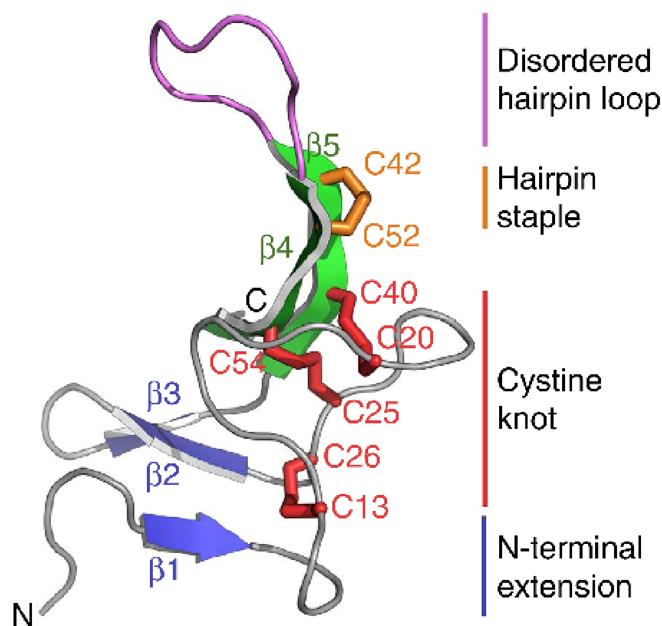

Fig. 1.5: Ribbon representation of rDc1a highlighting the five β-strands (b1–b5) and four di-sulphide bonds. The three di-sulphide bonds that form the ICK motif are shown in red while the fourth di-sulphide that staples the base of the disordered β4–β5 hairpin loop is highlighted in orange (adapted from Bende *et al.* 2014).

It consists of 56 amino acids including 8 half cysteine residues at the positions 12-25, 19-39, 24-53, 41-51. The structure of Dc1a includes a unique three-stranded beta-sheet derived from an extended N-terminal segment and a large inter cystine loop between Cys25 and Cys39 and a classical cystine knot motif (Fig. 1.5)

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(Bende *et al.* 2014). The toxin binds to the S1-S2 and S3-S4 loops in the domain II voltage-sensor of insect Na_V channels (i.e. receptor site 4) (Fig. 1.6).

It paralyses lepidopteran and dipteran larvae but is non-toxic to mice at 657 pmol/g (4.2 mg/kg) i.p. and 156 pmol/g (1.0 mg/kg) i.c.v. Paralysed insects ultimately die from secondary effects of starvation and dehydration (Krapcho *et al.* 1995). Incorporation of a transgene encoding this toxin into *Autographa californica* nuclear polyhedrosis virus enhances the efficacy of the virus in several lepidopteran hosts (Hughes *et al.* 1997). The American cockroach *Periplaneta americana* is largely resistant to the effects of Dc1a due to an unusual sequence within the domain II S1-S2 loop (Bende *et al.* 2014).

Fig. 1.6: Schematic representation of site of action of ISVP Dc1a on insect Na_V channel.

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1.9 Scope of the thesis

Control of insects-pests using conventional approaches of chemical pesticides has become a major challenge in modern agriculture. Transgenic fungi expressing insecticidal genes from heterologous origin provide an alternative method for insect pest control. Crop-growing countries have already demonstrated their efficacy by successfully adopting the *Bt* crops. The emergence of resistance in pest species and the adverse effects of chemical pesticides have triggered research to explore more insect-toxin transgenes.

Arthropods venoms are considered to be an arsenal of insecticidal peptides and have enormous biotechnological potential in the agrichemical fields. Plenty of these insecticidal peptides have been isolated from scorpions, spiders, and centipedes having bio-insecticidal properties including rapid kill speed, broad range activity against insect pests, low production costs, high potency, and lack of toxicity towards vertebrates. Mode of action in some of these peptides is unique, consequently, make them useful for the management of insect pests which have developed resistance to chemical pesticides.

The basis of this proposal is to exploit the diverse class of spider toxins, which include many insect-specific deadliest toxins that are produced mainly as defence molecules against predators or for hunting prey for food. These insecticidal toxins act primarily by targeting Na_v channels. However, the potency of different ISVPs varies based on their structural and amino acid content. Hence, there is a dire need of studies to find potent ISVPs that can be used directly as formulations or to increase the virulence of biocontrol agents. Upon exhaustive literature review, it was

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found that the venom of desert bush spider (*D. canities*) contains a 56 amino acid peptide known as Dc1a. It is one of the most potent insect-selective neurotoxins found in arthropod venoms (Krapcho *et al.* 1995). Its neurophysiological studies on housefly larvae and **d**orsal **u**npaired **m**edican (DUM) neurons of American cockroaches revealed excitation of sensory and neuromuscular preparations on the application of Dc1a (Bloomquist *et al.* 1996; Bende *et al.* 2014). Acute toxicity of Dc1a coupled with insensitivity against human Nav channels, could be exploited for augmenting the virulence of the biocontrol agents. Thus, studies on the expression of ISVP Dc1a to increase the virulence of entomopathogenic fungi is of utmost importance and to address concerns associated with chemical pesticides.

1.10 Hypotheses

- Treatment of recombinant Dc1a (rDc1a) will significantly reduce cell proliferation and viability of cultured cells of lepidopteran insects *in situ*.
- Injection-based bioassays with rDc1a will show a cessation of feeding or death in lepidopteran and dipteran insects-pests within 24-48 h.
- The fungal expression of Dc1a under the control of tissue-specific promoter PMCl1 will limit the expression to the hemocoel of the target insects.
- Lepidopteran and dipteran insects-pests infected with transgenic fungi will die early post topical infection compared to wild types.

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1.11 Aims of PhD work

Overall Aims

Considering the immense biotechnological potential of ISVP Dc1a in agrichemical fields, the overall aims of this research work were: (1) to evaluate the bio-insecticidal potential of rDc1a; (2) to improve the virulence of entomopathogenic fungi. *Escherichia coli* expression system was used to produce the recombinant protein, and *Agrobacterium tumefaciens* was exploited for gene transfer to the entomopathogenic fungi.

Specific Aims

The specific aims of this research work are:

Aim 1: To overexpress, purify spider toxin peptide Dc1a in *E. coli*, and its characterisation and structural studies.

Aim 2: To evaluate the insecticidal activity of rDc1a against cultured insect cells and insect pests *in situ*.

Aim 3: Heterologous expression of spider toxin peptide Dc1a in *Metarhizium* and *Beauveria* under the control of hemolymph specific promoter.

Aim 4. To evaluate the bio-insecticidal potential of transgenic fungi against insect pests.

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1.12 Outline of the thesis

The outline of the thesis is as follow:

Chapter 1 collates a review gathered through published literature on the crop losses due to arthropod pests, and food security. The literature emphasising the concerns with the use of synthetic chemical pesticides was highlighted. The role of various promising alternatives and their efficacy in modern agriculture was discussed. The heterologous expression of ion channel modulators peptides in *Metarhizium* and *Beauveria* and their potential to be used as bio-insecticide was discussed in detail.

Chapter 2 describes the experiments that were aimed to recombinantly express and purify the ISVP Dc1a from the periplasm of *E. coli*. Experiments related to characterisation studies were carried out to confirm the structural stability of rDc1a peptide. Bioassays conducted to study the bio-insecticidal potential of the rDc1a against cultured insect cells and insect pests *in situ* were described.

Chapter 3 focuses on the experiments related to cloning of the ISVP Dc1a in entomopathogenic fungi and its expression studies at a transcriptional and translational level under the control of PMCl1 promoter. This chapter also illustrates the experiments conducted to examine the bio-efficacy of transgenic fungi.

Chapter 4 summarises the research findings from the experimental chapters. Suggestions for future prospects to further improve the use of entomopathogenic fungi were highlighted.

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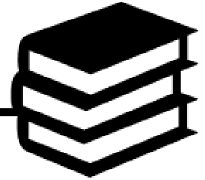
Chapter Two



Heterologous expression of a disulfide-rich insecticidal spider peptide under oxidising environment and its functional characterisation against insects of different orders

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2.1 Introduction

Herbivorous insects, especially those belonging to order lepidoptera cause a significant loss in crop yields and damage to stored grains (Oerke and Dehne 2004; Pimentel 2009; Boyer, Zhang and Lempérière 2012). In the context of human disease, dipteran insects are vectors for a multitude of animal and human pathogens (Benelli 2015). The preferred method for controlling pest populations still relies on chemical insecticides (Oerke 2006; Smith *et al.* 2013). However, extensive use of generic chemical insecticides to control insect pest population poses potential risks to human health and the environment because of the appearance of insect resistance. Besides this, issues with biodegradability and poor selectivity have negative impacts on the environment (Aktar, Sengupta and Chowdhury 2009) which consequently have led to cancellations and deregistration of chemical pesticides by regulatory authorities. Besides this, stringent registration requirements for new insecticides have significantly limited the arsenal of available insecticides (Bende *et al.* 2013) resulting in an urgent need to discover new and safe alternative methods for controlling insect pests.

Biological control methods such as insecticidal peptide toxins especially from spider venom have been the objects of increasing interest in the last decade and have been shown to be a promising alternative for replacing chemical agents (Maggio *et al.* 2010; Windley *et al.* 2012b; Smith *et al.* 2013). Most of these insecticidal peptides act by altering the function of presynaptic calcium, sodium, potassium, or chloride channels (King and Hardy 2013; Smith *et al.* 2013).

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Insecticidal spider venom peptides are considered as promising bioinsecticides because they are fully biodegradable, highly potent, and have no effects on beneficial species because of high specificity (De Lima *et al.* 2007; Schwartz *et al.* 2012; Windley *et al.* 2012b; King and Hardy 2013). Approximately 63 % of the identified toxins show specificity towards insects based on a "phyletic specificity" search from a curated database of spider toxins (ArachnoServer) (Herzig *et al.* 2010).

A study reported the discovery of 3 active insecticidal peptides designated DTX9.2, DTX11, and DTX12 (numbering based on the order of elution after rHPLC), isolated from the venom of the American desert bush spider (*Diguetia canities*) (Krapcho *et al.* 1995). The venom of *D. canities* was found to be potent in tobacco budworm, *Heliothis virescens* larvae, with DTX9.2 (Mu-diguetoxin-Dc1a, hereafter Dc1a) was the most potent of the toxins (PD_{50} = 0.38 nmol/g) (Krapcho *et al.* 1995). Similar toxicities (PD_{50} approx. 0.3-1.0 nmol/g) were observed in preliminary assays with cabbage looper, *Trichoplusia ni* and the beet armyworm, *S. exigua* larvae (Krapcho *et al.* 1995). Dc1a was also found potent against blowfly, *Lucilia cuprina* with an LD_{50} value of 231±32 pmol/g (Bende *et al.* 2014). The potency, and more importantly, insecticidal activity in lepidopteran and dipteran insects, has evoked strong commercial interest in this peptide as bioinsecticides.

Recombinant production of bioactive peptides using heterologous expression systems are an alternative choice rather than natural extraction from the organism. However, correct expression and folding of the desired peptide need to be ensured while choosing an expression system (Meng *et al.* 2011; Klint *et al.* 2013; Bende *et*

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al. 2015). The low cost, simplicity, speed-of-growth and wide-spread availability of bacterial-culturing facilities have made *E. coli* the host organism of choice for recombinant protein production. However, the reducing environment of *E. coli* cytoplasm can hinder the disulfide-bond formation and render cysteine-containing proteins prone to misfolding and aggregation (Berkmen 2012).

This chapter describes an approach for the expression of disulfide-rich spider toxin peptide Dc1a in the periplasm of *E. coli*, where the enzymes involved in disulfide bond formation are located (Klint *et al.* 2013). Functional characterisation and biological activities of the recombinant peptide was carried out by performing insect toxicity bioassays against lepidopteran and dipteran insects. The structural and functional information obtained from this study may aid in the future development of peptide-based bioinsecticides for crop protection in the future.

2.2 Materials and Methods

2.2.1 Chemicals, Reagents and Kits

Chemicals and reagents used in the present study were of molecular biology grade or analytical grade. List of all the chemicals, reagents and kits with particulars of the manufacturer is mentioned in the appendix.

2.2.2 Bacterial strains used for cloning and expression studies

DH5α competent *E. coli* cells from Novagen (Merck), Germany were used as cloning host and were maintained on **L**uria-**B**ertani (LB) broth. However, expression studies of the recombinant protein were carried out with NEB® Express competent *E. coli* cells obtained from New England Biolabs, USA, where **T**errific **B**roth (TB) was

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used to maintain high cell density during logarithmic growth phase for maximal recombinant protein production. The media composition of both LB and TB is listed in Table A7 (see appendix).

2.2.3 Standard molecular biology techniques

All standard molecular biology techniques were performed following the protocols described in Molecular Cloning: A Laboratory Manual: Fourth Edition (Sambrook and Russell 2001; Green and Sambrook 2012).

2.2.4 Gene synthesis

Dc1a toxin peptide (UniProtKB Accession No. P49126) comprises of 3 parts: signal peptide (1-17), propeptide (18-38), and mature toxin (39-94). The peptide sequence of the mature toxin was reverse translated to a nucleotide sequence using EMBOSS Backtranseq tool provided by the **E**uropean **B**ioinformatics **I**nstitute of the **E**uropean **M**olecular **B**iological **L**aboratory (EMBL-EBI) (www.ebi.ac.uk/Tools/st/emboss_backtranseq/) (Madeira *et al.* 2019). Nucleotide sequence obtained was codon-optimized for maximal expression in *E. coli* K12 strains using OptimumGene^{TM} algorithm of GenScript, USA. The codon usage quality was depicted using **G**raphical **C**odon **U**sage **A**nalyzer (gcua) version 2.0 (Sharp and Li 1987). The synthesised gene was sequenced and cloned between the *EcoRI* and *HindIII* restriction sites into a pUC57 vector (GeneScript, USA). The pUC57 vector harbouring Dc1a gene was transformed and maintained in DH5α competent *E. coli* cloning host for further use.

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2.2.5 Cloning strategy and Expression construct

The coding region of the synthesized Dc1a gene was amplified by **P**olymerase **C**hain **R**eaction (PCR) using the plasmid pUC57 Dc1a as a template. The oligonucleotides sequence and other components used for a 50 µL PCR reaction mixture for the amplification of the synthesised Dc1a gene are shown in Table 2.1 and Table 2.2, respectively.

Table 2.1: List of oligonucleotides used for amplification and sequencing (bacterial cloning).

Gene	Sequence
Dc1a	FP 5'-CGCCATATGGCGAAAGATGGTGATGTGGAGGG-3' RP 5'-CGCGGATCCTTAAACATCACGGCACACGCATTTGC-3'
pMAL sequencing	FP 5'-GGTCGTCAGACTGTCGATGAAGCC-3' RP 5'-TGTCCTACTCAGGAGAGCGTTCAC-3'

FP: Forward primer; RP: Reverse primer; *NdeI* and *BamHI* sites are shown as underlines in FP and RP, respectively; TTA: Stop codon included before *BamHI* site

Table 2.2: PCR reaction components (bacterial cloning).

Component	Volume (50 µl reaction)	Final concentration
5X Phusion HF Buffer	10.0	1X
10 mM dNTPs	1.0	200 µM
10 µM Forward Primer	2.5	0.5 µM
10 µM Reverse Primer	2.5	0.5 µM
Phusion DNA Polymerase, 2U/µl	0.5	0.02 U/µl
DMSO	1.5	3%
Template DNA	0.5	20 ng
Nuclease-free water	31.5	---

Mastercycler (Eppendorf, Germany) was used for the amplification of the synthesised Dc1a gene following the PCR condition presented in Table 2.3.

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Table 2.3: Amplification conditions used for Dc1a and pMAL sequencing.

Gene	PCR Conditions	Time
Dc1a	I. Initial Denaturation at 98°C	2 min
	II. 30 cycles	
	i) Denaturation at 98°C	30 s
	ii) Annealing at 69°C	30 s
	iii) Extension at 72°C	30 s
	III. Final Extension at 72°C	5 min
pMAL sequencing	I. Initial Denaturation at 98°C	2 min
	II. 30 cycles	
	i) Denaturation at 98°C	30 s
	ii) Annealing at 65°C	30 s
	iii) Extension at 72°C	30 s
	III. Final Extension at 72°C	5 min

Additional sequences were added at the 5' of the oligonucleotides, representing the sequences to be recognised by the restriction enzymes *NdeI* and *BamHI*, to facilitate insertion of the PCR fragment into the expression vector pMALp5X (Fig. 2.1).

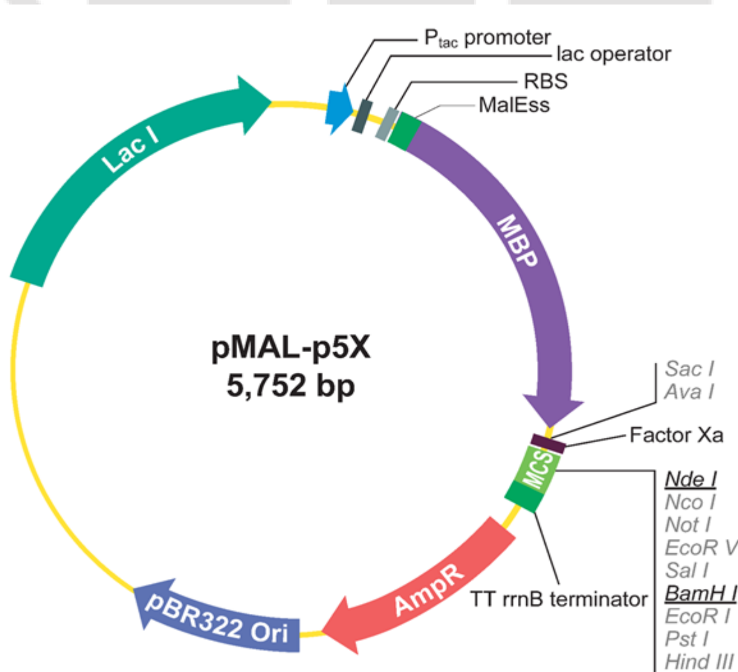

Fig 2.1: Pictorial representation of the pMAL-p5X expression vector map.

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This vector encodes an **i**so**p**ropyl β-d-1-**t**hio**g**alactopyranoside (IPTG) inducible Tac promoter, malE gene having a signal sequence for periplasmic expression and encoding for **M**altose **B**inding **P**rotein (MBP) fusion tag for purification and enhances solubility, and a factor Xa cleave site for removal of MBP purification tag from Dc1a toxin peptide. An ampicillin resistance gene is used as a selection marker. The cloning strategy used for periplasmic expression of a disulfide-rich peptide Dc1a in *E. coli* is represented in Fig. 2.2.

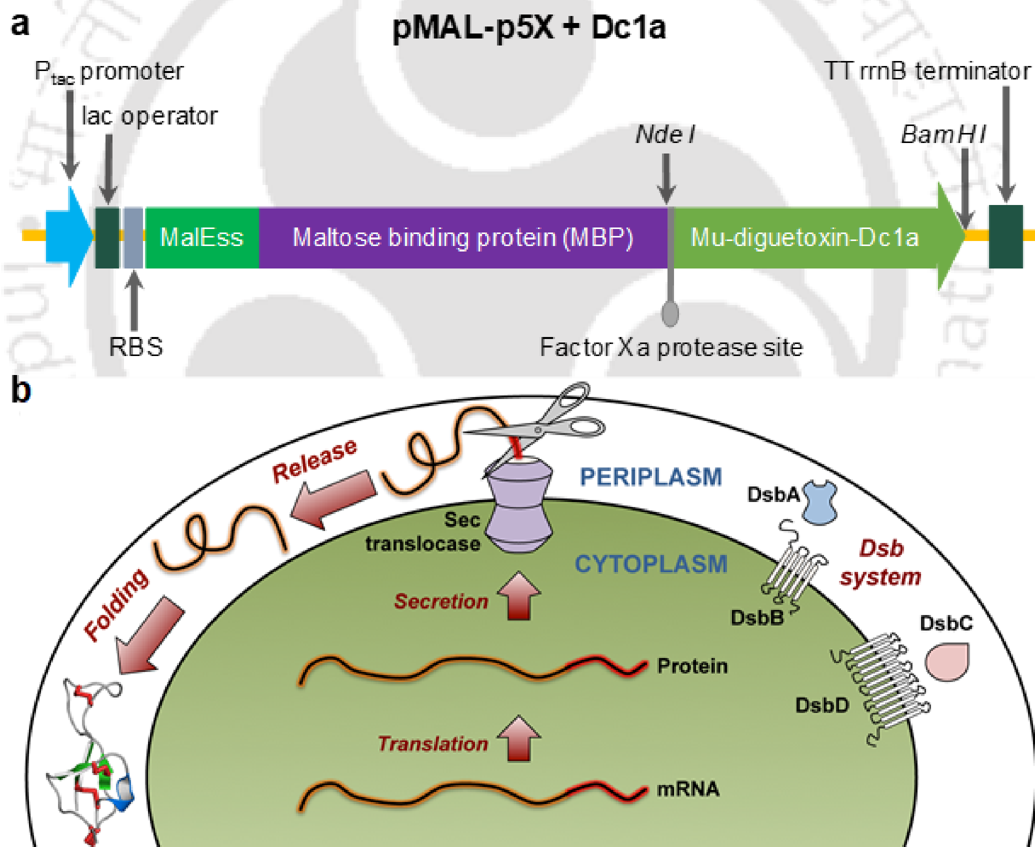

Fig 2.2: Illustration of the cloning strategy used. (a) Expression construct showing Dc1a gene was inserted downstream of the malE gene for periplasmic expression. (b) Schematic of the periplasmic expression system for production of disulfide-rich peptides in *E. coli.*

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For expression cassette construction, the 189 bp PCR product along with expression vector pMALp5X were subjected to restriction digestion with *NdeI* and *BamHI* enzymes at 37 °C for 2 h. Restriction fragments were separated and extracted from the 1 % agarose gel by Gel and PCR clean-up kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The purified fragments of gene and vector were ligated using T4 DNA ligase at 16 °C for 16 h. The ligated product was transformed into NEB® Express competent *E. coli* cells by standard heat shock method. Positive transformants were selected on LB agar plates containing ampicillin (100 µg/mL). Selected colonies were screened for positive clones by restriction digestion, colony PCR and sequencing. The clones with insert were stored as glycerol stock at -80 °C until further use.

2.2.6 Expression conditions optimization for rDc1a

Protein expression was carried out using pMAL™ Protein Fusion and Purification System kit as per the manufacturer's protocol with little modification. In summary, a single colony of *E. coli* NEB® Express containing the cloned expression cassette was grown in 5 mL of different growth media (see Table A7: AI, TB, 2XTY, and LB in appendix) in the presence of 100 µg/ml ampicillin at 37 °C with shaking at 200 rpm. MBP-toxin fusion protein expression was induced in late log phase (**o**ptical **d**ensity at 600 nm: OD_{600} ≈ 0.9-1.0) with different concentration of IPTG (0.0 mM, 0.05 mM, 0.1 mM, 0.5 mM) and cells were allowed to grow at 18 °C for a further 15 h. Cells were harvested by centrifugation at 8,000 × g for 20 min at 4 °C and washed cell pellet was re-suspended in 250 µL lysis buffer supplemented with 5 mg of lysozyme per litre of culture. After 30 min of incubation on ice, protease inhibitors:

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Benzamidine HCl and **p**henyl**m**ethyl**s**ulfonyl **f**luoride (PMSF) were added at a final concentration of 1 mM. The fusion protein from the bacterial periplasm was extracted by ultrasonication using short pulses of 3 sec (Vibra cell Sonicator, USA) on ice. The extract was clarified by centrifugation at 12000 × g for 30 min at 4 °C. The MBP-toxin fusion protein expression was verified by separating the total cell extract and total soluble extract on 16 % SDS-PAGE analysis (see Table A14 in appendix).

2.2.7 Large scale production of rDc1a and purification of the fusion protein

E. coli NEB® Express harbouring plasmid containing Dc1a expression cassette was grown in 1 L of TB in the presence of previously defined conditions and induced with 0.5 mM IPTG in late log phase (OD_{600} ≈ 0.9-1.0). The culture was shifted to 18 °C and allowed to grow for a further 15 h. Cells were harvested and re-suspended in 10 mL of lysis buffer supplemented with 5 mg of lysozyme per litre of culture. After 30 min of incubation on ice, protease inhibitors: Benzamidine HCl and PMSF were added at a final concentration of 1 mM. The extraction of the fusion protein from the bacterial periplasm was performed according to the optimised protocol discussed above. The extract was clarified by centrifugation at 12000 × g for 30 min at 4 °C and MBP-toxin protein was captured using amylose resin pre-equilibrated with column buffer. Non-specifically bound proteins were removed by washing followed by elution of the fusion protein with column buffer containing 10 mM maltose. The buffer composition of various buffers used for protein purification is mentioned in Table A14 (see appendix).

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2.2.8 In vitro folding of toxin peptide Dc1a

Protein containing fractions were detected by Bradford protein assay (as mentioned in section 2.2.9) and pooled before cleavage of the fusion protein by Factor Xa protease. The reaction was catalysed at 4 °C for 16 h and supplemented with 2 mM $CaCl_2$ to maintain Factor Xa protease activity, and 0.6 mM reduced, and 0.4 mM oxidised glutathione to promote proper folding of the toxin peptide. Approximately, 50 µg Factor Xa protease was used in a reaction containing 10 mg MBP-toxin fusion protein. **S**ize **e**xclusion **c**hromatography (SEC) was used for further purification of toxin from fusion malE tag. In brief, Factor Xa protease cleaved fusion protein samples were separated by the HiLoad 16/600 Superdex 200 pg SEC column (volume: 120 mL) using AKTA purifier 100 **F**ast **p**rotein **l**iquid **c**hromatography (FPLC) system (GE Healthcare Life Sciences, USA). The proteins were eluted at a flow rate of 1 mL/min and fractions of separated peaks were collected. Amicon® Ultra centrifugal filters of 3 kDa **n**ominal **m**olecular **w**eight **l**imit (NMWL) were used for the protein concentration of finally purified rD1a.

2.2.9 Protein estimation by Bradford's assay

The concentration of purified samples of rDc1a was estimated by Bradford protein assay (Bradford 1976). 5 µL of rDc1a sample was mixed with 200 µL of Bradford reagent and incubated for 10 min at room temperature. Absorbance was measured at 595 nm. A known concentration of **b**ovine **s**erum **a**lbumin (BSA) was used to generate a standard curve. The concentration of purified rDc1a was estimated by using the standard graph.

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2.2.10 Characterization studies of purified rDc1a

2.2.10.1 SDS-PAGE

Sodium **d**odecyl **s**ulfate-**p**oly**a**crylamide **g**el **e**lectrophoresis (SDS-PAGE) is routinely used to separate macromolecules based on molecular mass (Laemmli 1970). Samples of the fusion protein and purified rDc1a collected at various stages of purification were mixed with loading dye and boiled at 95 °C for 10 min. The denatured samples were loaded on 16 % SDS-PAGE gel (see Table A14 in appendix) and were separated in a Mini-Protean Tetra cell gel electrophoresis apparatus (Bio-Rad, USA). The gels were then either stained with silver staining solutions (Rabilloud 1992), or used immediately for Western blot analysis. Gel images were recorded using a Gel Doc XR^{+} Gel Documentation System (Bio-Rad, USA).

2.2.10.2 Western immunoblotting

Following SDS-PAGE, Power Blotter semi-dry transfer system (ThermoFisher Scientific, USA) was used to transfer proteins from gel to 0.45 µm pore size nitrocellulose membranes (Bio-Rad, USA). The process of electro-blotting was performed at 12 A for 15 min in Towbin transfer buffer. The transfer process followed by blocking the membrane with 10 mL of blocking solution for 2 h with gentle agitation at room temperature. The blocking step was followed by placing the membrane in the primary antibody, anti-Dc1a polyclonal antibody (GenScript, USA), at a dilution 1:2000 in blocking buffer and allowed to incubate overnight at 4 °C. Removing residual unbound primary antibody was then done by washing the

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membrane with 1x TBST 5 times, for 5 min each time, under shaking conditions. The membrane was then left in diluted HRP-conjugated secondary antibody, goat anti-rabbit antibody in blocking buffer at ratio 1:10000 (v/v), for 1 h at room temperature. The membrane was then briefly washed in distilled water. The specifically bound secondary antibody of the target protein was detected using Clarity Max ECL western blotting chemiluminescent substrate (Bio-Rad, USA). Detection solutions were mixed in a ratio of 1:1, gently poured over the membrane and incubated for 5-10 s. ChemiDoc XR^{+} Gel Documentation System (Bio-Rad, USA) was used to develop the membrane. The buffer composition of various buffers used for western blotting is mentioned in Table A14 (see appendix).

2.2.10.3 Mass spectrometry analysis

The quality of rDc1a purification and molecular mass determination by **M**atric **A**ssisted **L**aser **D**esorption/ **I**onization **T**ime **o**f **F**light (MALDI-TOF) mass spectrometry was carried out using the AutoFlex Speed instrument (Buker Daltonics, United States). The FPLC purified rDc1a samples were co-crystallized with α-**c**yano-4 **h**ydroxy**c**innamic **a**cid (CHCA, 5 mg/ml in 50/50 acetonitrile/H_2O) matrix (1:1, v/v) on MALDI target plate (Buker Daltonics, United States). The mass spectrum was acquired in positive reflector mode, and the results were analysed using FlexAnalysis 3.1 software (Buker Daltonics, United States).

2.2.10.4 Circular dichroism (CD) analysis

CD spectroscopy measures unequal absorption of left-handed and right-handed circularly polarised light arises because of structural asymmetry and is an

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excellent method for rapid evaluation of the secondary structure and folding of proteins derived using recombinant techniques. The functional characteristics of the refolded rDc1a were evaluated using CD analysis in JASCO J-815 spectro-polarimeter (Jasco Corporation, Japan). The CD spectrum was measured in the far UV region (190–250 nm) using a quartz cell of 0.1 cm pathlength, with an average of three scans at a scanning speed of 100 nm/min and 1 nm bandwidth. The concentration of peptide used for CD spectrum analysis was 10 μM in insect saline, pH 6.2 at room temperature. The **m**olar **r**esidual **e**llipticity (mre) was calculated from the values of ellipticity (θ) of the specified range of the wavelength. The per cent occurrence of different secondary structure in refolded rDc1a was estimated by uploading the mre values obtained to K2D3 server (http://k2d3.ogic.ca/) (Perez-Iratxeta and Andrade-Navarro 2008; Fu *et al.* 2011).

2.2.10.5 SWISS-MODEL homology modelling

The primary sequence of the mature toxin including the four vestige amino acids was submitted to the SWISS-MODEL, a protein structure homology-modelling server (https://swissmodel.expasy.org/) (Waterhouse *et al.* 2018). Template search with BLAST and HHBits were performed against the SWISS-MODEL template library to get a suitable template for modelling. The template's quality was checked after target-template alignment. The template with the highest quality was selected for model building. Models were built using ProMod ver. 3. The model quality was assessed using the QMEAN scoring function. The quaternary structure annotation of the template was used to model the target sequence in its oligomeric form.

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2.2.11 Cytotoxicity effects of the recombinant purified peptide toxin against cultured cells in vitro

National Centre for Cell Science (NCCS) Pune, India supplied cultured cells of fall armyworm Sf21 ovary (*Spodoptera frugiperda*) (Vaughn *et al.* 1977; O'Reilly, Miller and Luckow 1994) and human U-937 myeloid leukemia (*Homo sapiens*) (Sundström and Nilsson 1976).

Sf21 insect ovary cells were cultured in a TNM-FH medium (modified Grace's insect medium) as monolayer and suspension culture; however, U-937 human myeloid leukaemia cells were grown in RPMI 1640 medium as a suspension culture. Media were supplemented with 10 % (v/v) heat-inactivated fetal bovine serum and 1X antibiotic-antimycotic solution. Cultured insect ovarian cells were maintained at 27 °C in non-humidified BOD incubator. In contrast, human myeloid leukaemia cells were incubated at 37 °C in a humidified incubator with 5 % CO_2 saturation. Cultures were maintained at a cell density between 1 X 10^5 and 2 X 10^6 viable cells/mL and sub-culturing to the fresh medium was routinely done in T-25 flasks every 3 to 4 days (depending on cell density).

2.2.11.1 MTT cytotoxicity assay

Cultured cells of fall armyworm Sf21 ovary and human U-937 myeloid leukaemia were used for examining the cytotoxicity effects of rDc1a on cell growth by a colourimetric 3-(4,5-di**m**ethyl**t**hiazol-2-yl)-2,5-diphenyl **t**etrazolium bromide (MTT) assay. This assay is based on the cellular conversion of a tetrazolium salt into a formazan product that is easily detected using a 96-well plate reader. Therefore,

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the MTT assay is simple and sensitive in reflecting cellular survival and cellular growth, useful in cytotoxicity, and apoptosis assays.

rDc1a was initially diluted in **p**hosphate **b**uffer **s**aline (PBS) (see Table A14 in appendix) and then serial dilutions in final concentration from 1×10^{-9} to 10×10^{-6} M were prepared in serum-free cell culture medium in 96-well microtiter plates (Thermo Fisher Scientific, USA). Sf21 cells (5×10^{4} cells/well) and U-937 (1×10^{4} cells/well) were added into each well containing various concentrations of rDc1a and final volume of 200 µL was made using complete TNM-FH insect and RPMI-1640 mammalian cell culture medium, respectively. Cytotoxicity effects were recorded for 24 h, 48 h, 72 h and 96 h. Then, 20 µL MTT solution (0.5 mg/mL) was added into each well, followed by incubation at 27 °C for 4 h. The microtiter plate was centrifuged at 250 ×g for 5 min at 4 °C. 100 µL of **dim**ethyl **s**ulf**o**xide (DMSO) was added, and the OD_{570} was measured by a microplate reader (Tecan Infinite® 200 PRO). Experiments were performed in triplicate and % viability was calculated.

2.2.11.2 Immunofluorescence assay

Sf21 (2×10^{5} cells/well) and U-937 (1×10^{5} cells/well) cells were fixed and permeabilised in cold 4 % paraformaldehyde for 30 min and allowed to adhere to microtiter plate. Cells were washed in PBS three times. Nonspecific-binding sites were blocked by incubating the cells in blocking solution (1 % gelatin in PBST) for 30 min at room temperature (RT). Cells were then incubated with rDc1a (10 µM) for 12 h at 4 °C. After that, cells were washed three times with PBS and incubated with

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rabbit anti-Dc1a polyclonal antibody (1:500 dilution) for 1 h at RT. Then cells were washed three times with PBS and incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:2000 dilution) for 1 h at RT. Cells were washed three times with PBS before being stained with Alexa Fluor 594 membrane and Hoechst nuclear stains. Finally, plates were washed and observed using an inverted fluorescence microscope (Nikon Ti-S) at 40 × magnification.

2.2.11.3 Cell cycle analysis

Sf21 (2×10^5 cells/well) and U-937 cells (1×10^5 cells/well) were treated with 10 µM concentrations of rDc1a for 48 h. The cells were harvested and washed with cold PBS. Dropwise cold 70 % ethanol was added to the treated cells and incubated for 30 min to 1 h at 4 °C for fixation. The cells were centrifuged at 850 ×g to remove ethanol and washed twice with PBS. The cells were re-suspended in 500 µL of PBS and incubated with 5 µL RNase (20 µg/mL) for 30 min at RT. The cells were incubated with **p**ropidium **i**odide (PI) at a final concentration of 50 µg/mL for 1 h in the dark. The cell cycle distribution for **f**orward **s**catter (FS), and **s**ide **s**catter (SS) of the cells were determined using **F**luorescence-**a**ctivated **c**ell **s**orter (FACS) Calibur (BD Biosciences, USA). Cell cycle analysis was carried out using ModFit LT software and the percentage of cells in the different phases of the cell cycle were calculated.

2.2.11.4 Mitochondrial potential

Disruption of **m**itochondrial **m**embrane **p**otential (MMP) is considered as a pre-apoptotic indicator that can be monitored by using

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tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a membrane potential sensitive dye (Ko *et al.* 2014; Soto-Cerrato *et al.* 2015; Saha *et al.* 2016). Sf21 (2×10^5 cells/well) cells were incubated with rDc1a (10 μM) for 48 h, and the JC-1 dye was used to stain the cells. The cells were immediately observed under an inverted fluorescence microscope (Nikon Ti-S) at 40 × magnification.

2.2.11.5 Microscopic analysis using Phase Contrast microscopy and FESEM

The ultra-morphology of the rDc1a treated Sf21 (2×10^5 cells/well) cells were examined using phase contrast microscopy and **f**ield **e**mission **s**canning **e**lectron **m**icroscopy (FE-SEM) (Carl Zeiss, SIGMA, USA). The cells pellet was washed with PBS and then fixed in 500 μL of 4 % paraformaldehyde and 2.5 % glutaraldehyde for 24 h at 4 °C. The pellet was again washed with filtered PBS and finally, ethanol dehydrated with gradients of 30 %, 50 %, 75 %, 90 % and 100 %, for 10 min each. The pellets were then smeared evenly on a glass coverslip and dried in the desiccator up to a critical point. The samples were sputter-coated with gold film using a sputter coater (SC7620 "Mini", Polaron Sputter Coater, Quorum Technologies, Newhaven, England) before FE-SEM analysis.

2.2.12 Insect bioassays to determine bio-insecticidal potential of the recombinant purified peptide toxin

Insect bioassays were carried out at National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India. The bio-insecticidal potential of the purified recombinant peptide Dc1a was determined by injection and oral toxicity bioassays against dipteran and lepidopteran insect pests.

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Dipteran insect selected in the present study includes housefly (*Musca domestica*) which was reared in standard insectary conditions at 25±2 °C and ambient humidity (40–60 %) with a photoperiod of 14 h light and 10 h darkness. Housefly larvae (Maggots) were reared on an artificial diet (see Table A11 in appendix). Adults of housefly were housed in cages (50 X 50 X 50 cm) and provided with honey-soaked cotton wicks. Water in cages was provided in cotton wicks as drinking sites.

Lepidopteran insects selected in the present study includes tobacco cutworm (*Spodoptera litura*), and cotton bollworm (*Helicoverpa armigera*). Larvae of the selected lepidopteran insects were reared on castor leaves up to second instar and thereafter shifted to artificial diet (see Table A12 in appendix). Adults were housed in cages (50 X 50 X 50 cm) and provided with honey-soaked cotton wicks. Standard insectary conditions at 25±2 °C and ambient humidity (40–60 %) with a photoperiod of 14 h light and 10 h darkness was provided.

2.2.12.1 Injection bioassays against lepidopteran insect pests

To assess the lethal activities of rDc1a against tobacco cutworm (*S. litura*), fall armyworm (*S. frugiperda*) and cotton bollworm (*H. armigera*), injection-based insect bioassays were performed. Third to fourth-instar larvae were injected with 5 µl of an aqueous solution containing varying doses (0.1-2.0 µg/larvae) of rDc1a dissolved in 1X PBS. Controls were injected with 5 µl 1X PBS. Injections were made using 1.0 ml B-D Ultra-Fine insulin syringe with a fixed 31G needle into the ventrolateral thoracic region. For each dose, 30 larvae were injected and mortality was recorded at 12, 24, 36 and 48 h post-injection including control. To estimate

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LD_{50} values, mortality at 48 h was used and survival data were analysed using Probit analysis (Finney 1952), and plotted using Graphpad Prism (ver. 6).

2.2.12.2 Diet based bioassays against dipteran insects-pests

Droplet-feeding assays were conducted to assess the oral activity of rDc1a towards adults of housefly (*M. domestica)*. Adults were fed once with a 5 µl droplet containing 1 to 20 µg/fly of rDc1a in 1X PBS and 10 % sucrose. Control adults were fed on droplets containing 1X PBS and 10 % sucrose solution. Treated adults were placed in ventilated plastic jars (250 mL) with standard artificial diet after consumption of the droplet. To encourage droplet consumption, adults were starved for approx. 6-8 h prior to feeding. Survival was recorded once daily after droplet feeding for up to 72 h and survival data were analysed as discussed above.

2.2.13 *Data analysis and image processing*

Mortality data analysis and interpretations were carried out using a statistical tool comprising GraphPad Prism version 6.0. T-test was performed out using Microsoft Excel, $*P < 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. All the DNA sequencing were carried out using Sangers' chain-terminating dideoxynucleotides method on DNA Analyzer 3730xl (Applied Biosystems) from Eurofins Genomics, India. All the agarose and SDS-PAGE gel images were recorded using Gel Doc XR$^+$ Gel Documentation System (Bio-Rad, USA) and processed in Image Lab version 6.0.1 software for Windows (Bio-Rad, USA).

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2.3 Results and discussion

Numerous bioactive peptides have been isolated from the venoms of spiders (Schwartz *et al.* 2012; Windley *et al.* 2012a; King and Hardy 2013; Smith *et al.* 2013). These peptides act on neuronal ion channels and receptors (De Lima *et al.* 2007), but very few have a high degree of phyletic selectivity. With respect to their application in agriculture, insecticidal toxins that could specifically target insect pests would be highly desirable. Besides this, obtaining enough toxin peptide from the original source to perform functional, structural, and biochemical characterisation is one of the major challenges in this field. To overcome this problem, various heterologous expression systems were used previously for the recombinant production of small peptide toxins with disulfide bridges, including bacterial systems (mostly *E. coli*) (Park, Hausdorff and Miller 1991; Li *et al.* 2000; Maggio and King 2002a; Tedford *et al.* 2004a), yeast (Anangi *et al.* 2007), and cultured insect cells (Escoubas *et al.* 2003). Among these, *E. coli* is by far the first choice due to its ease of handling, manipulation, reasonable product/cost ratio, rapid growth and involves simple plasmid constructs (Gopal and Kumar 2013; Rosano and Ceccarelli 2014). However, when a recombinant protein is expressed in *E. coli,* the intracellular cytoplasmic microenvironment keeps cysteine residues in the reduced state, which may lead to protein aggregation. In attempting to solve these problems, the present study describes a strategy for efficient production of the ISVPs in *E. coli* expression system.

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2.3.1 Gene synthesis and expression construct

OptimumGene™ algorithm of GenScript, USA, helped to optimise various parameters that play a critical role in optimal gene expression. The parameters considered during optimisation include codon usage bias, GC content, mRNA secondary structure, RNA instability motif (ARE), etc. The **C**odon **A**daptation **I**ndex (CAI) for optimal expression in *E. coli* was adjusted to 0.97, where CAI of 1.0 is considered perfect, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level. The **g**uanine-**c**ytosine (GC) content was adjusted to 54.54 %, where the ideal percentage range of GC content is between 30-70 %. The amino acid sequence of the mature toxin is illustrated in Fig. 2.3 (a) and nucleotide information of the codon-optimised gene sequence is mentioned in Table A16 (see appendix). The graphical representation of the relative adaptiveness which measures the quality of codon usage the CAI is depicted in Fig. 2.3 (b) whereas the results of the optimal distribution of codon usage bias after OptimumGene™ optimisation with GC content adjustment is shown in Fig. 2.3 (c).

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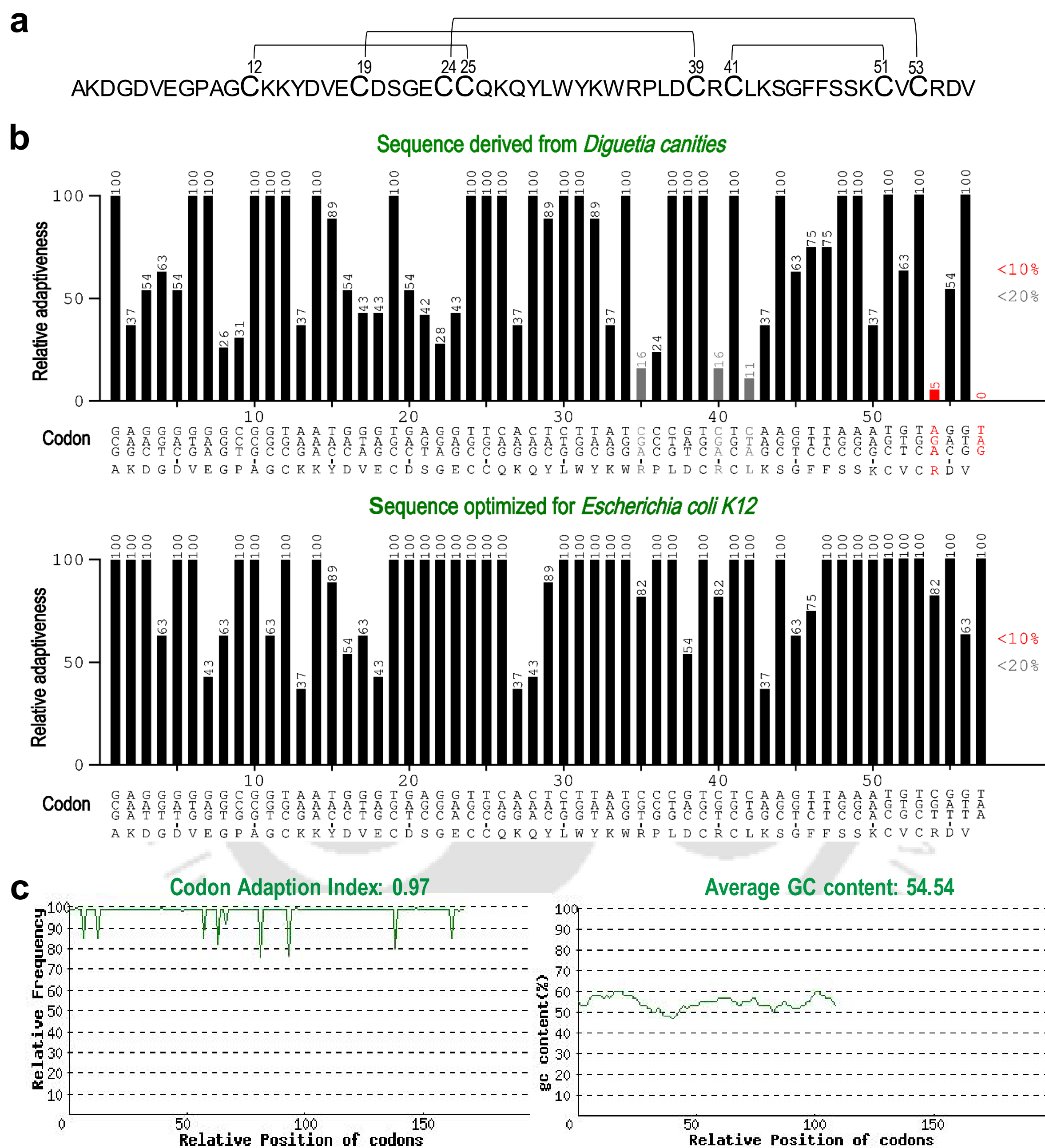

Fig. 2.3: Graphical illustration of Codon optimisation. (a) The peptide sequence of mature toxin of Dc1a. (b) Illustration of the change in relative adaptiveness index in *E. coli* after optimization. (c) Optimal distribution of codon usage bias after OptimumGene™ algorithm and GC content adjustment.

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Following codon optimisation and chemical synthesis, Dc1a gene was amplified and successfully cloned between the designated restriction sites into the expression vector pMALp5X. The release of 189 bp fragment upon restriction digestion and amplification of the 189 bp product following the colony PCR were separated on 1.2 % agarose gel confirmed the presence of synthesised Dc1a gene (Fig. 2.4, a). Sequencing using gene-specific and pMAL sequencing primers (Table 2.1), further confirmed the insertion of Dc1a gene inside pMALp5X vector. The electropherogram sowing the DNA sequencing results of cloned Dc1a gene is shown in Fig. 2.4 (b).

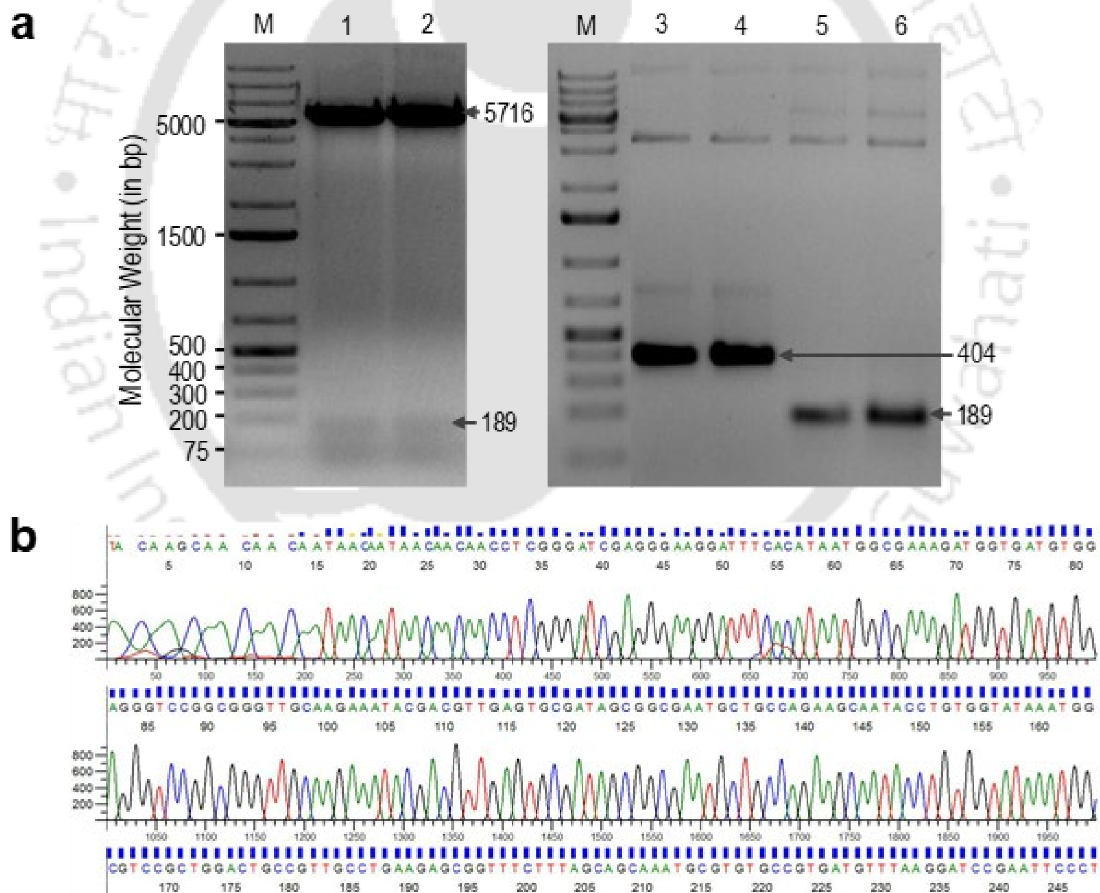

Fig. 2.4: Illustration of clone confirmation and DNA sequencing. (a) Agarose gel electrophoresis illustrating clone confirmation in pMALp5X using restriction digestion and colony PCR. (b) Electropherogram showing the DNA sequencing results of cloned Dc1a.

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The nucleotide sequence deduced after sequencing results is mentioned in Table A17 (see appendix). Phusion High-fidelity DNA polymerase was used for proofreading amplification in the cloning of expression constructs. The confirmed clones with insert were stored as glycerol stock at -80 °C until further use.

2.3.2 Production of rDc1a

The reducing environment of *E. coli* cytoplasm hinder the disulfide-bond formation, and cysteine-containing proteins are prone to misfolding and aggregation (Berkmen 2012). Therefore, Dc1a was cloned and allowed to express in such a manner to secrete the fusion protein into the oxidising environment of *E. coli* periplasm after translation. The MalE signal sequence was removed during this process, releasing the fusion protein into the periplasm where the Dsb machinery (DsbA, DsbB, DsbC, and DsbD) can assist with disulfide-bond formation (Klint *et al.* 2013). To enhance the expression yields and influence solubility, MBP tag was incorporated at the N-terminus of the protein of interest (Vu *et al.* 2014).

Following the above strategy, rDc1a peptide was successfully expressed using a novel approach that addresses the challenge of correctly folding disulfide-rich spider toxins in *E. coli*. Out of the different IPTG concentrations used for the expression of MBP-Dc1a fusion protein, 0.5 mM IPTG concentration was found optimum at a reduced temperature of 18 °C post-induction (Fig. 2.5, a). Additionally, the expression in TB media was found optimum to express the protein at high levels (Fig 2.5, b).

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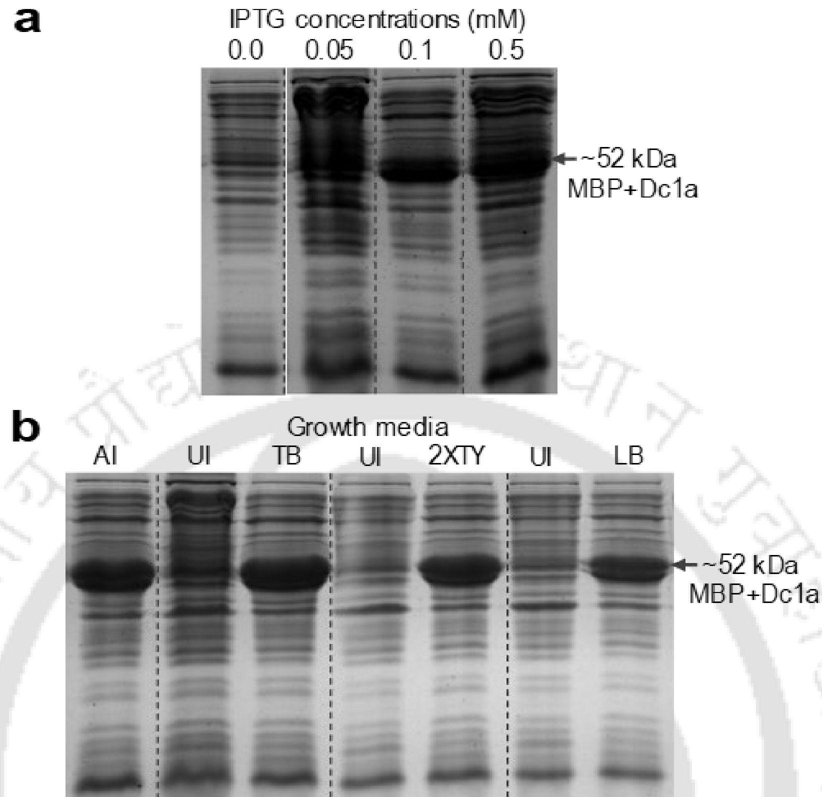

Fig 2.5: SDS-PAGE illustrating expression of fusion protein MBP-Dc1a in *E. coli* (NEB Express) using (a) different IPTG concentrations (0.0 mM, 0.05 mM, 0.1 mM, and 0.5 mM), and (b) different growth media used (UI: Un-induced sample, AI: Auto induction broth, TB: Terrific broth, 2XTY: 2XTY broth, and LB: Luria-Bertani broth).

Purification of MBP-Dc1a is illustrated in Fig. 2.6. On comparison of lanes 1 and 2 (Fig. 2.6), reveals over-expression of the MBP-Dc1a fusion protein after IPTG induction. The fusion protein is highly soluble as most of the MBP-Dc1a fusion protein was found in the soluble fraction following cell lysis. Significantly less amount of the MBP-Dc1a fusion protein was lost during application of the soluble cell fraction to an amylose affinity column (Fig. 2.6, lane 3) or during a subsequent wash step (Fig. 2.6, lane 4). A large amount of MBP-Dc1a fusion protein having a molecular mass of ~52 kDa was eluted from the column with 10 mM maltose (Fig. 2.6, lane 5 a-c).

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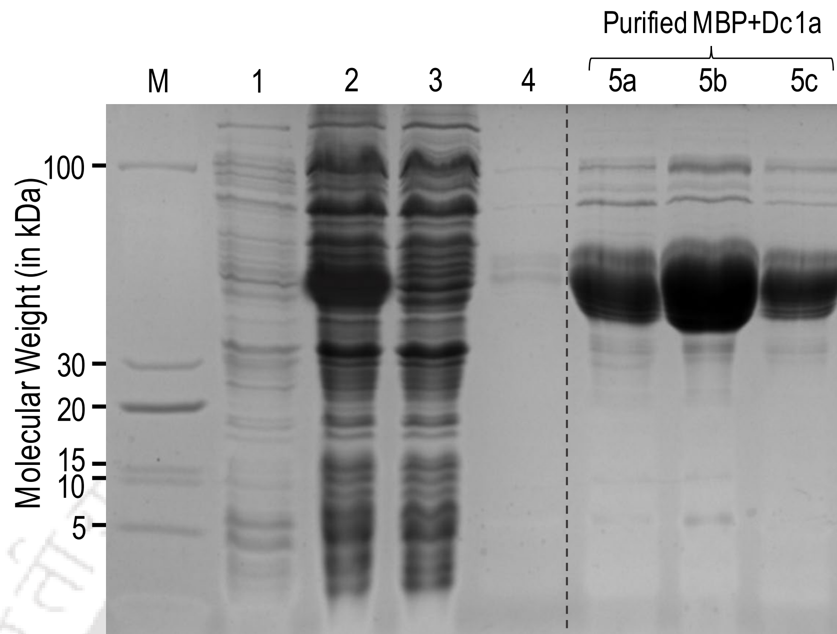

Fig 2.6: SDS-PAGE illustrating different steps in the purification of rDc1a. lane M: Molecular weight markers; lane 1: *E. coli* cell extract before IPTG induction; lane 2: *E. coli* cell extract after IPTG induction (the MBP-rDc1a fusion protein is evident at ~52 kDa); lane 3: Non-specific proteins flow through after loading the cell lysate on amylose resin; lane 4: Eluate after washing amylose resin with column buffer (see Table A14 in appendix); lane 5 (a-c): Fusion protein eluate 1-3 after washing amylose resin with 10 mM maltose in column buffer.

Most of the fusion protein could be successfully cleaved with factor Xa protease to release Dc1a (Fig. 2.7 (b), lanes 6 and 7). The Dc1a liberated by factor Xa protease cleavage of the fusion protein was purified via AKTA purifier 100 FPLC system to obtain three major peaks (Fig. 2.7, a). Based on the elution profile of HiLoad 16/600 Superdex 200 pg SEC column, (www.gelifesciences.co.jp/catalog/pdf/18110052.pdf) and separation on SDS-PAGE gel (Fig. 2.7, b), peak 3 was verified to be containing purified Dc1a peptide fractions (Fig. 2.7 (b), Lane 8, c) having a molecular mass of ~6.8 kDa. It was further concentrated using centrifugal filters of 3 kDa NMWL, to yield a brighter band having purity (>95 %) as illustrated in Fig. 2.7, lane 9 with no loss of peptide in the flow through (Fig. 2.7, Lane 10).

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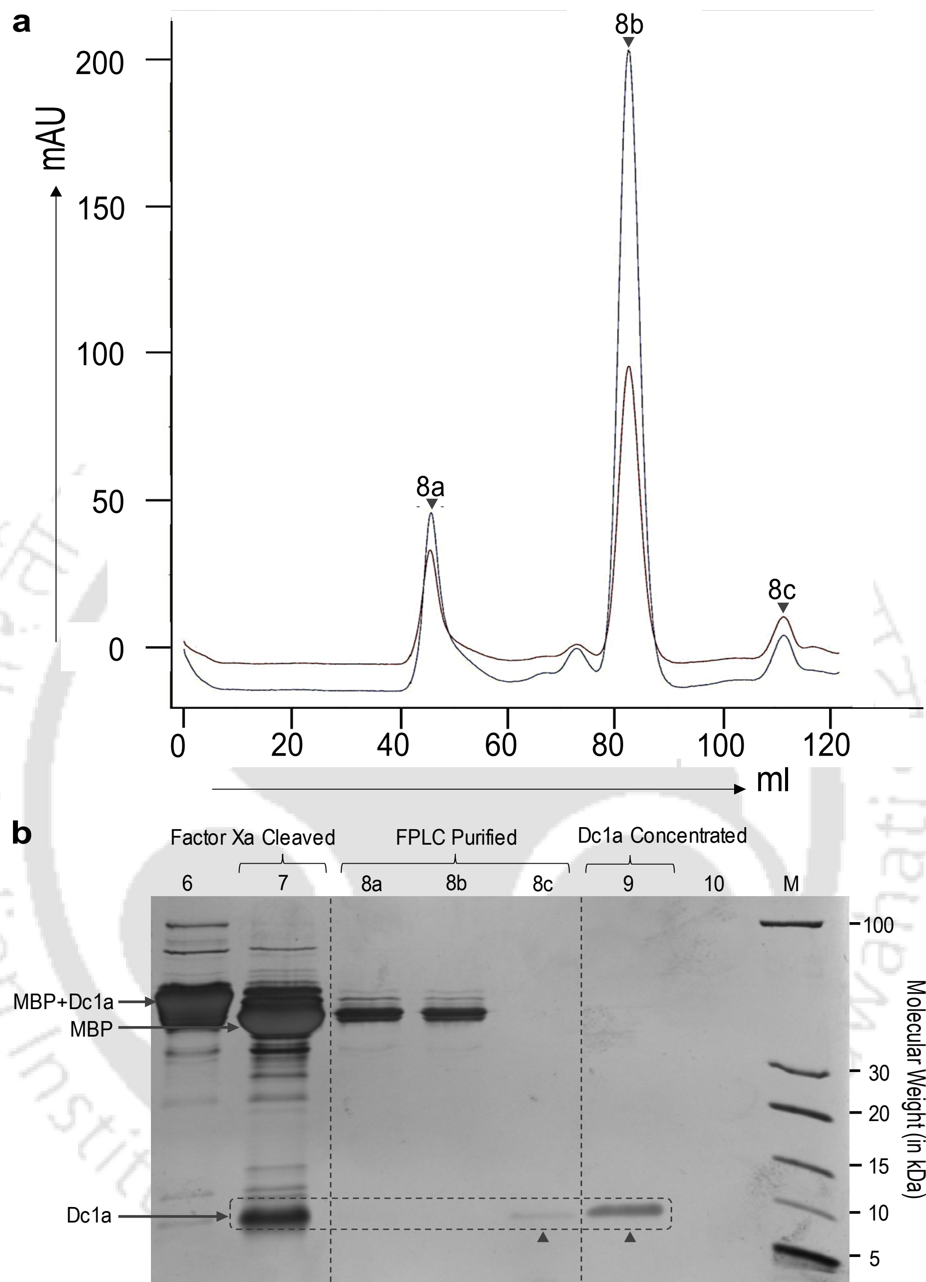

Fig 2.7: (a) Typical chromatograph of elution profile of cleaved MBP-Dc1a fusion protein with Factor Xa protease using FPLC Akta Purifier-100, (b) SDS-PAGE illustrating purification of rDc1a following FPLC. Lane 6, Concentrated fusion protein sample before factor Xa protease cleavage; lane 7, Fusion protein sample after factor Xa protease cleavage showing cleavage of fusion protein to MBP and rDc1a; lane (8, a-c): FPLC eluate peaks collected after size exclusion chromatography corresponding to fusion protein, MBP and rDc1a respectively; lane 9: concentrated Peak 3 corresponding to final purified rDc1a.

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2.3.3 Standard curve and Protein estimation

Bradford assay was used to estimate the concentration of the protein based on the known concentrations (0.05-1 mg/L) of the BSA (Bradford 1976). A standard curve was plotted between the known concentrations of BSA vs absorbance at 595 nm (see Fig. A1 in appendix). The calculated concentration of rDc1a after purification was equal to 0.4276 mg/mL or 62.15 µM.

2.3.4 MALDI-ToF MS analysis and mass determination

The MALDI-ToF MS analysis of purified rDc1a showed the intact molecular mass, 6867.93 Da in linear positive mode (Fig. 2.8, a) similar to the predicted theoretical mass of 6873.90 Da and apparent molecular mass observed by SDS-PAGE analysis. MALDI-TOF MS analysis further confirmed the purity of the purified rDc1a as a single peak is observed. The theoretical MALDI-TOF MS peptide mass results were not shown. Chromatographic and mass spectrometry analyses demonstrated the homogeneity and mono-dispersity of the purified protein. Size exclusion chromatography coupled with FPLC Akta Purifier 100 provided an alternate way to purify short peptides compared to rHPLC.

2.3.5 Secondary structural studies using CD analysis

The CD spectra of rDc1a is an important parameter in confirming that the expressed peptide was a functional toxin (Fu *et al.* 2011). The secondary structure composition of rDc1a was confirmed by CD analysis (Fig. 2.8, b).

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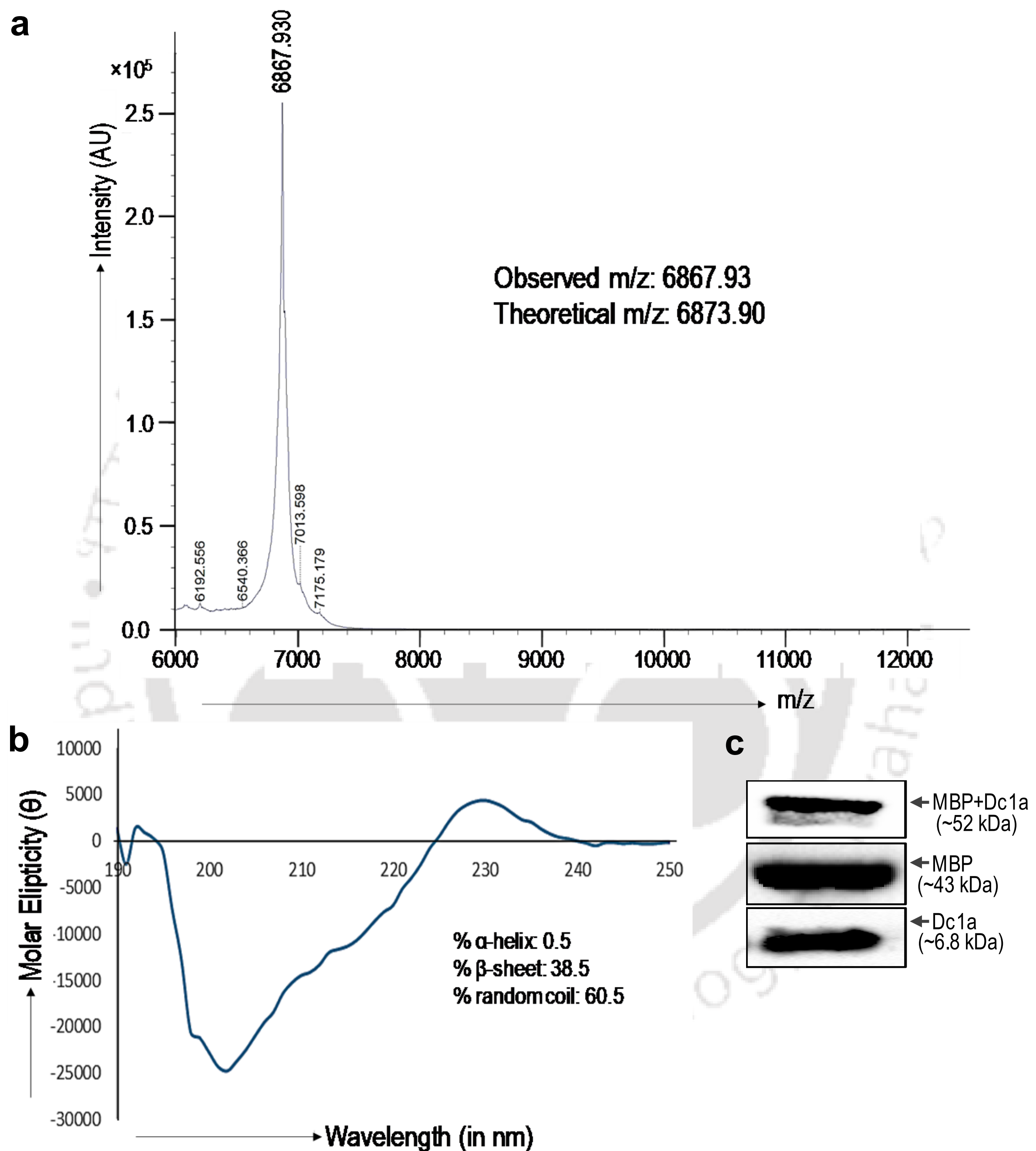

Fig 2.8: Illustration of the characterisation studies carried using purified rDc1a. (a) Typical MS Spectrum of purified rDc1a (Observed m/z: 6867.93, Theoretical m/z: 6873.90). (b) CD spectra of purified rDc1a, and (c) Western Blot illustrating MBP-Dc1a fusion protein, MBP and rDc1a.

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The CD spectrum of rDc1a was analysed using the K2D3 server (Perez-Iratxeta and Andrade-Navarro 2008), where the data points were compared with the available solution structure of Dc1a (Bende *et al.* 2014) and secondary structure predicted using PSIPRED v3.3 (Jones 1999). The PSIPRED prediction of % secondary structures of rDc1a are showed in Table 2.4.

Table 2.4: % secondary structures predicted using PSIPRED v 3.3.

% α-helix	% β-sheet	% random coil
0.5	38.5	60.5

The graphical representation of predicted secondary structure of purified rDc1a using PSIPRED v 3.3 is shown in Fig. 2.9. The CD analysis using K2D3 server also showed that 5 % of the residues form α-helix, 37.5 % residues give rise to β-strands and 57.5 % of the residues form loops. Therefore, secondary structure prediction analysed by K2D3 gave similar results to those of PSIPRED prediction and CD. Secondary structure prediction using PSIPRED and K2D3 server confirmed that the recombinant toxin was structurally stable. It is evident from the secondary structural studies using CD and PSIPRED server results that *in vitro* protease digestion of fusion protein in the presence of 0.6 mM reduced and 0.4 mM oxidised glutathione helped to promote proper folding of the toxin peptide rDc1a (Klint *et al.* 2013; Bende *et al.* 2014).

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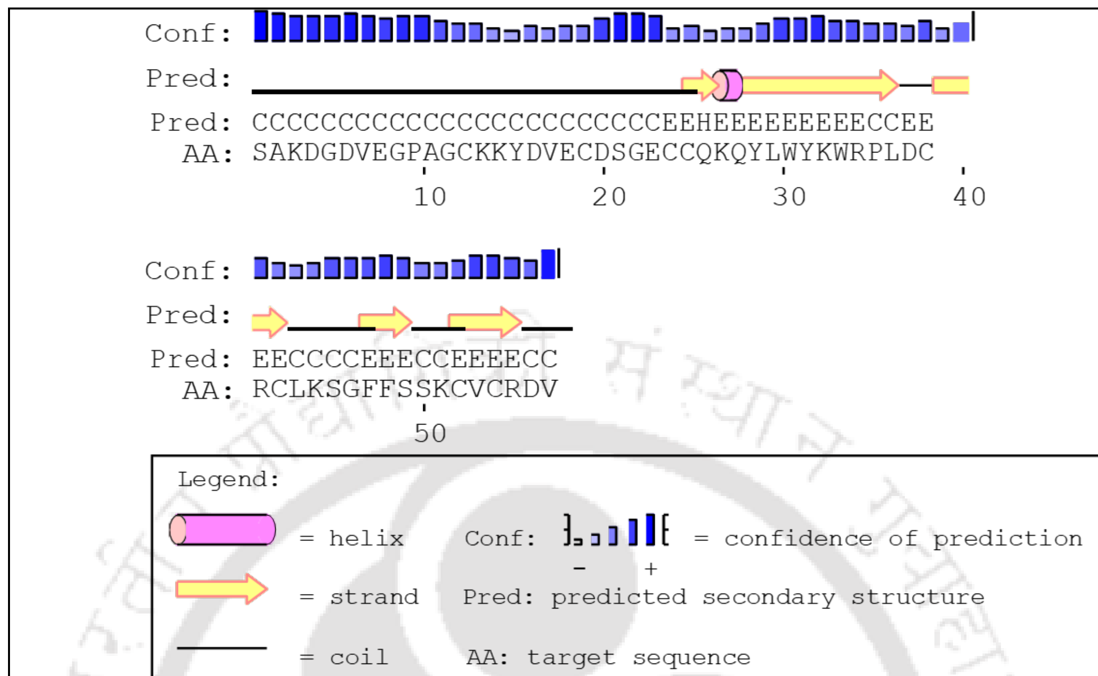

Fig. 2.9: Graphical representation of predicted secondary structure of purified rDc1a using PSIPRED v 3.3.

2.3.6 SDS PAGE and Western immunoblotting

The SDS-PAGE analysis of the purified rDc1a protein showed a single band, indicating that the final rDc1a preparation was mostly free from contaminating proteins (>95 %) (Fig 2.7, b). The purified fraction of MBP-DTX, MBP and Dc1a were subjected to western blot analysis using anti-MBP monoclonal antibody and anti-Dc1a toxin polyclonal antibody. The primary antibodies were able to specifically bind to the respective purified fraction after developing the membranes with chemilucent agent from Bio-Rad, USA (Fig. 2.8, c).

2.3.7 SWISS-MODEL homology modelling

Structural modelling studies of recombinant Dc1a having four vestige amino acid at N-terminus using SWISS-MODEL homology modelling further confirmed that it

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indeed adopts the correctly-reticulated native fold when compared to the solution structure of the Dc1a (Fig. 2.10, a) (Bende et al. 2014; Waterhouse et al. 2018). SWISS-MODEL used PDB ID 6a95 as a template for modelling the structure of expressed rDc1a (Fig. 2.10, b). Alignment results of the SWISS modelled structure with the solution structure of Dc1a are shown in Fig. 2.10, c.

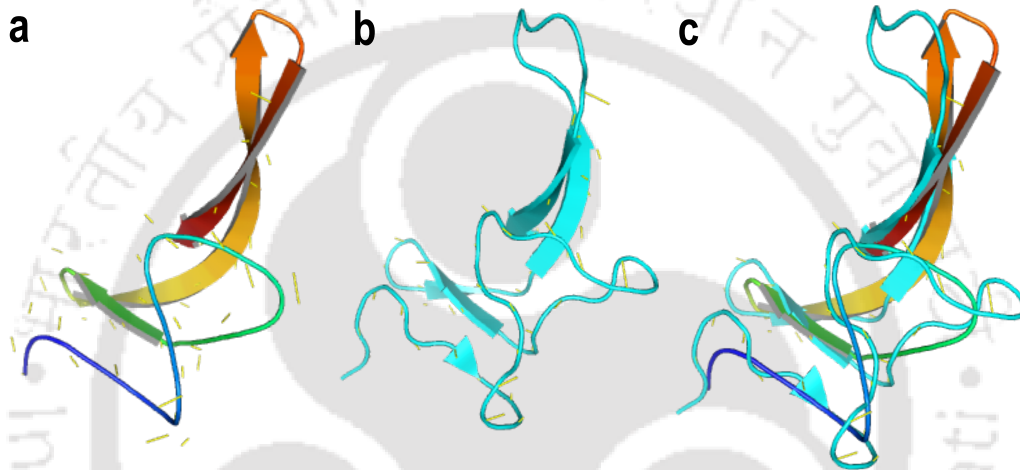

Fig. 2.10: Structural modelling. (a) SWISS-MODEL structure of expressed Mu-diguetoxin-Dc1a. (b) Solution structure of Mu-diguetoxin-Dc1a toxin, PDB ID: 2mi5. (c) Alignment of SWISS modelled structure with solution structure to predict the structural stability using Pymol Molecular Graphics System, Schrödinger, LLC (PyMOL 2017)**.**

2.3.8 Cytotoxicity effects of rDc1a on Sf21 and U-937cells

In this study, insect Sf21 cells and human U-937 cells were used to examine the effects of rDc1a on cell proliferation by a colourimetric MTT assay. Cultured cells were treated with five different concentrations of rDc1a and results were observed for 24 h, 48 h, 72 h and 96 h. The results showed that rDc1a inhibited the growth of insect Sf21 cells in a dose-dependent manner, with a significant reduction in the cell proliferation above 1 μM Dc1a concentration after 72 h post-treatment compared to

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control cells. Data analysis revealed an IC_{50} (**i**nhibitory **c**oncentration, i.e. concentration required for 50 % inhibition *in vitro*) of 7.08 μM rDc1a at 96 h (Fig. 2.11, a). In contrast, rDc1a showed no cytotoxicity effect on cultured human U-937 cells (Fig. 2.11, b). The experiments were performed in triplicate and repeated two times to confirm this result. It clearly demonstrated the selectiveness of spider toxin Dc1a to insect cells. Cytotoxicity assay using cultured Sf9 insect cells and MCF-7 human cells demonstrated that the toxin AaIT had specific toxicity against insect cells but not human cells. Only 0.13 μM rAaIT was needed to kill 50 % of cultured insect cells while as much as 1.3 μM toxin had absolutely no effect on human cell (Ji et al. 2002). However, rBmKIT accelerated the growth of Sf9 cells in a dose-dependent manner, with a growth rate of approximately 32.7 % under the maximal concentration of 13.0 μM rBmKIT (Fu et al. 2011).

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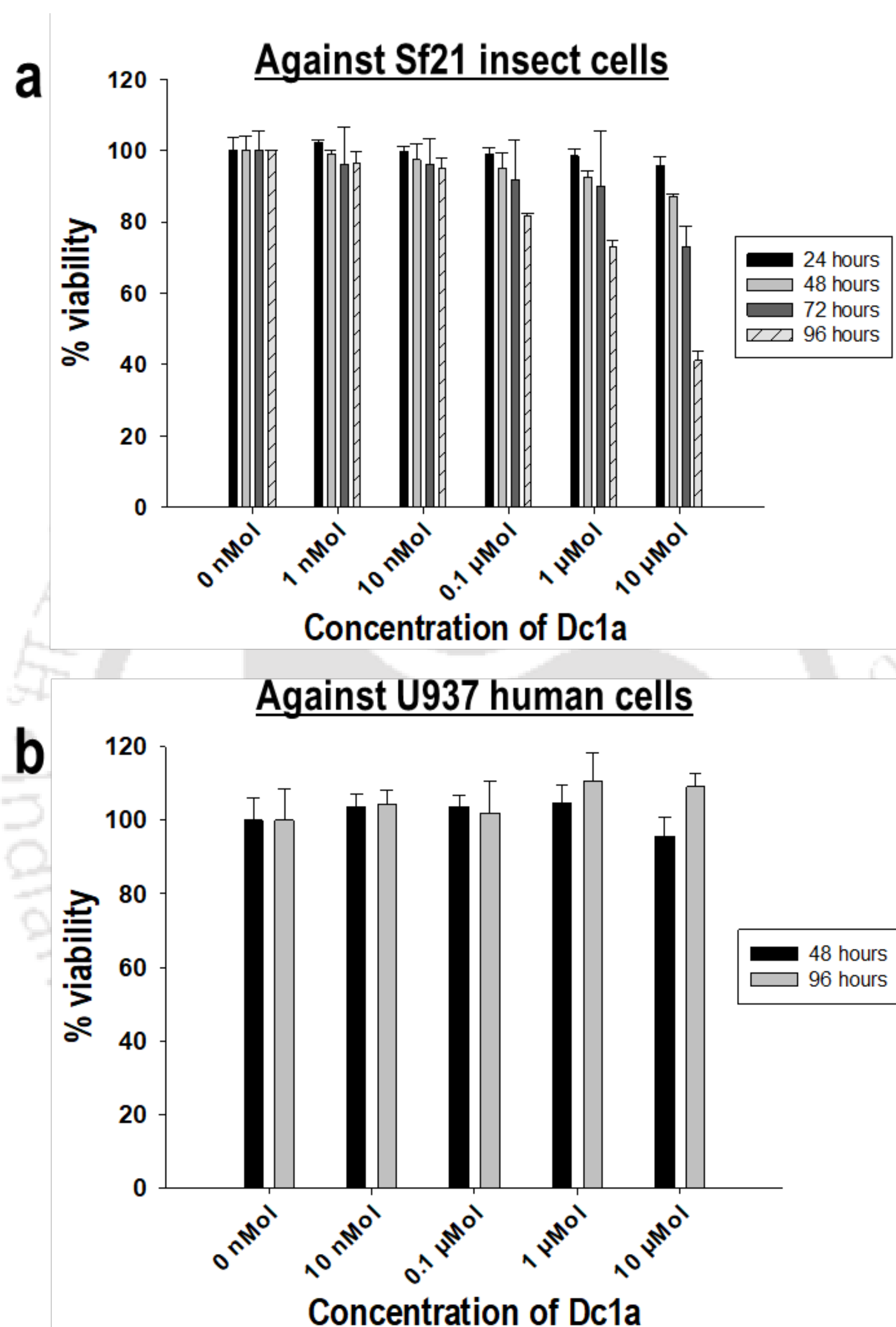

Fig. 2.11: Dose and the time-dependent response of rDc1a against cultured insect and human cells. (a) Sf21 cells were treated with purified rDc1a toxin up to 96 h, and cell viability were determined by the MTT colourimetric growth assay. The relationship between concentrations of rDc1a and percentage viability of cells was calculated by SigmaPlot software version 12. (b) shows cytotoxicity effects of rDc1a against human myeloid cells U-937 which were used as control cell lines.

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2.3.9 Localization studies of rDc1a on Sf21 and U-937cells

Immunofluorescence assay was performed to localise the site of action of rDc1a in Sf21 cells. Inverted fluorescent microscopy analysis clearly showed localisation of rDc1a on the membrane of Sf21 cells. Particularly, FITC conjugated secondary antibody recorded green spots in the merged image of fluorescence studies indicated that rDc1a was located on the insect Sf21 cell membrane (Fig. 2.12). However, no green fluorescence spots of rDc1a were observed on the human U-937 cell membrane, which further confirmed the selectiveness of spider toxin Dc1a to insect cells. Similarly, confocal microscopy analysis showed co-localization of tetrodotoxin (TTX) and rBmK IT on the membrane of Sf9 cells give support of the view that rDc1a could bind to and act on the Na_V channels on the Sf21 cells.

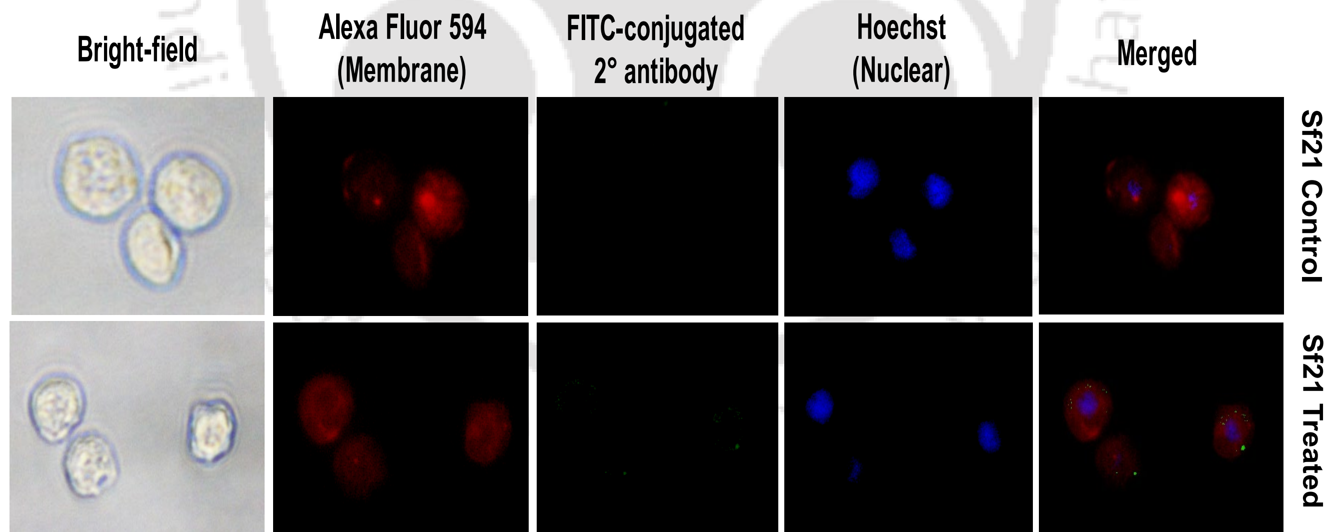

Fig. 2.12: Immunofluorescence localisation of rDc1a in Sf21 cells. Sf21 cells were incubated with 10 μM rDc1a toxin for 12 h. Then cells were stained with rabbit anti-Dc1a 1° antibody followed by incubation with goat anti-mouse IgG FITC. Fluorescence spots indicated the specificity of rDc1a for Na_V channels on the insect cell membrane.

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2.3.10 Cell cycle arrest and apoptosis studies using FACS

Cell cycle analysis by quantitation of DNA content was one of the earliest applications of flow cytometry. The cell cycle progression and apoptosis after rDc1a treatment (10 μM) to Sf21 cells was studied using PI (Fig. 2.13, a). It seems that the toxin rDc1a causes the arrest in G2/M phase as in treated cells total percentage of cells are significantly higher compared to control cells (Table 2.5). Besides this, reduction of the red fluorescence and enhancement of the green fluorescence signals of the JC-1 dye were observed in Sf21 cells (Fig. 2.13, b), indicating the disruption of mitochondrial potential in the presence of 10 μM rDc1a. Similar results were obtained when HeLa cells were treated with diphenylethylenediamine-based potent anionophores resulted in a stepwise reduction of the red fluorescence signals of the JC-1 dye in a concentration-dependent manner, indicating the disruption of mitochondrial potential (Akhtar et al. 2018). Cell cycle arrest studies using PI and change in mitochondrial potential using JC-1 provided evidence of early-stage apoptosis caused by rDc1a.

Table 2.5: Cell cycle arrest using PI arrest insect cells in G2/M phase compared to human cells.

Cell cycle Phase	Sf21		U-937	
	Control	rDc1a treated	Control	rDc1a treated
G0/G1	32.51 %	11.50 %	36.22 %	39.60 %
S	32.39 %	6.14 %	50.50 %	46.49 %
G2/M	35.20 %	82.36 %	13.28 %	13.91 %

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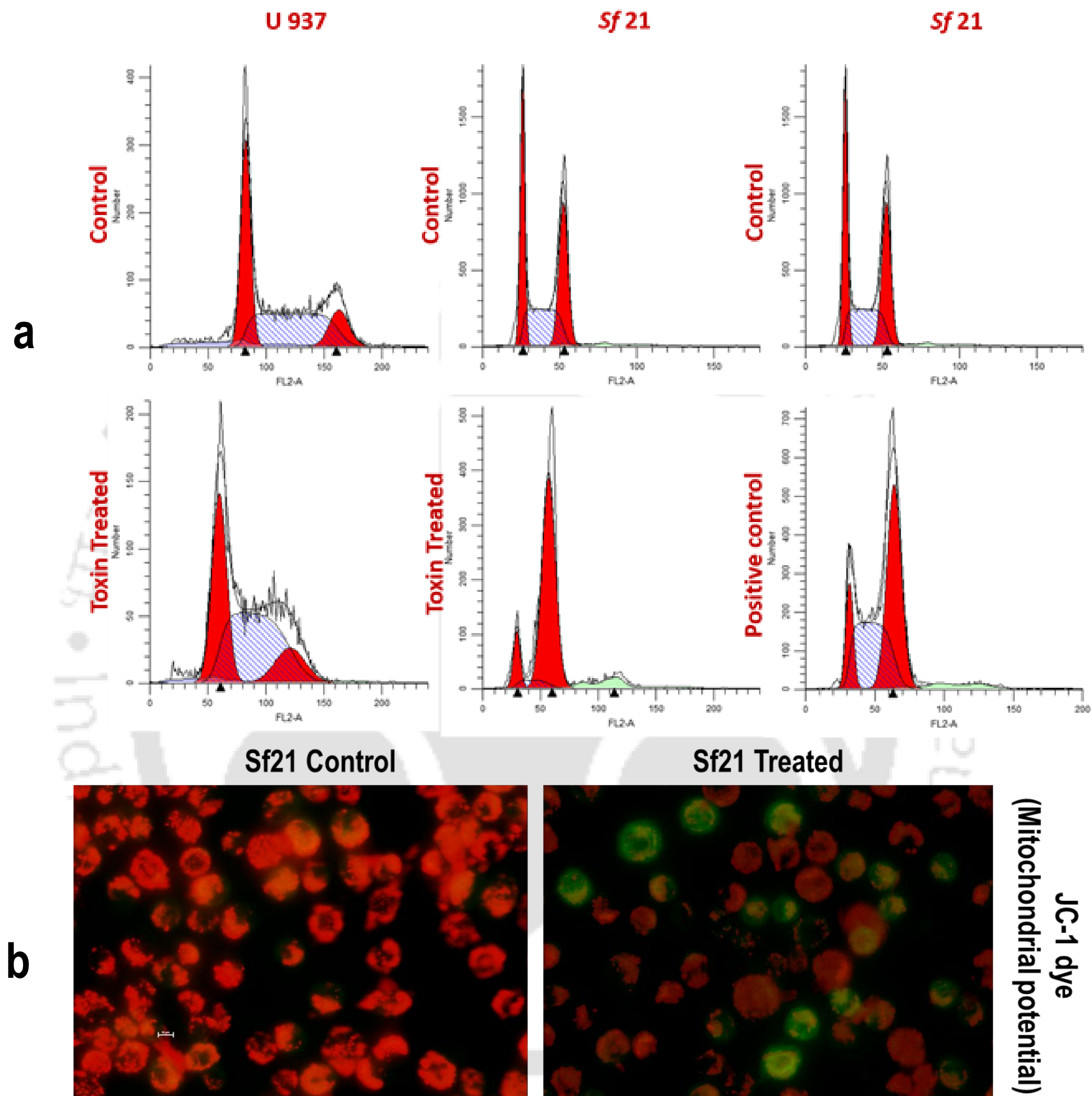

Fig. 2.13: Mitochondrial health and apoptosis studies using rDc1a. (a) Cell cycle progression studies with 10 μM rDc1a toxin at 48 h post-treatment were carried out using propidium iodide in FACS Calibur and data was fitted in Modfit Varity software. (b) Mitochondrial potential of rDc1a treated Sf21 cells were examined with membrane-permeant JC-1 dye.

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2.3.11 Microscopy analysis of rDc1a induced apoptosis in Sf21 cells

Sf21 insect cells treated with rDc1a toxin peptide showed significant morphological shape deformities such as irregular shaped cells, blebbing of the membrane and formation of apoptotic cell structures under phase contrast microscope at 40 X (Fig. 2.14). The results provide further support to our previous studies of mitochondrial-mediated apoptosis in lepidopteran cell lines (Tseng, Wu and Hou 2008). The morphological analysis using FESEM microscopy confirmed the cell death due to ionic pores formation in the plasma membrane leading to cell swelling and death upon exposure to Dc1a toxin (Fig. 2.14). No cytotoxicity effect was observed against cultured human myeloid cells U-937. The apoptosis is marked by the cell shrinkage causing detectable changes in cell dimensions (Wang et al. 2008). Cytotoxic study of neurotoxin AaIT1 from *A. australis* on Sf9 cell line and swainsonine on Sf21 cell lines demonstrated that small intrusion in the plasma membrane caused the cells to swell and resulting in membrane blebbing (Ji et al. 2002; Singh and Kaur 2013).

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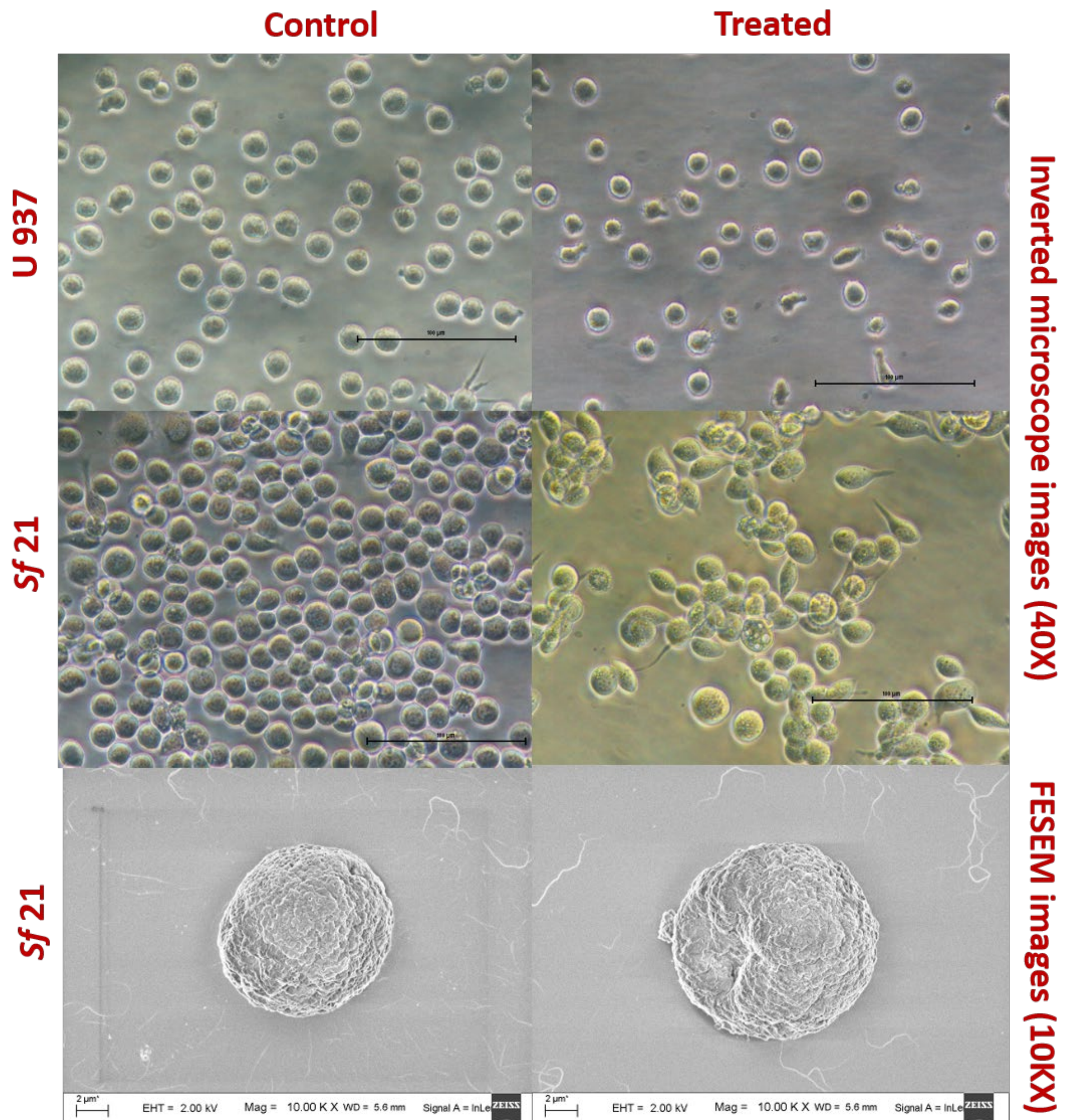

Fig. 2.14: Morphological analysis of *rDc1a* toxin (10 µM) induced apoptosis in *Sf* 21 cells using an inverted microscope (40X) and FESEM (10KX).

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2.3.12 Insect bioassays to determine the bio-insecticidal potential of the recombinant purified peptide toxin

In vivo insecticidal activity of purified rDc1a protein was determined using an intra-hemocoel injection bioassay against two major lepidopteran insect pests: *H. armigera,* and *S. litura.* Injection bioassays demonstrated that rDc1a was toxic to lepidopteran larvae causing a spastic paralysis with 50 % paralytic doses (LD_{50}) for *S. litura* was 0.416 nmol/g (Fig. 2.15 and 2.16, a) and for *H. armigera* was 0.397 nmol/g (Fig. 2.15 and 2.16, b), conclude that rDc1a has a similar lethality to agricultural pests when compared with native toxin. Larvae injected with rDc1a toxin all displayed paralysis within 30 sec (little mobility and almost a complete absence of feeding). Mortality was observed up to 48 h of the assay. The effects of rDc1a were dose-dependent, with mortality after 24 h ranging from 75 % at 2.0 µg toxin/larvae to 20 % at 0.1 µg toxin/larvae.

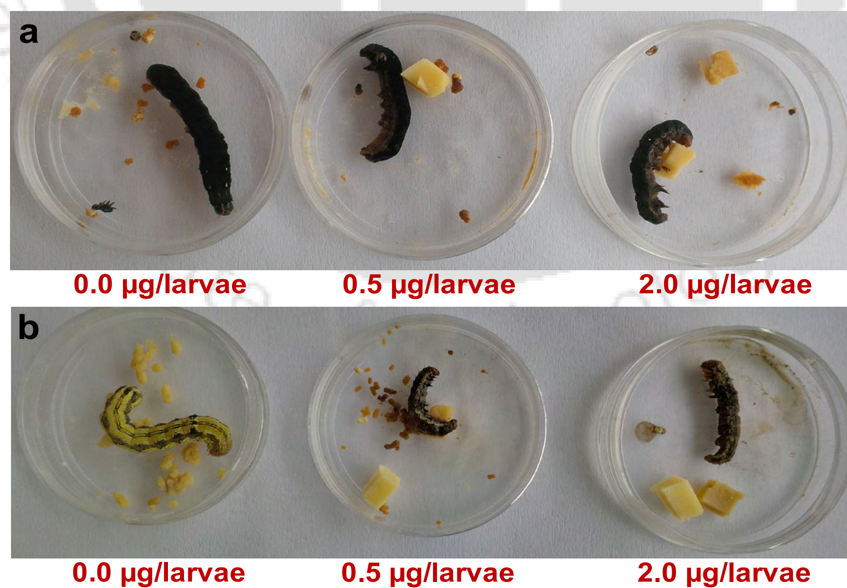

Fig. 2.15: Acute toxicity of rDc1 showing lethal effects against two major lepidopteran insect pests. (a) *S. litura,* and (b) *H. armigera* (mortality was determined after 24-48 h of injection). (N=10 insects per dose).

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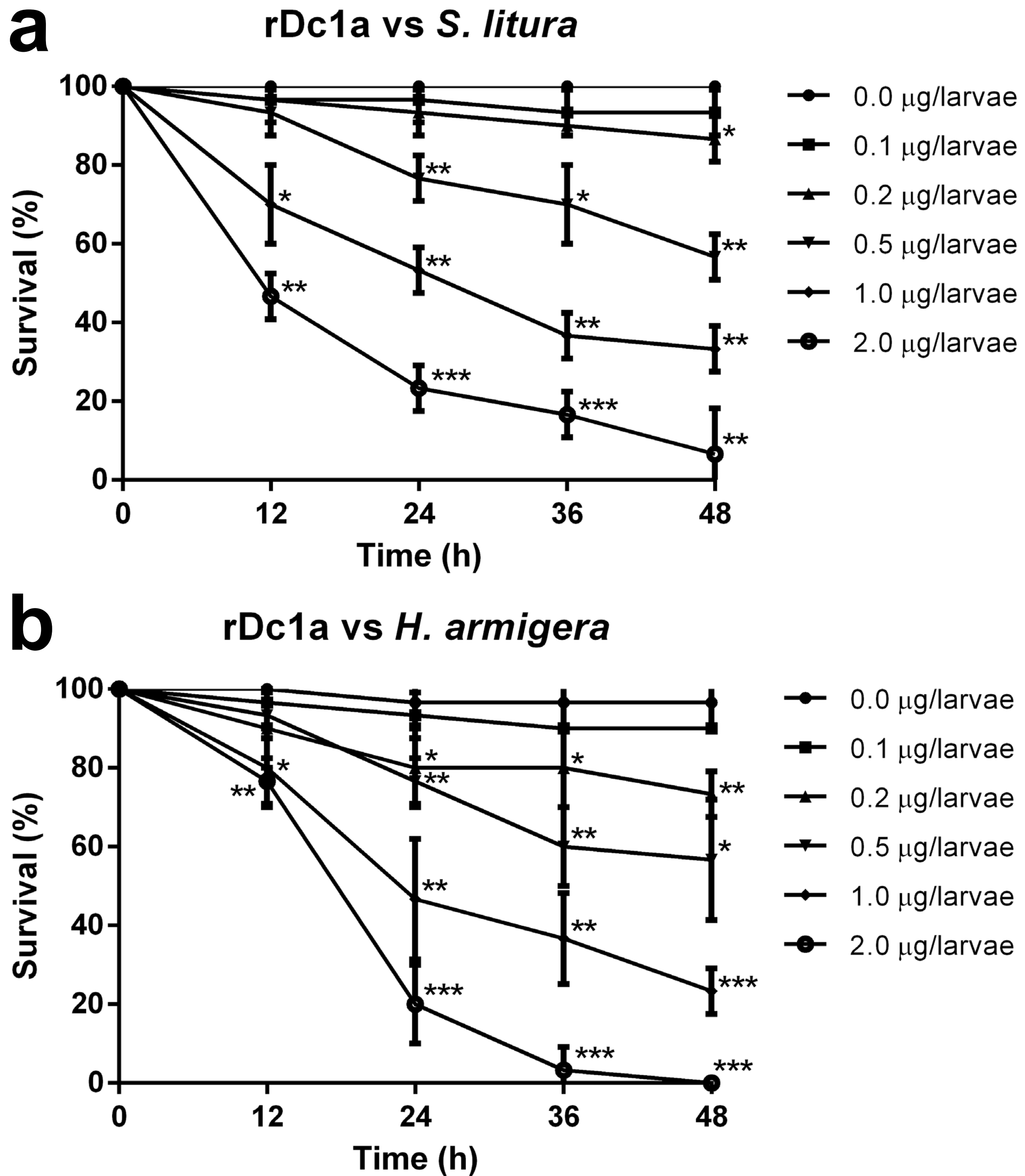

Fig. 2.16: Mortality data were fitted using Probit analysis and LD_{50} values were calculated. Survival analysis showing the effect of rDc1a against (a) Third to fourth instar larvae of *S. litura*, and (b) Third to fourth instar larvae of *H. armigera.* (N=10 insects per dose). **T-test using Microsoft Excel, *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001.**

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Diet based toxicity of rDc1a was determined against adults of *M. domestica*, a major vector for human pathogens. Venom peptides were dissolved in 5 % sucrose solution, and droplet-feeding assays were conducted. Significant oral activity (>75 % mortality at 48 h post-feeding) was observed with LD_{50} of 85.39 nmol/g (Fig. 2.17). The reported oral toxicity revealed that it is 171-fold less toxic to injection bioassays carried out against *M. domestica* (Bende et al. 2014). The oral potency was much lower than injection potency because of the fact that orally administered venom peptides have to first cross the epithelium of the insect midgut before reaching their molecular targets in the insect nervous system (Guo, Herzig and King 2018).

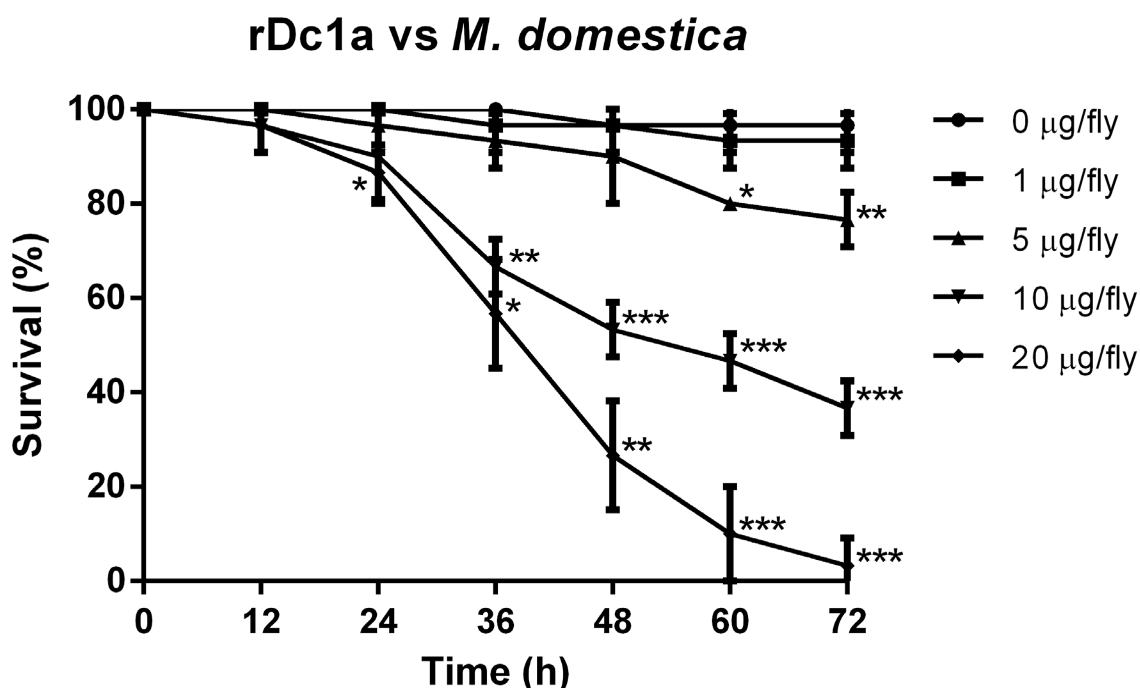

Fig. 2.17: Mortality data were fitted using Probit analysis and LD_{50} values were calculated. Survival analysis showing the effect of rDc1a against adults of *M. domestica.* (N=10 insects per dose). **T-test using Microsoft Excel, *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001.**

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In vivo insecticidal activity of purified rDc1a protein and LD_{50} values calculated using an intra-hemocoel injection bioassay and droplet-feeding assays is summarised in Table 2.6.

Table 2.6: Summary of the insect bioassays with rDc1a against lepidopteran and dipteran insect pests.

S. No.	Insect pest	LD_{50}
1.	Tobacco cutworm (*S. litura*)	2.86 µg/g insect weight or 416.28 pMol/g insect weight
2.	Cotton bollworm (*H. armigera*)	2.73 µg/g insect weight or 397.76 pMol/g insect weight
3.	Housefly (*M. domestica*)	580.06 µg/g insect weight or 85.39 nmol/g insect weight

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2.4 Conclusion

Recombinant anti-insect selective spider peptide toxins modulate voltage-gated ion channels, thus considered as an attractive alternative to chemical insecticides for efficient and environmentally safer means of insect pest control. In the present study, the codon-optimized gene of spider venom peptide, Mu-diguetoxin-Dc1a, was successfully expressed as a soluble fusion protein in the periplasm of E. coli. Purified fractions of recombinant Dc1a toxin were finally characterised using MALDI-TOF mass spectrometry, CD spectroscopy and western blotting. Structural studies of the Dc1a peptide confirm that it indeed adopts the correctly-reticulated native fold. *In situ* cytotoxicity effect on lepidopteran *S. frugiperda* (Sf21) insect tissue cells revealed a significant reduction in cell proliferation. Injection bioassays demonstrated that rDc1a was toxic to lepidopteran larvae causing a spastic paralysis having similar lethality compared with the native toxin. The reported oral toxicity against dipteran adults revealed that it is 171-fold less toxic to injection bioassays. The selected ISVP Dc1a have high potency, environmental stability, insect specificity and novel molecular target, all ideal attributes for development as bioinsecticides. As the arsenal of chemical insecticides is diminishing rapidly due to the development of insect resistance, significant high-level toxicity of rDc1a toxin peptide towards lepidopteran and dipteran insects *in vivo* renders it as a potential candidate for incorporation in recombinant baculovirus based insecticides and to construct transgenic plants.

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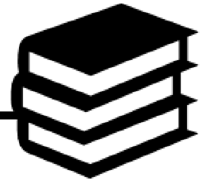
Chapter Three



Functional expression of ISVP Dc1a in Metarhizium and Beauveria, and evaluating the bio-insecticidal potential of genetically modified fungi against insects of different orders

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3.1 Introduction

Crop protection strategies using biological control offers a promising area and an attractive alternative for insect pest management. Bacteria, viruses, and fungi that cause diseases to insects have been studied and evaluated for many years, but very few of them are widely used as bioinsecticides. Fungal entomopathogens, *Beauveria bassiana* and *Metarhizium anisopliae*, have tremendous potential for use as pest biological control agents (Lacey *et al.* 2015; Lovett and St Leger 2017), particularly as alternatives to chemical pesticides. One of the main advantages of using entomopathogenic fungi in insect control is that these fungi can infect all stages of the insects, including larvae and adults (de Paula *et al.* 2008; Pereira *et al.* 2009; Santos *et al.* 2009). Furthermore, no cases of resistance of insects to entomopathogenic fungal infections have been reported to date. However, the relatively slow action of fungal pathogens in killing insect pests and requirement of high conidial dose, compared with chemical insecticides, has hampered their widespread application in the field (Farenhorst and Knols 2007; Fang, Azimzadeh and Leger 2012). It is a major deterrent to their commercial use and large-scale application, and thus they are unable to compete with faster-acting and cheaper chemical insecticides (Leger and Wang 2010).

A better understanding of fungal pathogenesis in insects and the introduction of genetic manipulation techniques is allowing construction of transgenic strains with improved efficacy. *M. anisopliae* was engineered to express AaIT, a NaV channel

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blocker from scorpion venom, improved fungal performance against the lepidopteran *Manduca sexta* and the malaria vector *Anopheles gambiae* by 22-fold and 9-fold, respectively (Wang and St Leger 2007). Similarly, a strain of *M. pingshaense* expressing the spider toxin Hybrid was highly effective against wild-caught, insecticide-resistant *A. gambiae* (Bilgo *et al.* 2017). The transgenic strain of *B. bassiana* Bb-Cyt2Ba significantly reduced the survival and fecundity of *A. aegypti* and *A. albopictus* larvae with LT_{50} decreased by 42 % and 33 %, respectively, compared with the WT strain.

Hence, the study presented in this chapter was aimed to improve and examine the efficacy of genetically engineered *M. anisopliae* and *B. bassiana* against insect pests of different orders after the expression of highly insecticidal spider-venom peptide Dc1a.

3.2 Materials and Methods

3.2.1 Chemicals, Reagents and Kits

Chemicals and reagents used in the present study were of molecular biology grade or analytical grade. List of all the chemicals, reagents and kits with particulars of the manufacturer is mentioned in the appendix.

3.2.2 Microbial strains and media

DH5α competent *E. coli* cells from Novagen (Merck), Germany were used as cloning host and were maintained on LB broth. However, gene cassette transfer to fungal entomopathogens was carried out with the help of *Agrobacterium tumefaciens* strain EHA105 which was kindly provided by Dr. Lingaraj Sahoo,

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Professor, Department of Biosciences & Bioengineering, Indian Institute of Technology, Guwahati. The strain EHA105 was maintained on **Y**east **P**eptone **D**extrose (YPD) broth. The genotype information of both DH5α and *Agrobacterium tumefaciens* strain EHA105 is mentioned in Table A2 (see appendix).

Wild type cultures of insect pathogenic fungi *B. bassiana* strain 984 and *M. anisopliae* strains 892 and 3310 were purchased from the **M**icrobial **T**ype **C**ulture **C**ollection (MTCC), Chandigarh, India. The culture of *B. bassiana* was maintained on **S**abouraud **d**extrose **a**gar (SDA) and conidia were obtained by growing the fungus on SDA for 7-10 days at 25±1 °C. Likewise, cultures of *M. anisopliae* were maintained on **P**otato **d**extrose **a**gar (PDA) at 25±1 °C. *M. anisopliae* strain 892 produced conidia by growing the culture on PDA for 7-10 days; however, *M. anisopliae* 3210 produced conidia within 5-7 days on PDA. Detailed strain information and the media composition used for maintaining the fungal cultures are highlighted in Table A3 and Table A6 (see appendix), respectively.

3.2.3 Standard molecular biology techniques

All standard molecular biology techniques were performed following the protocols described in Molecular Cloning: A Laboratory Manual: Fourth Edition (Sambrook and Russell 2001; Green and Sambrook 2012).

3.2.4 Gene and amino acid information

The nucleotide sequence of the MCl1 gene of *M. anisopliae* (GenBank accession no. DQ238489.1) and the amino acid sequence of the *D. canities* (Desert

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bush spider) toxin (UniProtKB accession no. P49126) were retrieved from the NCBI website (https://www.ncbi.nlm.nih.gov).

3.2.5 Gene synthesis

The peptide sequence of Dc1a toxin was reverse translated to a nucleotide sequence using EMBOSS Backtranseq tool provided by the EBML-EBI (www.ebi.ac.uk/Tools/st/emboss_ backtranseq/) (Madeira *et al.* 2019). Nucleotide sequence obtained was codon-optimized for maximal expression in *Metarhizum* and *Beauveria* strains using OptimumGene^{TM} algorithm of GenScript, USA. The codon usage quality was depicted using gcua version 2.0 (Sharp and Li 1987). The synthesised gene was sequenced and cloned between the *BamHI* and *EcoRI* restriction sites into a pUC57 vector (GeneScript, USA). The pUC57 vector harbouring Dc1a gene was transformed and maintained in DH5α competent *E. coli* cloning host for further use.

3.2.6 Cloning strategy and vector construction

The expression cassette was composed of four genes encoding: ISVP Dc1a, MCl1 signal peptide (help in expression of Dc1a in the secretory form), MCl1 promoter (restrict the expression of Dc1a toxin in the hemolymph), and MCl1 partial gene sequence (for homologous recombination after fungal transformation). The cloning strategy of the expression cassette construction is summarized in Fig. 3.1

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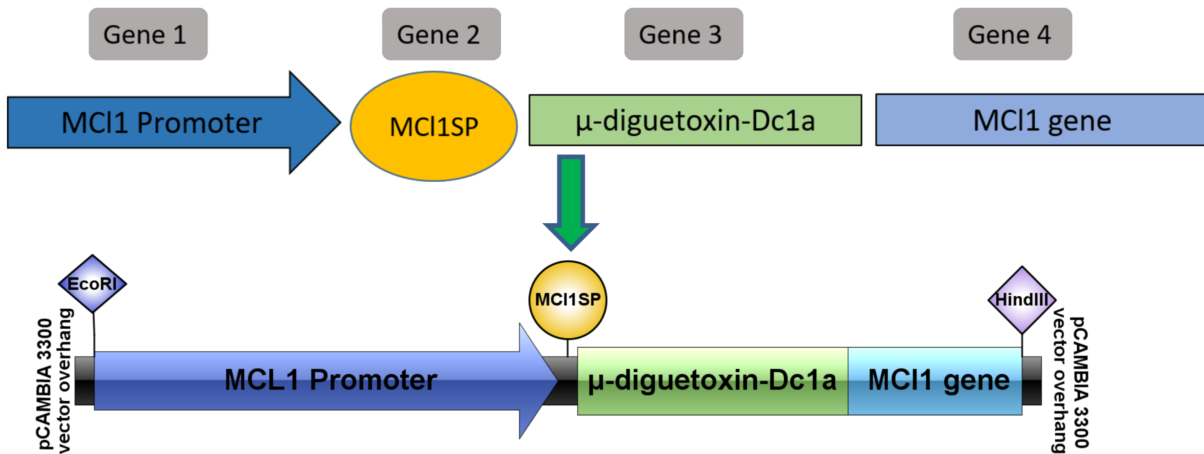

Fig 3.1: Illustration of the cloning strategy used for expression of Dc1a gene in *Metarhizium* and *Beauveria*, inserted downstream of the MCl1 signal sequence for targeted secretion of insecticidal venom peptide from fungal entomopathogen using hemolymph-specific promoter PMCl1.

3.2.6.1 Amplification conditions for gene cassette

The coding region of the synthesized Dc1a gene was amplified by PCR using the plasmid pUC57 Dc1a as a template. Similarly, the PMCl1 promoter, MCl1 gene signal peptide and MCl1 partial gene sequences were amplified using the genomic DNA of *M. anisopliae* strain 892 as a template. The oligonucleotides sequence and other components used for a 50 µL PCR reaction mixture are shown in Table 3.1 and Table 3.2, respectively. Mastercycler (Eppendorf, Germany) was used for the amplification following the PCR condition presented in Table 3.3. Additional overlap sequences were added at the 5' of the oligonucleotides, to facilitate insertion of the PCR fragment into the pCAMBIA3300 *Agrobacterium* binary vector.

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Table 3.1: List of oligonucleotides used for amplification and sequencing (fungal cloning).

Gene	Sequence
MCl1 promoter (PMCl1)	FP 5'-*ggaaacagctatgaccatgattac*gaattcaatcatgcagcgctatgagagc-3' RP 5'-ccgaagaaagttcacgcatgatggtctagggaacgga-3'
MCL1 SP	FP 5'-tccgttccctagaccatcatgcgtgaactttcttcgg-3' RP 5'-caacatcgccatccttagctgccgacgccagggccag-3'
Dc1a	FP 5'-ctggccctggcgtcggcagctaaggatggcgatgttg-3' RP 5'-gttgagcagggcggcaggatcctgttaaacatcgcggcagacgcac-3'
MCl1 partial gene	FP 5'-gtgcgtctgccgcgatgtttaacaggatcctgccgccctgctcaac-3' RP 5'-*cgttgtaaaacgacggccagtgcc*aagcttggcatcagagccagcaccggtg-3'

FP: Forward primer; RP: Reverse primer; *EcoRI* and *HindIII* sites are shown as underlines in FP and RP, respectively; atg and taa: Start and stop codons are highlighted in Blue colour, pCAMBIA3300 vector overlap sequences are shown in italics

Table 3.2: PCR reaction components (fungal cloning).

Component	Volume (50 µl reaction)	Final concentration
5X Phusion HF Buffer	10.0	1X
10 mM dNTPs	1.0	200 µM
10 µM Forward Primer	2.5	0.5 µM
10 µM Reverse Primer	2.5	0.5 µM
Phusion DNA Polymerase, 2U/µl	0.5	0.02 U/µl
DMSO	1.5	3 %
Template DNA	0.5	20-50 ng
Nuclease-free water	31.5	---

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Table 3.3: Amplification conditions used for amplification of gene cassette and sequencing.

Gene	PCR Conditions	Time
PMCI1 promoter	I. Initial Denaturation at 98 °C	2 min
	II. 30 cycles	
	i) Denaturation at 98 °C	45 s
	ii) Annealing at 71 °C	45 s
	iii) Extension at 72 °C	2 min
	III. Final Extension at 72 °C	5 min
MCI1 signal sequence	I. Initial Denaturation at 98 °C	2 min
	II. 30 cycles	
	i) Denaturation at 98 °C	45 s
	ii) Annealing at 65 °C	45 s
	iii) Extension at 72 °C	30 s
	III. Final Extension at 72 °C	5 min
Dc1a mature toxin gene sequence	I. Initial Denaturation at 98 °C	2 min
	II. 30 cycles	
	i) Denaturation at 98 °C	30 s
	ii) Annealing at 62 °C	30 s
	iii) Extension at 72 °C	30 s
	III. Final Extension at 72 °C	5 min
MC11 gene partial sequence	I. Initial Denaturation at 98 °C	2 min
	II. 30 cycles	
	i) Denaturation at 98 °C	45 s
	ii) Annealing at 67 °C	45 s
	iii) Extension at 72 °C	1 min
	III. Final Extension at 72 °C	5 min

Amplicons were separated individually and extracted from the 1 % agarose gel by Gel and PCR clean-up kit (Macherey-Nagel, Germany) following the manufacturer's instructions (Fig. 3.2).

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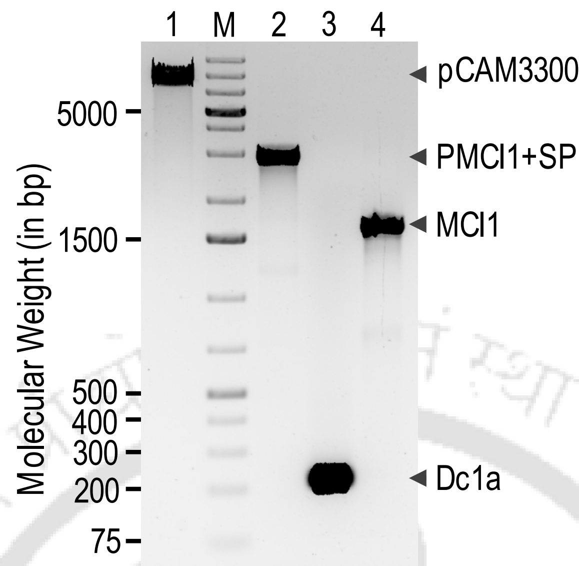

Fig. 3.2: Agarose gel electrophoresis illustrating PCR amplified fragments after gel purification. lane M: Gene ruler 1 kb plus DNA ladder; lane 1: pCAMBIA 3300 vector; lane 2: PMCI1 promoter; lane 3: Dc1a gene optimised for fungal expression; lane 4: MCI1 gene.

The purified fragments were introduced between the *EcoRI* and *HindIII* sites in pCAMBIA3300 vector using Gibson cloning assembly (NEB, USA). Gibson Assembly employs three enzymatic activities in a single-tube reaction: 5′ exonuclease, the 3′ extension activity of a DNA polymerase and DNA ligase activity (Gibson *et al.* 2009). The 5′ exonuclease activity chews back the 5′ end sequences and exposes the complementary sequence for annealing. The polymerase activity then fills in the gaps in the annealed regions. A DNA ligase then seals the nick and covalently links the DNA fragments together. The assembly reaction was facilitated in the 5X isothermal reaction buffer at 50 °C for 45 min and the product pCAM-bar-PMCI1-SP-Dc1a-MCI1 was transformed into DH5α competent cells using standard heat shock method. The whole reaction is summarized in Fig. 3.3.

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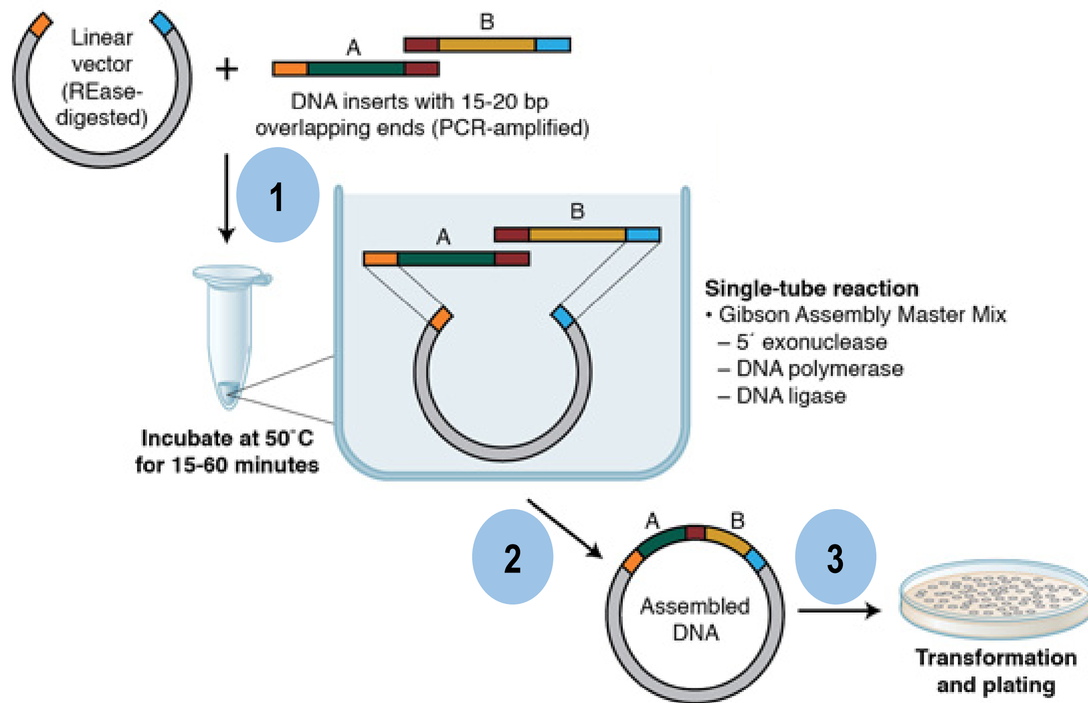

Fig. 3.3: Schematic representation of Gibson cloning steps. 1: Linearized pCAMBIA300 vector was mixed with purified DNA encoding sequence of PMCI1 promoter, MCI1 signal sequence, Dc1a gene, 2: Samples were incubated in a thermocycler at 50°C for 60 minutes to assemble DNA fragments. 3. Assembled DNA was transformed to DH5α competent *E. coli* cells and selection of transformants on LB agar media plates with suitable antibiotics.

The pictorial representation of pCAM-bar-PMCI1-SP-Dc1a-MCI1 used for transformation is represented in Fig. A2 (see appendix). Positive transformants were selected on LB agar plates containing kanamycin (50 μg/mL). Selected colonies were screened for positive clones by colony PCR and sequencing. The clones with full gene cassette insert were further transformed into *A. tumefaciens* strain EHA105 by electroporation using Gene Pulser Xcell Electroporation System by Bio-Rad (USA). Positive transformants were checked by colony PCR using gene-specific primers and stored as glycerol stock at -80 °C until further use.

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3.2.7 Gene transfer strategies

3.2.7.1 *Agrobacterium*-mediated gene transfer to *M. anisopliae*

Transformation of *M. anisopliae* was carried out as described by Covert *et al.* (2001) with modifications. Positive transformants harbouring full gene construct in *A. tumefaciens* strain EHA105 were grown at 29 °C for 18 h in LB medium that was supplemented with kanamycin (50 µg/mL) and rifampicin (10 µg/mL). The culture was diluted to an OD_{660} of 0.15 in 5 mL of induction medium (IM) containing 200 µM acetosyringone (AS, a virulence gene inducer) (see Table A8 in appendix). The cells were further grown under the same conditions until an OD_{660} of 0.6–0.8 was reached.

Meanwhile, *Metarhizium* conidia from a 10-12-days old culture plate were harvested into 5 ml of 0.05 % sterile Triton-X 100 (Triton-X helps to separate conidia). The mixture was vortexed for 2 min then filtered through sterile glass wool to remove the mycelial mass. The conidia concentration was calculated using a hemocytometer and readjusted to a final concentration of 1×10^6 conidia/mL with induction media.

Conidial suspension (100 µL) of *Metarhizium* (1×10^6 conidia/mL) was mixed with the equal volume of *Agrobacterium*-pCAM-bar-PMCI1-SP-Dc1a-MCI1 culture (OD_{600} ~ 0.4–0.8) and plated on nitrocellulose membrane (0.45 µm) for co-cultivation in dark. Following co-cultivation at 29 °C for 48 h, the membranes were transferred to **C**zapek-**d**ox **a**gar (CDA) minimal medium plates that contained glufosinate ammonium (250 µg/mL) as the selection agent for fungal transformants and

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cefotaxime (300 μg/mL) to inhibit the growth of *A. tumefaciens* strain EHA105. The media composition of CDA is given in Table A9 (see appendix). Putative transformants, visible 7 days later, were transferred to PDA plates and were sub-cultured for 2-3 generations for genomic stability of the gene constructs. Positive transformants were finally screened after genomic DNA isolation and PCR amplification was carried out using Dc1a gene-specific primers.

3.2.7.2 Protoplast based gene transfer to *B. bassiana*

B. bassiana 984 culture was grown in SDB medium at 27±1 °C with shaking for 36 h. Mycelia were collected and washed with 20 mM phosphate buffer. Protoplasts were released from mycelia after treatment with lysing enzyme (10 mg/mL) in the buffer containing 20 mM phosphate buffer, 0.7 M KCl and 10 mM DTT. Cell debris was removed using centrifugation at very less rpm (~ 50 rpm/min). Protoplasts were resuspended in ice-chilled 1 M sorbitol and incubated with a vector containing gene construct pCAM-bar-PMCI1-SP-Dc1a-MCI1 for half an hour on ice before electroporation using Gene Pulser Xcell Electroporation System by Bio-Rad (USA). The product was plated on to CDA medium (see Table A9 in appendix) containing 200 μg/mL glufosinate ammonium as a selection marker. Agar overlay was done after 12-16 h of incubation at 28 °C with fresh CDA medium containing 250 μg/mL glufosinate-ammonium. Transformants started appearing after 5-6 days. Putative transformants with fast growth were sub-cultured onto fresh CDA medium plates for 2-3 generations for genomic stability of the gene constructs and then screened of positive recombinants after DNA isolation.

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3.2.8 Fungal DNA extraction

Genomic DNA was extracted using the CTAB method (Zhang *et al.* 1996) with necessary modifications. Briefly, the mycelia collected from the transformants with fast growth were ground using mortar-pestle in liquid nitrogen into a fine powder. Mycelium powder approx. 200 mg was suspended in 1000 µL of DNA extraction buffer. To the above suspension, 200 µL of 5 M NaCl and 100 µL of 10 % **c**e**t**yltrimethyl**a**mmonium **b**romide (CTAB) were added. The resulting mixture was incubated in a water bath for 15 min at 65 °C with occasional inversion. An equal volume of Chloroform: Isoamyl alcohol (24:1; v/v) was added to the lysed mixture and centrifuged at 12,000 × g for 15 min. The upper aqueous layer was collected gently and incubated with an equal amount of isopropanol overnight at −20 °C for precipitation. Post incubation, tubes were centrifuged at 10,000 × g for 10 min (4 °C), followed by washing of upper aqueous phase with 70 % ethanol. Finally, the pellet was dissolved in nuclease-free water (30 µL) and stored at −20 °C till further use.

DNA encoding sequence of Mu-diguetoxin-Dc1a toxin gene was amplified using gene-specific primers from the isolated DNA for screening the positive clones. PCR products from each of the transformants (Ma 892-Dc1a, Ma 3210-Dc1a, Bb 984-Dc1a) were electrophoresed on an agarose gel. DNA bands were excised and sequenced to confirm the presence of ISVP transgene in transformed *Metarhizium* and *Beauveria* strains.

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3.2.9 Characterization of expression of ISVP Dc1a

Putative transformants of *M. anisopliae* (Ma 892-Dc1a, Ma 3210-Dc1a) and *B. bassiana* (Bb 984-Dc1a) were individually inoculated into conical flasks containing 30 mL SDB media and incubated at 28 °C for 36-40 h at 120 rpm. *B. bassiana*, mycelial inoculum (~0.1 g wet weight) from SDB cultures were further incubated at 28 °C for up to 12 h in 10 mL of *Antheraea assamensis* hemolymph. The same amount of mycelia were also collected before incubation and used as a control. The filtrates were collected at different time intervals (0 min, 20 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h) and expression studies were carried out using RT-PCR and Western blot analysis. However, in the case of *M. anisopliae* strain 892 and 3210, putative transformants were induced for fixed 12 h in 10 mL of *A. assamensis* hemolymph for expression studies using RT-PCR and Western blot analysis.

3.2.9.1 Reverse transcription PCR analysis

The liquid nitrogen grounded samples were collected from different putative transformants (100-200 mg) and used for RNA isolation using RNAiso Plus (Takara, Japan) as per the manufacturer protocol. The precipitated RNA was dissolved in **di**e**thyl** **p**yro**c**arbonate (DEPC) water and the concentration was quantified using nanodrop (Implen NanoPhotometer). To check the integrity of RNA, samples were run on the agarose gel electrophoresis. RNA samples were digested with DNase I (Promega) as per the manufacturer's protocol before cDNA synthesis. Samples were further used for cDNA synthesis using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Japan) following the manufacturer's protocol. PCR

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amplification using gene-specific Dc1a RT-PCR primers (FP: CTTCGGTTCTCGCCCTTTC, RP: ACTTCAGGCAACGGCAGTC) was done to check the amplification of Dc1a gene from isolated cDNA of different transformants of *Metarhizium* and *Beauveria*.

3.2.9.2 Western immunoblotting

Tricine SDS-PAGE is used to separate peptides having low molecular mass (Schägger 2006). The filtrates collected at different time intervals after hemolymph induced samples were collected at various stages of purification were mixed with loading dye, and boiled at 95 °C for 10 min. The denatured samples were loaded on 16 % Tricine SDS-PAGE gel and were separated in a Mini-Protean Tetra cell gel electrophoresis apparatus (Bio-Rad, USA). The gels were then used immediately for western blot analysis. The buffers used for Tricine SDS-PAGE are shown in Table A14 (see appendix).

Following SDS-PAGE, Power Blotter semi-dry transfer system (ThermoFisher Scientific, USA) was used to transfer protein from gel to 0.22 µm pore size nitrocellulose membranes (Bio-Rad, USA). The process of electro-blotting was performed at 12 A for 25 min in Towbin transfer buffer. The transfer process followed by blocking the membrane with 10 mL of blocking solution for 2 h with gentle agitation at room temperature. The blocking step was followed by placing the membrane in the primary antibody, Anti-Dc1a polyclonal antibody (GenScript, USA), at a dilution 1:2000 in blocking buffer and allowed to incubate overnight at 4 °C. Removing residual unbound primary antibody was then done by washing the

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membrane with 1x TBST 5 times, for 5 min each time, under shaking conditions. The membrane was then left in diluted HRP-conjugated secondary antibody, goat anti-rabbit antibody in blocking buffer at ratio 1:5000 (v/v), for 1 h at room temperature. The membrane was then briefly washed in distilled water. The specifically bound secondary antibody of the target protein was detected using Clarity Max ECL Western Blotting Chemiluminescent Substrate (Bio-Rad, USA). Detection solutions were mixed in a ratio of 1:1, gently poured over the membrane and incubated for 5-10 s. ChemiDoc XR+ Gel Documentation System (Bio-Rad, USA) was used to develop the membrane. The buffers used for immunoblotting are shown in Table A14 (see appendix).

3.2.10 Insect bioassays against tobacco cutworm and housefly

Insect bioassays against dipteran and lepidopteran insect pests were carried out at National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India.

Lepidopteran insect selected in the present study includes tobacco cutworm (*S. litura*). Larvae of the selected lepidopteran insect were reared on castor leaves up to second instar and thereafter shifted to artificial diet (see Table A12 in appendix). Adults were housed in cages (50 X 50 X 50 cm) and provided with honey-soaked cotton wicks. Standard insectary conditions at 25±2 °C and ambient humidity (40–60 %) with a photoperiod of 14 h light and 10 h darkness was provided.

Dipteran insect selected in the present study includes housefly (*M. domestica*) which was reared in standard insectary conditions at 25±2 °C and ambient humidity (40–60 %) with a photoperiod of 14 h light and 10 h darkness.

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Housefly larvae (Maggots) were reared on an artificial diet (see Table A11 in appendix). Adults of housefly were housed in cages (50 X 50 X 50 cm) and provided with honey-soaked cotton wicks. Water in cages was provided in cotton wicks as drinking sites.

The virulence of *M. anisopliae* strains 892-Dc1a, 3210-Dc1a and *B. bassiana* 984-Dc1a, and their respective wild type was assayed using topical application of conidial suspension. Conidia from a 15-days old culture plate of each transformed strain *Metarhizium anisopliae* (Ma 892-Dc1a, Ma 3210-Dc1a), and *Beauveria bassiana* (Bb 984-Dc1a) were harvested by scraping off the contents with sterile micropipette tips into 5 mL of 0.05 % sterile tween-20. Mycelial mass was removed by passing the conidial suspension through glass wool. Similarly, conidia from wild type (WT) strains were also collected. The concentration was calculated using a hemocytometer and microscope (at 40 X magnification (Olympus, Japan). For infecting the *S. litura* larvae and adults of *M. domestica*, the final concentration was re-adjusted as shown in Table 3.4. Before infection, the viability of conidia was assessed on SDA slides. Germination was checked after incubation of slides for 16-20 h at 25±2 °C under a light microscope (Nikon, Japan).

For bioassays with *S. litura*, newly moulted second instar larvae (N=10) were dipped in the conidial suspension (Table 3.4) of transformed and wild type strains of *Metarhizium* and *Beauveria* for 30 sec each to become contaminated. Control insects were treated with 0.05 % tween 20 solution. Treated larvae were then placed in clean ventilated dishes with sterile filter paper and maintained at 25.0±2 °C, 70-90

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% humidity with a photoperiod of 14 h light and 10 h darkness. The insects were fed with artificial diet and mortality at every 24 h was recorded.

Table 3.4 Concentrations of conidia used for insect bioassays.

Strain	Strain designation	Conidia concentration used for bioassay (per mL)
M. anisopliae 892	Ma 892 WT	2×10^8
	Ma 892-Dc1a	2×10^8
M. anisopliae 3210	Ma 3210 WT	1×10^8
	Ma 3210-Dc1a	1×10^8
B. bassiana 984	Bb 984 WT	5×10^8
	Bb 984-Dc1a	5×10^8

Ma: *Metarhizium anisopliae*, Bb: *Beauveria bassiana*, WT: Wild type

In the case of bioassays against *M. domestica*, conidial suspension of transformed and wild type strains of *Metarhizium* and *Beauveria* were mixed with sesame oil to obtain 10 % oil formulations. 5 mL of the formulations were pipetted evenly over a 98 cm^2 piece of filter paper resulting in densities of ~1×10^7 conidia per cm^2. The impregnated papers were left to dry at 70 % humidity for 48 h and were then placed on the inside of a test tube (height 15.0 and diameter 3.0 cm). The mouth of the tube was closed with black cloth. For infection with the fungus, cold anaesthetized (4 °C) adults of *M. domestica* (N=10), were released inside this tube for 5 h. Formulation of 0.05 % Tween-20 containing 10 % sesame oil was used as a control. After 6 h, flies were released into 250 mL aerated plastic containers and provided with honey and water (soaked into cotton balls). The design of the experiment and cages used to perform housefly bioassays is illustrated in Fig. 3.4. The containers were maintained at 25±2 °C with 70-90 % humidity and a photoperiod of 14 h light and 10 h darkness.

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Mortality was recorded every 24 h. To confirm mortalities were due to *Metarhizium* and *Beauveria* infection, dead insects were transferred to a Petri dish containing a wet tissue paper to provide humidity for fungal growth (Fig. 3.4 (d) and Fig 3.5).

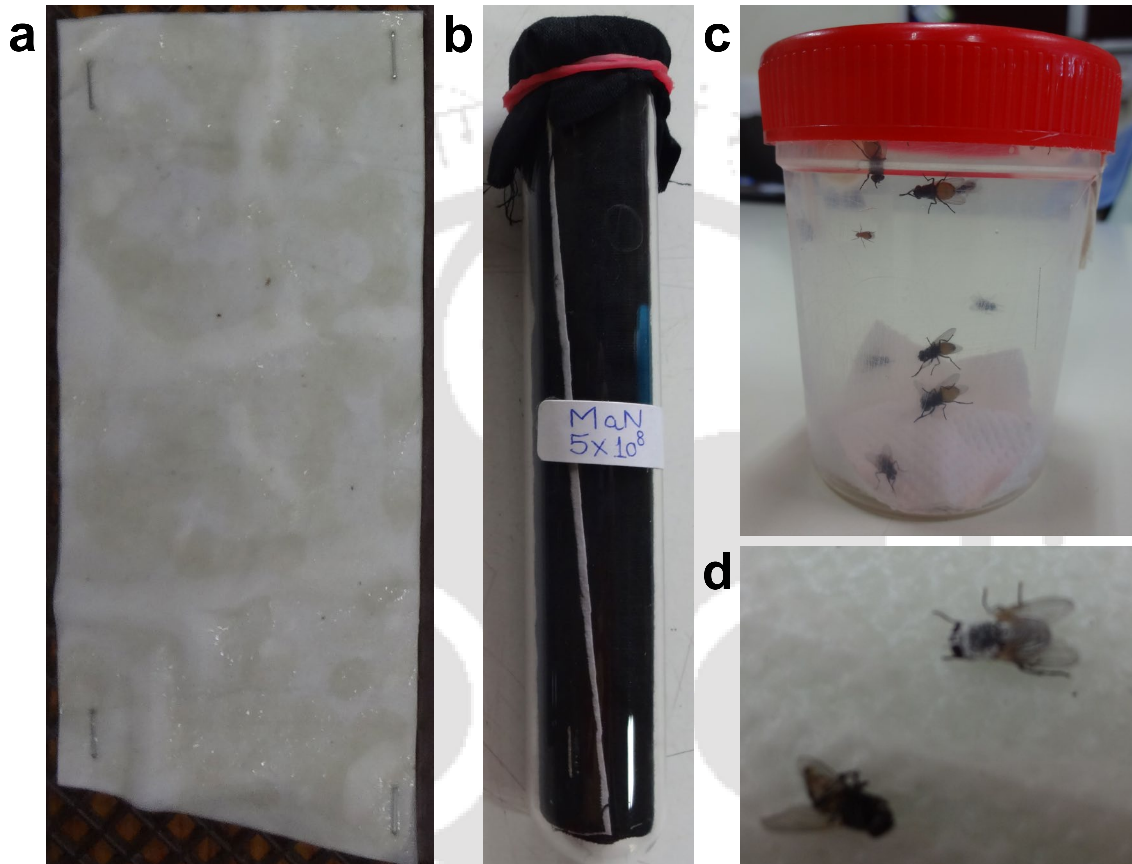

Fig 3.4: Design of the Housefly bioassays (N=10 insects per treatment). (a) shows a sheet of paper used to spread conidial suspension. (b) shows test tube used for infecting houseflies from one of the treatments (c) shows the plastic cages used for the bioassay. (d) shows dead houseflies from one of the treatments with a wet tissue paper for providing humidity for fungal growth

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Fig 3.5: Tobacco cutworm bioassays (N=10 insects per treatment) shows a Petri dish containing dead larvae from one of the treatments with a wet tissue paper beneath castor leaf for providing humidity for fungal growth.

3.2.11 *Data analysis and image processing*

Mortality data analysis and interpretations were carried out using a statistical tool comprising of GraphPad Prism version 6.0. Bioassay experiments were performed in triplicate and each treatment consists of 10 insects. Survival data was analysed using Probit analysis (Finney 1952), and LT_{50} values were calculated.

All the DNA sequencing were carried out using Sangers' chain-terminating dideoxynucleotides method on DNA Analyzer 3730xl (Applied Biosystems) from Eurofins Genomics, India. All the agarose and SDS-PAGE gel images were recorded using Gel Doc XR^{+} Gel Documentation System (Bio-Rad, USA) and processed in Image Lab version 6.0.1 software for Windows (Bio-Rad, USA).

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3.3 Results and discussion

The slow action of insect-pathogenic fungi on target pests is one of the limitations to their commercialisation and large-scale application as biocontrol agents (Wei *et al.* 2017). Some studies have shown that the insect-killing efficacy of *B. bassiana* is considerably improved by recombination with the exogenous *A. aegypti* TMOF gene, scorpion neurotoxin AaIT gene (Wang and St Leger 2007; Deng *et al.* 2019), cuticle degradation protease PR1A gene (Lu *et al.* 2008), or vegetative insecticidal proteins of *B. thuringiensis* (Qin *et al.* 2010).

Designing suitable genetic engineering methods always represents a challenge. Another important factor to be considered is the selection of promoter to direct expression of the transgene of interest. In attempting to solve these problems, the present study describes an optimised methodology for the targeted secretion of insecticidal venom peptide from fungal entomopathogen using development stage-specific promoter. In this study, PMCl1 promoter was used to direct expression of ISVP to the insect hemolymph (Wang and St. Leger 2006). The ISVP Dc1a gene was stably introduced into the genome of *B. bassiana* and *M. anisopliae* to enhance its virulence against dipteran and lepidopteran insects.

3.3.1 Gene synthesis and expression construct

OptimumGene^{TM} algorithm of GenScript, USA, was used to optimise various parameters that play a critical role in optimal gene expression. The parameters considered in this study include codon usage bias, GC content, mRNA secondary structure, RNA instability motif (ARE), etc. The CAI for optimal expression in

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Metarhizium and *Beauveria* was adjusted to 0.87 and 0.81 respectively, where CAI of 1.0 is considered perfect, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level. The GC content was adjusted to 57.36 %, where the ideal percentage range of GC content is between 30-70 %. The nucleotide information of the codon-optimised gene sequence is mentioned in Table A16 (see appendix). The graphical representation of the relative adaptiveness which measures the quality of codon usage the CAI is depicted in Fig. 3.6 (b) whereas the results of the optimal distribution of codon usage bias after OptimumGene^{TM} optimisation with GC content adjustment are shown in Fig. 3.6 (c).

Following codon optimisation and chemical synthesis, expression construct encoding PMCl1 promoter, MCl1 signal peptide, ISVP Dc1a gene, and MCl1 gene partial sequence were successfully cloned between the designated restriction sites into a fungal expression vector pCAMBIA3300 using Gibson cloning (Fig. 3.7, a). The resulting gene cassette was designated as pCAM-bar-PMCl1-SP-Dc1a-MCl1. The clones were confirmed by amplifying individual components of the gene cassette with respective primers and sequencing. The electropherogram showing the DNA results of sequencing results of Dc1a in pCAMBIA 3300 shuttle vector is illustrated in Fig. 3.7, b. The nucleotide sequence of cloned Dc1a gene deduced from sequencing results confirmed that there is no mutation in the cloned sequence of Dc1a gene (see Table A17 in appendix). The confirmed clones with gene cassette were stored as glycerol stock at -80 °C until further use.

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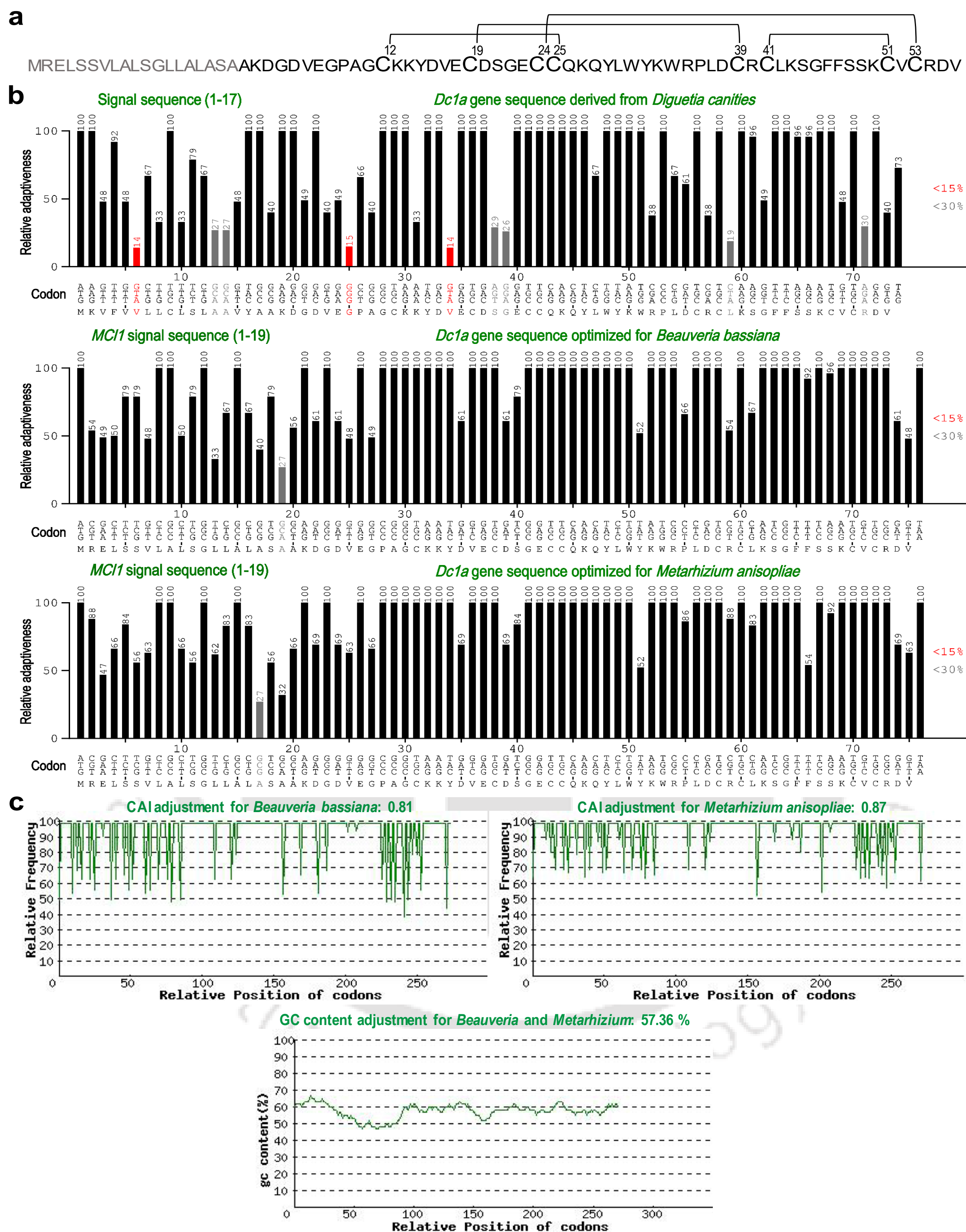

Fig. 3.6: Gene synthesis and OptimumGene™ Optimization. (a) The peptide sequence of Dc1a toxin including signal peptide. (b) The graphical representation of the relative adaptiveness index in *Metarhizium* and *Beauveria*. (c) Optimal distribution of codon usage bias in *Metarhizium* and *Beauveria* after OptimumGene™ optimisation and GC content adjustment.

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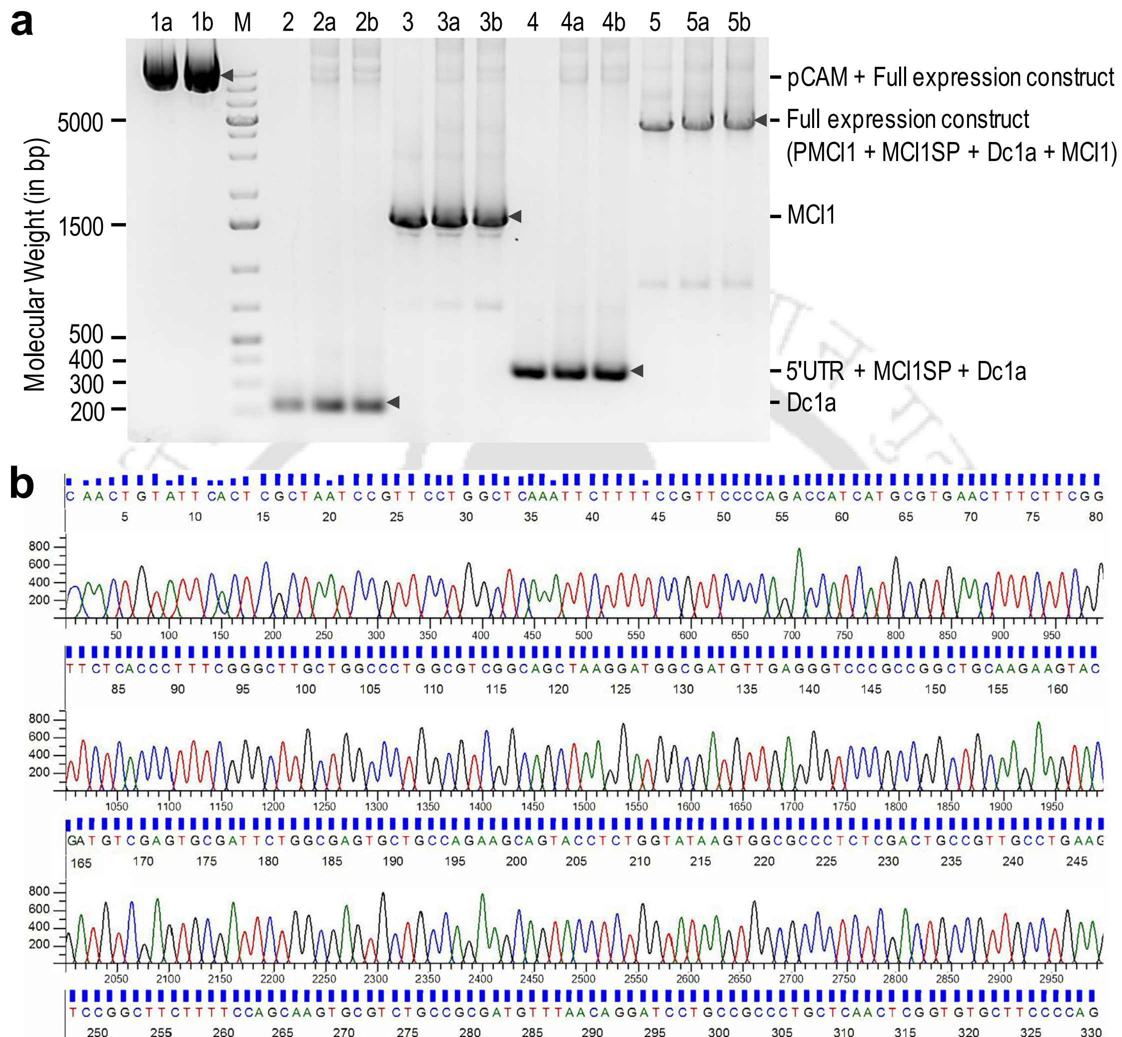

Fig. 3.7: Illustration of clone confirmation and DNA sequencing. (a) Colony PCR by amplifying individual components of the gene cassette with respective primers. lane M: Gene ruler 1 kb plus DNA ladder; lane 1 (a and b): pCAMBIA3300 vector containing cloned gene construct pCAM-bar-PMCl1-SP-Dc1a-MCl1; lane 2 to 5: PCR amplification using different gene cassette primers, and (b) Electropherogram showing the DNA results of sequencing results of Dc1a.

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3.3.2 Genetic transformation and characterization of transgenic fungal entomopathogens

Over the past few years, transformation methods such as protoplast electroporation, *Agrobacterium*-mediated genetic transformation, and biostatic transformation have been assessed for fungal transformation. All techniques need case-by-case optimisation prior to application to fungal strains of interest (Meyer 2008). In this current study, the efficacy of protoplast as well as *Agrobacterium* to transfer the specially designed expression construct pCAM-bar-pMCL1-Dc1a-MCl1 into *M. anisopliae* strains 892, 3210 and *B. bassiana* strain 984 was assessed. Using the promoter of MCL1 for genetically engineering *Metarhizium* and *Beauveria*, the expression of the target genes can be limited to the hemocoel of the target insects, ensuring the targeted expression of the ISVPs in genetically engineered fungal strains (Wang and St. Leger 2006). As a result, it will restrict the release of ISVPs into the environment, thereby engineered fungus would not interfere with non-target insects beyond their host range.

Protoplast were released from the mycelia of *B. bassiana* strain 984 using the lysing enzyme from *Trichoderma harzianum* (Fig. 3.8). It comprised of mixture of isoenzymes (β-glucanase, cellulase, protease, and chitinase) with β-1, 3 glucanase activity and hydrolyzes fungal cell wall oligosaccharides to glucan for protoplast preparation. It was observed that protoplast-based gene transfer followed by electroporation worked efficiently for *B. bassiana* strain 984. On the contrary,

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Agrobacterium-mediated gene transfer strategy has shown higher transformation efficiency in *M. anisopliae* strains 892 and 3210 (data not shown).

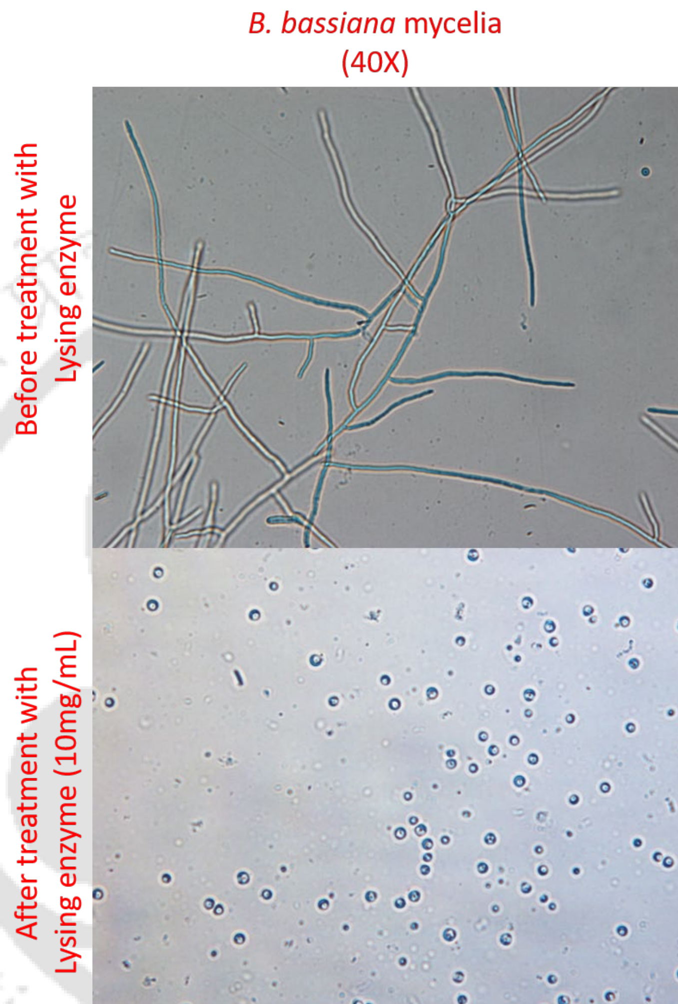

Fig. 3.8: Illustration of *B. bassiana* 984 mycelia treated with lysing enzyme for protoplast release using Phase contrast microscopy at 40X (Nikon TiS, Japan).

In total 4 transformants were selected for *Beauveria* and 7 transformants for *Metarhizium*. Genomic DNA isolation from selected transformants of *Metarhizium* and *Beauveria* was done and 0.8 % agarose gel electrophoresis showed bright bands of gnomic DNA from transformants with significant yield (Fig. 3.9). PCR

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amplification using Dc1a gene-specific primers confirmed the genetic integration of gene cassette into the genome of *Beauveria* and *Metarhizium* strains (Fig. 3.10).

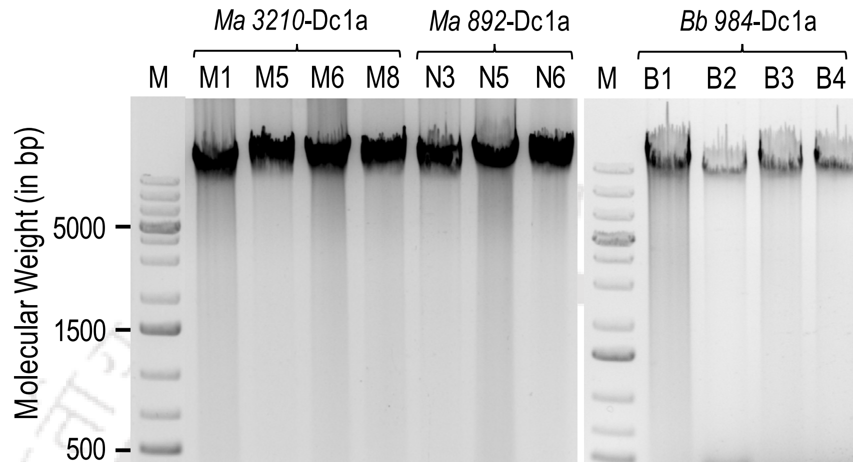

Fig. 3.9: Agarose Gel Electrophoresis illustrating. lane M: Gene ruler 1 kb plus DNA ladder; lane M1,3,6,8: shows genomic DNA isolation from positive transformants of *M. anisopliae* 3210-Dc1a; lane N3,5,6: shows genomic DNA isolation from positive transformants of *M. anisopliae* 892-Dc1a, and lane B1-B4: show genomic DNA isolation from positive transformants of *B. bassiana* 984-Dc1a.

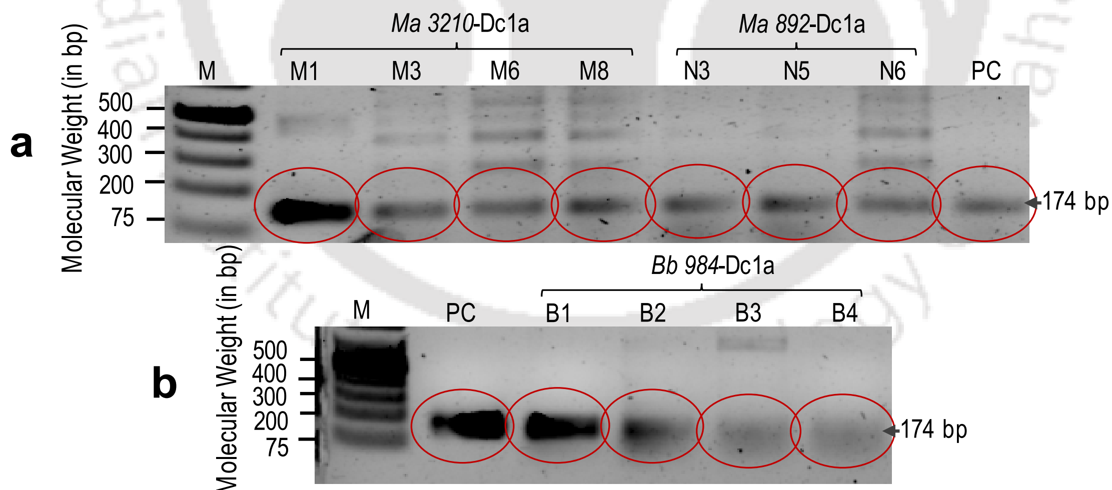

Fig. 3.10: Agarose Gel Electrophoresis illustrating: (a) lane M: Gene ruler 1 kb plus DNA ladder; lane M1,3,6,8: shows Dc1a gene amplification from genomic DNA isolation from positive transformants of *M. anisopliae* 3210-Dc1a, and lane N3,5,6: shows Dc1a gene amplification from genomic DNA isolation from positive transformants of *M. anisopliae* 892-Dc1a, and (b) lane M: Gene ruler 1 kb plus DNA ladder; lane B1-B4: shows Dc1a gene amplification from genomic DNA isolated from positive transformants of *B. bassiana* 984-Dc1a.

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3.3.3 Expression analysis using RT-PCR and Immunoblotting

Genetically stable transformants were chosen, and Dc1a expression was confirmed at the RNA and protein level using RT-PCR and western blotting. To confirm this, ISVP Dc1a gene in transgenic *Metarhizium* and *Beauveria* strains were induced by insect hemolymph. In the case of *M. anisopliae* strains 892 and 3210, expression studies were performed at 12 h. However, for *B. bassiana* strain 984, RT-PCR was performed from isolated RNA samples collected at 0 min, 20 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h after incubation with *A. assamensis* hemolymph. All transgenic *M. anisopliae* strains (Ma 892-Dc1a and Ma 3210-Dc1a) (Fig. 3. 11), and *B. bassiana* strain (Bb 984-Dc1a) showed higher expression of transgene Dc1a started within 20 min of induction and moderated until 12 h (Fig. 3.12).

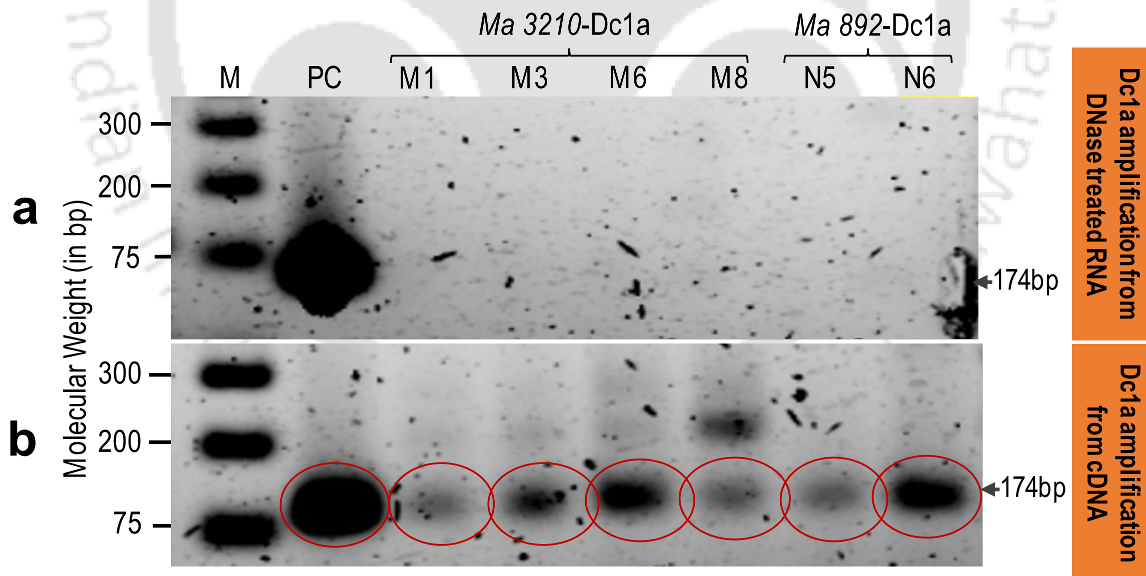

Fig. 3.11: Agarose Gel Electrophoresis illustrating Dc1a gene amplification from positive transformants of *M. anisopliae* 3210-Dc1a (lane M1,3,6,8) and *M. anisopliae* 892-Dc1a (lane N5,6) (a) amplification from DNase treated RNA of positive transformants, and (b) amplification from cDNA of positive transformants.

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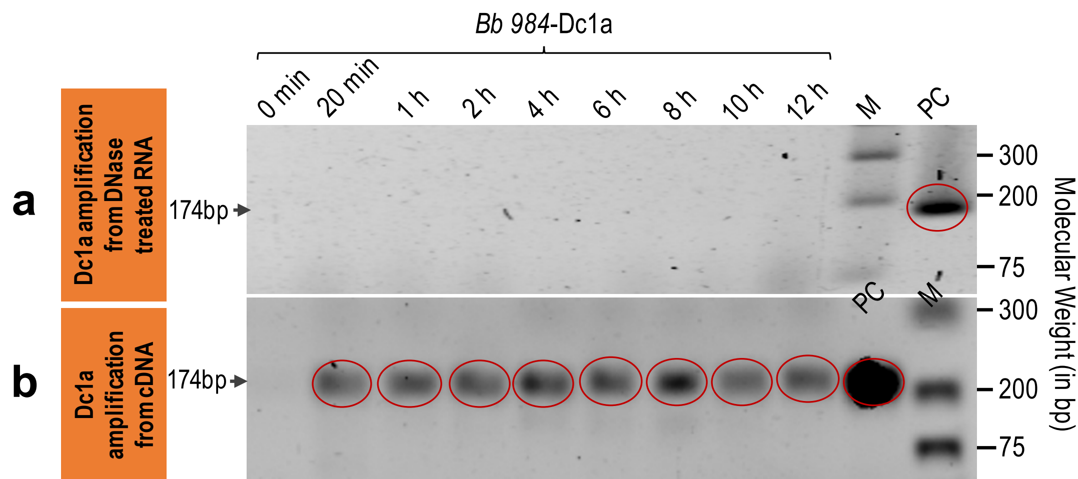

Fig. 3.12: Agarose Gel Electrophoresis illustrating Dc1a gene amplification from positive transformant of *B. bassiana* 984-Dc1a. (a) shows amplification from DNase treated RNA of positive transformant. (b) shows amplification from cDNA of positive transformants. Samples were induced for different time periods (20 min up to 12 h).

Similar results with immunoblotting studies were observed where Dc1a specific antibody confirmed the expression of transgene Dc1a after 20 min and moderated up to 12 h after incubation with *A. assamensis* hemolymph (Fig. 3.13, a and b).

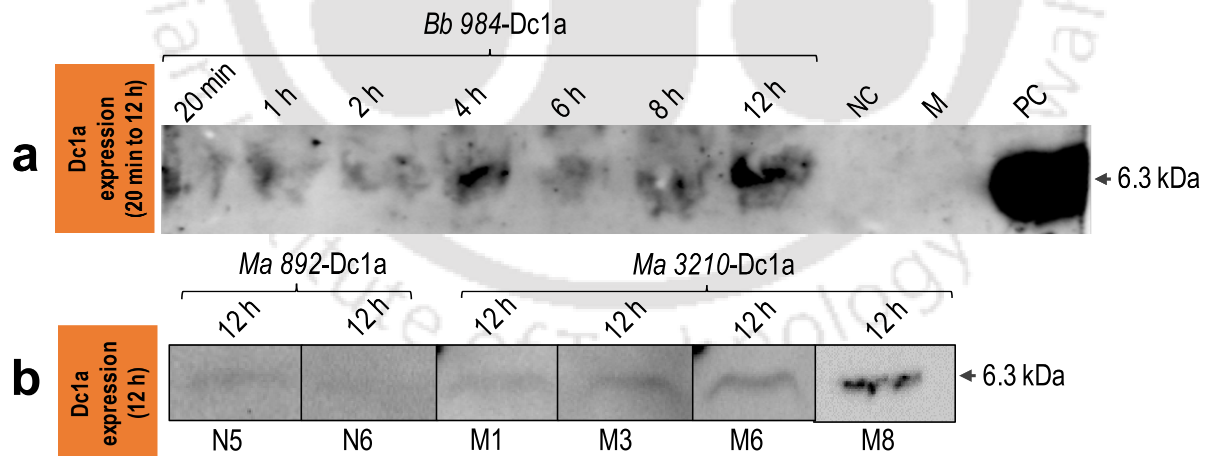

Fig. 3.13: Western blot analysis. (a) shows expression of spider toxin Dc1a in positive transformant of *B. bassiana* 984-Dc1a (20 min to 12 h). (b) shows the expression of spider toxin Dc1a in positive transformants of *M. anisopliae* 892-Dc1a (N5, N6), *M. anisopliae* 3210-Dc1a (M1, M3, M6, M8). All expression analysis studies in *M. anisopliae* were carried at 12 h after induction. Polyclonal antibody to Dc1a was used for detection. All protein samples were run on 16 % SDS-PAGE (Tris-Tricine) and blotted to nitrocellulose membrane (0.45 µm).

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3.3.4 Transgenic strains of Metarhizium and Beauveria were more virulent to lepidopteran and dipteran insects

Bio-insecticidal potential of transgenic strains of *Metarhizium* and *Beauveria* were determined against larvae of *S. litura* and adults of *Musca domestica.* The virulence of positive transformants of *M. anisopliae* WT stains 892, 3210 and *B. bassiana* strain 984 were compared with transgenic strains and the median lethal time LT_{50} was calculated. Majority of the *S. litura* larvae and housefly adults started dying from day 2 of the treatment. ~98 % of the dead larvae showed conidiation of *Metarhizium*; however, for *Beauveria* ~80 % showed conidiation under humid conditions confirming that the reason for the death of insects was a fungal infection. After 6–8 days of treatment, fungal growth was visible on treated insect pests, whereas there was no fungal growth on control insects (Fig 3.14).

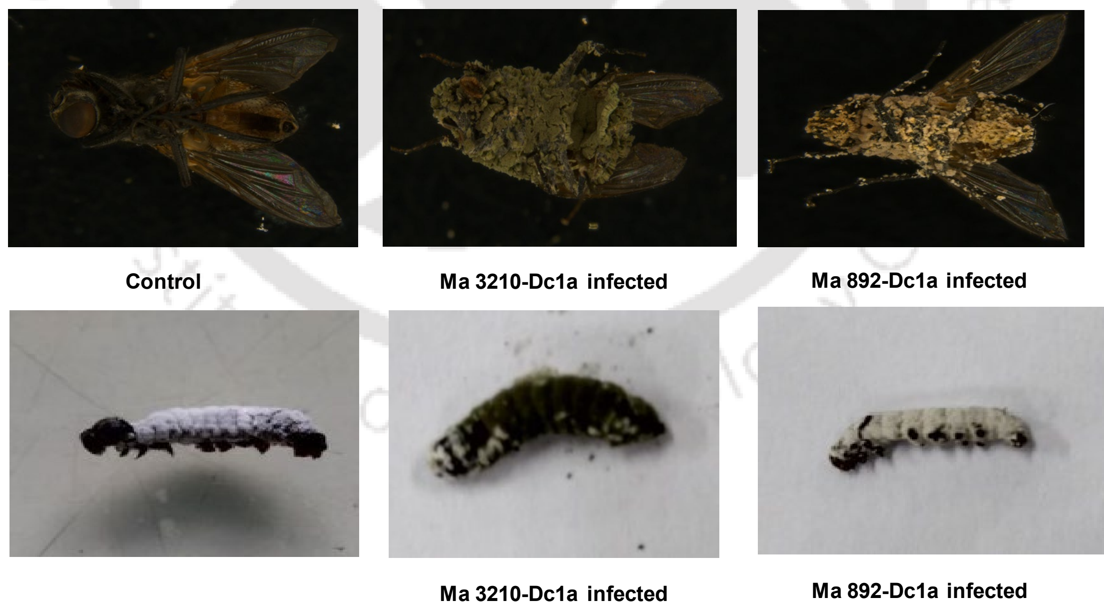

Fig. 3.14: Effect of topical application of transgenic *Metarhizium and Beauveria* conidial suspension. (a) *M. domestica,* and (b) *S. litura.* For each treatment, N=10 insects were used and all the experiments were performed in triplicate.

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All of the strains expressing ISVP Dc1a killed insects much faster than the isogenic wild-type strain. *M. anisopliae* 892-Dc1a, *M. anisopliae* 3210-Dc1a and *B. bassiana* 984-Dc1a reduced the LT_{50} by 28 % (Fig. 3.15, a), 32 % (Fig. 3.15, b) and 24 % (Fig. 3.15, c) in *S. litura,* respectively. Similarly, *M. anisopliae* 892-Dc1a, and *M. anisopliae* 3210-Dc1a reduced the LT_{50} by 21 % (Fig. 3.16, a) and 30 % (Fig. 3.16, b) in *M. domestica*, respectively. The LT_{50} values for each of the transgenic strains of *Metarhizium* and *Beauveria* strains are listed in Table 3.5.

Table 3.5 Summary of the insect bioassays performed with wild type and transgenic *Metarhizium* and *Beauveria* strains against lepidopteran and dipteran insect pests.

S. No.	Strain	LT_{50} (in days)	Decrease in LT_{50} (%)
Tobacco cutworm (S. litura)			
1.	Ma 892 WT	5.097±0.138	27.74-fold
2.	Ma 892-Dc1a	3.683±0.126	
3.	Ma 3210	4.523±0.159	31.51-fold
4.	Ma 3210-Dc1a	3.098±0.157	
5.	Bb 984	6.074±0.165	23.89-fold
6.	Bb 984-Dc1a	4.623±0.206	
Housefly (M. domestica)			
7.	Ma 892 WT	4.439±0.155	21.42-fold
8.	Ma 892-Dc1a	3.488±0.142	
9.	Ma 3210 WT	4.025±0.144	29.52-fold
10.	Ma 3210-Dc1a	2.837±0.147	

Ma: *Metarhizium anisopliae*, Bb: *Beauveria bassiana*, WT: Wild type, LT_{50}: Time taken to kill 50 % of the test population

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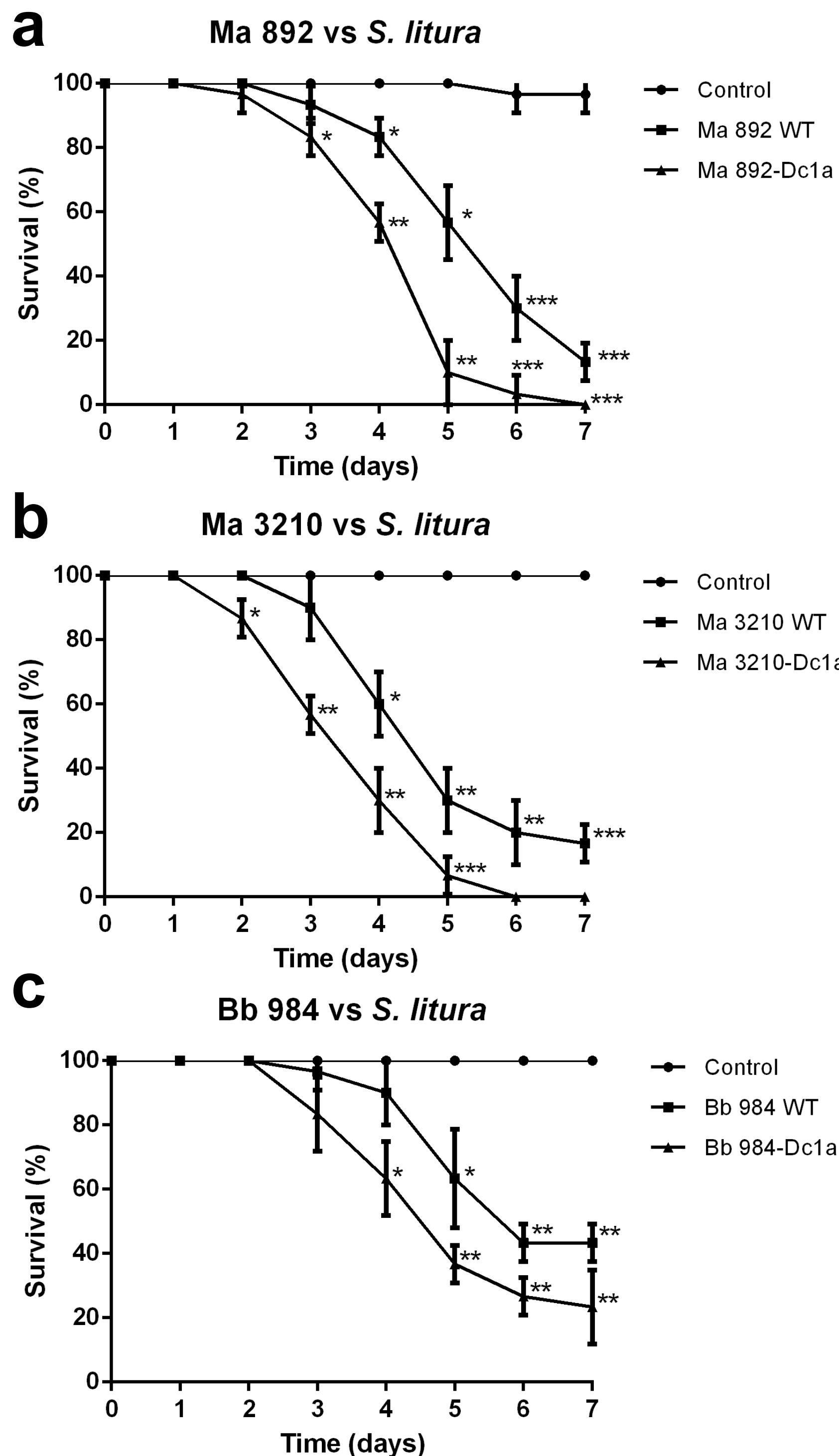

Fig. 3.15: Mortality data were fitted using Probit analysis and LT_{50} values were calculated. Survival analysis showing effect against second instar larvae of *S. litura* with (a) *M. anisopliae* 892, (b) *M. anisolpiae* 3210 and (c) *B. bassiana* 984. For each treatment, N=10 insects were used and all the experiments were performed in triplicate. T-test using Microsoft Excel, *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001.

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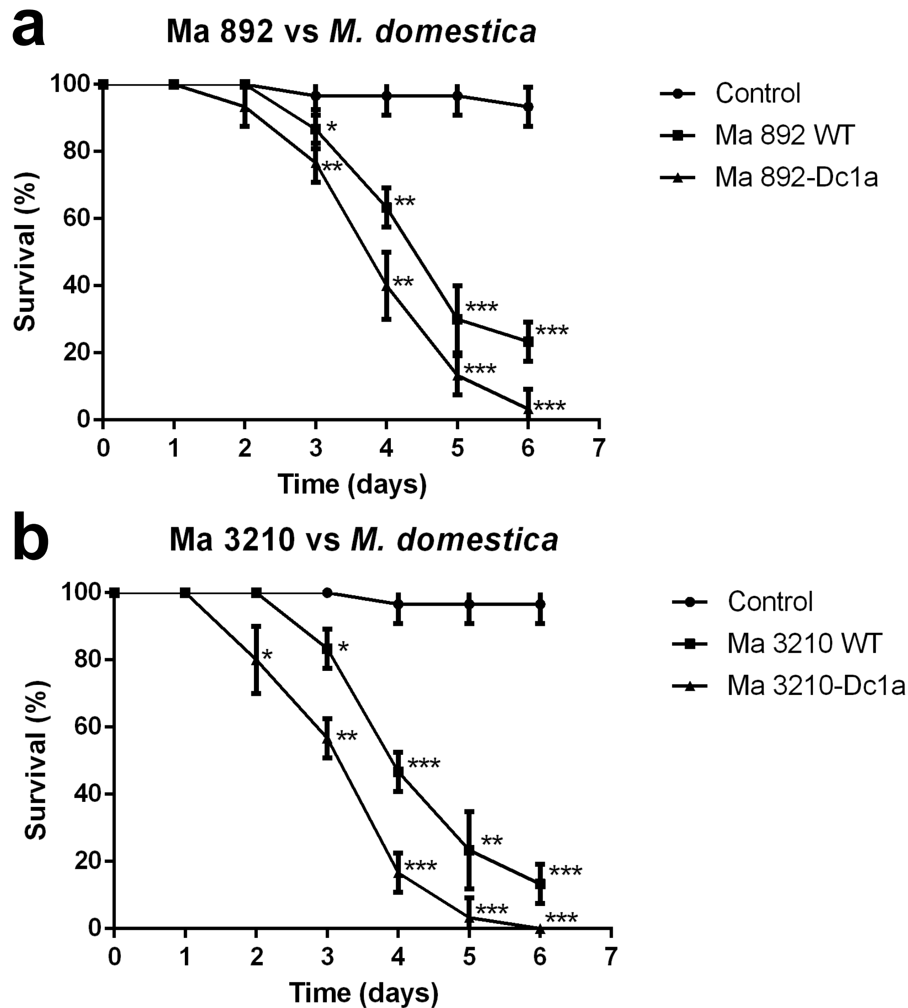

Fig. 3.16: Mortality data were fitted using Probit analysis and LT_{50} values were calculated. Survival analysis showing effect against adults of *M. domestica* with (a) *M. anisopliae* 892, and (b) *M. anisopliae* 3210. For each treatment, N=10 insects were used and all the experiments were performed in triplicate. T-test using Microsoft Excel, *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001.

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3.4 Conclusion

More than 1000 known species of entomopathogens are major mortality factors for insects. Some of these entomopathogens offer environmental advantages in addition to the ability to tackle insect populations that have become resistant to chemical insecticides. However, fungal pathogens have been underused because of their longer kill times compared to chemical insecticides and inconsistency in results. In this study, we significantly enhanced the virulence of *Metarhizium* and *Beauveria* strains by engineering them to express ISVP Dc1a. Although these transgenic strains need to pass stringent ecological laws and field trials, current data suggest that they will be safe under field conditions. Furthermore, expression of the ISVP was limited to the hemocoel of infected insects due to the use of the insect-hemolymph specific promoter, PMCl1. This will restrict the release of ISVP Dc1a into the environment and affect non-targeted insects. The recombinant ISVP-expressing entomopathogenic strains produced in this study are more virulent than the wild-type strains with reduced kill time.

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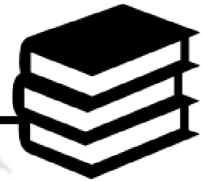
Chapter Four



Significant findings and future prospects

Indian Institute of Technology Guwahati

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4.1 Summary of findings

The long-term goal of the research described in this thesis was to develop environmentally friendly mycoinsecticides for the control of insect pests and vectors of human diseases. The first part of the research was focused on production, characterisation, and evaluating the insecticidal activity of recombinant venom peptide Dc1a against lepidopteran and dipteran insects. The second part of the thesis was focused on genetic engineering and testing of fungal entomopathogens that express insecticidal venom-peptide Dc1a.

4.1.1 Recombinant production and characterisation of rDc1a

The first objective of this thesis was the recombinant production of ISVP Dc1a. *E. coli* has been extensively used from a long time for overproduction of recombinant proteins. However, the expression of disulfide-rich proteins in the cytoplasm presents a significant challenge due to reducing environment. As described in Chapter 2, an efficient *E. coli* recombinant expression system was developed to overcome these difficulties. Recombinant Dc1a was successfully produced under oxidised conditions in the periplasm of *E. coli.* rDc1a was purified with >95 % purity using a combination of MBP amylose affinity and size exclusion chromatography. Size exclusion chromatography coupled with FPLC Akta Purifier 100 provided an alternate way to purify short peptides compared to rHPLC. Characterisation studies using MALDI-TOF mass spectrometry, Circular dichroism (CD) and Western Blotting further confirm the production and structural integrity of rDc1a. The production and characterisation methods established in the current study

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will serve as a basis for further improvements in the recombinant production of Dc1a.

4.1.2 Insecticidal activity and cytotoxicity assays of recombinant Dc1a

The second objective of this thesis was to investigate the insecticidal activity and cytotoxicity assays of ISVP rDc1a against dipteran and lepidopteran insect pests. Injection bioassays with two of the major lepidopteran insect pests larvae: tobacco cutworm *(S. litura*), cotton bollworm and (*H. armigera*) revealed that rDc1a has a similar lethality to agricultural pests when compared with a native toxin (Krapcho et al. 1995). Droplet-feeding assays showed a significant level of oral toxicity against adults of dipteran insect vector of human diseases: housefly (*M. domestica*). In contrast, rDc1a showed ~1000-fold less cytotoxicity effects towards cultured insect cells *in situ* compared to lepidopteran pests *in vivo*. Only a few rDc1a toxin peptide molecules were able to efficiently bind with the voltage-gated sodium channel on insect membrane, which is evident from the immunofluorescence results.

4.1.3 Generation and characterisation of transgenic fungal entomopathogens

The key objective of this thesis was to express ISVP Dc1a in selected *Beauveria* and *Metarhizium* strains using genetic engineering techniques. As described in Chapter 3, protoplast and *Agrobacterium*-mediated gene transfer strategies were used to transfer the specially designed expression construct pCAM-bar-pMCL1-Dc1a. Using the promoter of MCL1 for genetically engineering *Metarhizium* and *Beauveria*, the expression of the target genes was limited to the

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hemocoel of the target insects, ensuring the targeted expression of the ISVPs in genetically engineered fungal strains. It was observed that protoplast-based gene transfer followed by electroporation worked efficiently for *B. bassiana* strain 984. On the contrary, *Agrobacterium*-mediated gene transfer strategy has shown higher transformation efficiency in *M. anisopliae* strains 892 and 3210. Dc1a was successfully transformed into *Beauveria* and *Metarhizium* strains and fungal genome integration of transgene was confirmed using PCR. Genetically stable transformants were chosen and Dc1a expression was confirmed by western blotting with specific antibodies. A universal DNA extraction buffer and the isolation protocol were optimised having high yield efficiency from diverse types of sample. Liquid nitrogen-based lysis method for DNA and RNA isolation has been used successfully with good yields. Hemolymph induced expression of transgene Dc1a started within 20 min of induction and moderated until 12 h.

4.1.4 Insecticidal activity of genetically engineered fungal strains

The fourth and last objective of this thesis was to evaluate the improved insecticidal potency of *Beauveria* and *Metarhizium* strains expressing ISVP Dc1a. Bioassays with tobacco cutworm (*S. litura*) larvae and housefly (*M. domestica*) adults revealed that the reduced time was required for achieving mortality. Laboratory trials demonstrated that transgenic *Beauveria* and *Metarhizium* expressing ISVP are significantly more virulent than the wild-type fungus, with drastically reduced LT_{50} values against dipteran and lepidopteran insect pests. The LT_{50} values for GM *Beauveria* and *Metarhizium* expressing ISVP Dc1a will be 20–30 fold lower than wild-type strains.

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4.2 Future prospects

The production and characterisation methods established in the current study will serve as a basis for further improvements in the recombinant production of Dc1a. As the arsenal of chemical insecticides is diminishing rapidly due to the development of insect resistance, significant high-level toxicity of rDc1a toxin peptide towards lepidopteran crop pests: *H. armigera* and *S. litura in vivo* render it as a potential candidate for incorporation in recombinant baculovirus based insecticides and to construct transgenic plants. On the contrary, it was found that the *in situ* cytotoxicity assays against lepidopteran Sf21 cell lines were significantly less compared to the *in vivo* studies. There might be many possible reasons for the same as lack of suitable cell receptors on the cultured insect cell membrane or selectivity towards a particular type of cells or development of resistance. Therefore, the scientific justification and molecular mechanism behind this can be explored in future.

Besides this, information available for production and implementation of fungal biopesticides will be useful for the successful deployment of GM entomopathogenic fungi. It would be informative to conduct studies with a combination of GM entomopathogenic fungi and conventional insecticides against resistant strains of crop pests. Furthermore, multi-insecticidal transgene approach having different molecular target can be exploited in future to enhance the virulence of entomopathogenic fungi further. This approach will have the advantage of reduced chances of development of target-site resistance. In the end, evaluation of

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the efficacy of GM strains in the field under realistic environmental conditions will be the decisive step towards the successful deployment of these bioinsecticides for insect pest control.

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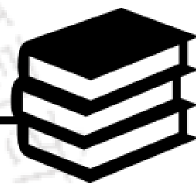
Appendices



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Chemicals, reagents and kits

Chemicals and reagents used in the present study were of molecular biology grade or analytical grade or cell culture grade supplied by following mentioned manufactures:

New England Biolabs (USA): Phusion® High-Fidelity DNA Polymerase, Restriction enzymes (*NdeI* and *BamHI*), T4 DNA ligase, Gibson Assembly® Cloning kit components and pMAL™ Protein Fusion and Purification System kit were procured from New England Biolabs, USA.

pMAL™ Protein Fusion and Purification System kit contains a pMAL-p5X and pMAL-c5X vectors for periplasmic and cytoplasmic expression of fusion proteins respectively, Amylose resin affinity matrix used for the purification of proteins fused to maltose-binding protein (MBP), Factor Xa Protease for removal of MBP tag, Anti-MBP Monoclonal Antibody as a murine anti-maltose binding protein antibody which is covalently linked to horseradish peroxidase and a glycerol suspension of *E. coli* ER2523 (NEB Express) cells.

Gibson Assembly® Cloning kit contains T5 exonuclease which creates single-stranded 3' overhangs and facilitates the annealing of fragments that share complementarity at one end (overlap region), Phusion DNA polymerase fills in gaps within each annealed fragment by adding the complementary nucleotides, and Taq DNA ligase seals nicks in the assembled DNA. The reaction is facilitated in the 5X isothermal reaction buffer (25 % PEG-8000, 500 mM Tris-HCl pH 7.5, 50 mM MgCl2, 50 mM DTT, 1 mM each of the 4 dNTPs, and 5 mM NAD).

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Thermo Fisher Scientific: O'GeneRuler™ 100 bp, 1 kb Plus DNA ladder, and PageRuler™ Unstained Low Range Protein Ladder were obtained from Thermo Fisher Scientific, USA. Cell culture essentials like Gibco™ Fetal Bovine Serum, E.U.-approved, South America origin, Antibiotic-Antimycotic (100X), Nunc™ MicroWell™ 96-Well and Nunc™ EasYFlask™ Cell Culture were also supplied by Thermo Fisher Scientific, USA.

Bio-Rad (USA): Precision Plus Protein™ Dual Xtra Pre-stained protein standards and Nitrocellulose membrane (0.45 µm) Bio-Rad, USA were used in Western blot analysis.

HiMedia Laboratories (India): All the media components, molecular biology chemicals and reagents otherwise unless stated were supplied by HiMedia Laboratories, India.

GE Healthcare Life Sciences (USA): Pre-packed HiLoad 16/600 Superdex 200 pg size exclusion chromatography (SEC) column having **m**olecular weight **r**ange (Mr) from Mr 10,000–600,000 kDa was kindly provided from Dr. B. Anand, Associate Professor, Department of Biosciences & Bioengineering, Indian Institute of Technology, Guwahati.

Sigma-Aldrich (USA): TNM-FH and RPMI-1640 media used for maintenance of cultured cells were procured from Sigma-Aldrich (USA). Sigma-Aldrich, USA supplied MALDI matrix α-cyano-4 hydroxycinnamic acid (CHCA), and 4-Hydroxy-3,5-dimethoxy-cinnamic acid (Sinapic acid) used for mass spectrometry analysis. Bradford reagent used for protein estimation and Glufosinate-ammonium

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PESTANAL® selection marker used for fungal screening were also supplied by Sigma-Aldrich, USA.

Merck (Germany): Amicon® Ultra centrifugal filters, Membrane 3 kDa **N**ominal **M**olecular **W**eight **L**imit (NMWL) for protein concentration and absolute ethanol were purchased from Merck-Millipore, Germany. Analytical and HPLC grade reagents were procured from Merck, India.

GenScript (USA): Anti-Dc1a polyclonal antibody was raised in New Zealand rabbit against a chemically synthesised antigen from GenScript, USA.

CST Signaling Technology (USA): Mouse anti-goat IgG-HRP and anti-rabbit IgG-HRP, secondary monoclonal antibodies, conjugated to horseradish peroxidase (HRP) for chemiluminescent detection were kindly provided by Dr. Anil Mukund Limaye, Associate Professor, Department of Biosciences & Bioengineering, Indian Institute of Technology, Guwahati.

Integrated DNA Technologies (IDT) (USA): Oligonucleotide primers used to amplify custom synthesised Dc1a gene, PMCl1 promoter, MCl1 gene signal peptide, MCl1 gene and sequencing were from Integrated DNA Technologies (IDT), USA. As per the quality control information of the manufacturer, the primers were re-suspended in nuclease-free water to a final concentration of 100 mM.

Macherey-Nagel (Germany): NucleoSpin® Plasmid, and Gel and PCR Clean-up kits were procured from Macherey-Nagel, Germany.

Takara Bio (Japan): PrimeScript™ 1st strand cDNA Synthesis Kit, RNAiso Plus and TaKaRa Ex Taq were procured from Takara Bio, Japan.

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Tarsons (India): Micropipette tips, microcentrifuge tubes, Petri dishes and other plastic wares were procured from Tarson Products Pvt. Ltd., India.

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Table A1: List of Plasmid vectors

Name	Use	Promoter	Selection marker (s)	Cloning Site
pMALp5X (NEB, USA)	Bacterial expression Vector	P_{tac}	Amp^R	*E. coli* K12 codon-optimized Dc1a gene was cloned between *BamHI and XhoI* sites
pAL1 (FGSC, USA)	Fungal expression Vector	Pccg-1	Amp^R Blp^R	Fungal codon-optimized Dc1a gene was cloned between *NcoI and EcoRI* sites
pCAMBIA 3300	Fungal expression vector	CaMV35S	Kan^R Blp^R	Fungal codon-optimized Dc1a gene was cloned between *EcoRI and HindIII* sites

Table A2: List of Bacterial strains and genotype information

Strain	Genotype
E. coli DH5α (Novagen)	*F⁻ en6fdA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17($r_K^-m_K^+$), λ⁻*
E. coli NEB Express (New England Biolabs)	*fhuA2 [lon] ompT gal sulA11 R(mcr73::miniTn10--Tet^S)2 [dcm] R(zgb-210::Tn10--Tet^S) endA1 D(mcrC-mrr)114::IS10*
A. tumefaciens strain EHA105	*C58 (rif R) Ti pEHA105 (pTiBo542DT-DNA) Succinamopine*

Table A3: List of Fungal strains

Strain No.	Type	Source/Isolated from	Medium
MTCC No. 892	*M. anisopliae* ATCC® 26852™	*Pyrausta nubilalis*, Czechoslovakia	Potato Dextrose Agar
MTCC No. 3210	*M. anisopliae* E/98/1	Cricket, Jorhat, Assam, India	Potato Dextrose Agar
MTCC No. 984	*B. bassiana* ATCC® 7159™	Laboratory contaminant	Sabouraud dextrose agar

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Table A4: List of Cultured Insect and Human cells

Cells	Source	Doubling time	Appearance	Medium
Sf 21	*Spodoptera frugiperda* ovary tissue cells	24-30 h	Monolayer or Suspension	Complete TNM-FH
U-937	*Homo sapiens* myeloid leukaemia cells	48-72 h	Suspension	Complete RPMI-1640

Table A5: List of Lepidopteran and dipteran insects

NBAIR No.	Insects	Order
NBAII-MP-NOC-01	*Helicoverpa armigera*	Lepidopteran
NBAII-MP-NOC-02	*Spodoptera litura*	Lepidopteran
---	*Spodoptera frugiperda*	Lepidopteran
---	*Musca domestica*	Dipteran
---	*Aedes aegypti*	Dipteran

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Table A6: Growth media used for the maintenance of microbial cultures

Growth medium	Ingredients	Weight (g/L)
Luria Bertani Agar (LBA)	Tryptone	10.0
	Yeast Extract	5.0
	NaCl	10.0
	Agar	15.0
Yeast Peptone Dextrose Broth (YPDA)	Yeast Extract	10.0
	Peptone	20.0
	Dextrose	20.0
	Agar	15.0
Sabouraud dextrose agar (SDA) pH 5.6±0.2	Mycological Peptone	5.0
	Dextrose	20.0
	Yeast Extract	5.0
	Agar	15.0
Potato Dextrose Agar (PDA) pH 5.6±0.2	Potato (scrubbed and diced)	200.0
	Dextrose	20.0
	Agar	15.0

Sterilised the media by autoclave for 15 min at 121 °C and allowed to cool before the addition of appropriate antibiotics (if desired)

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Table A7: Different media used for expression of rDc1a

Growth medium	Ingredients	Weight (g/L)
Luria Bertani Broth (LB)	Tryptone	10.0
	Yeast Extract	5.0
	NaCl	10.0
	Glycerol	8.0 ml
Terrific Broth (TB)	Tryptone	11.8
	Yeast Extract	23.6
	Potassium Hydrogen Phosphate, dibasic	9.4
	Potassium Phosphate, monobasic	2.2
	Glycerol	8.0 ml
2XTY Broth (2XTY)	Tryptone	16.0
	Yeast Extract	10.0
	NaCl	5.0
	Glycerol	8.0 ml
Auto-Induction Broth (AI)	Tryptone	10.0
	Yeast Extract	5.0
	Ammonium sulphate	3.3
	Potassium Phosphate, monobasic	6.8
	Sodium Phosphate, dibasic	7.1
	Glucose	0.5
	Lactose	2.0
	Magnesium sulfate	0.15
	100X Trace elements	0.0044
	Glycerol	8.0 ml

Sterilised the media by autoclave for 15 min at 121 °C and allowed to cool before the addition of desired antibiotics (if desired)

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Table A8: Induction media used for *Agrobacterium*-mediated transformation

Growth medium	Ingredients	Weight (mM)
Induction medium (IM)	Potassium phosphate dibasic	10.0
	Potassium phosphate monobasic	10.0
	Sodium chloride	2.5
	Magnesium sulphate	2.0
	Calcium chloride	0.7
	Ferrous sulphate	9.0 µM
	Ammonium sulphate	4.0
	Glucose	10.0
	2-[N-**m**orpholino] **e**thane**s**ulfonic acid (MES), pH 5.3	40.0
	Glycerol	0.5 %
	Acetosyringone	200.0 µM

Sterilised the media by autoclave for 15 min at 121 °C and allowed to cool before the addition of acetosyringone and MES

Table A9: Selection media used during fungal transformation

Growth medium	Ingredients	Weight (g/L)
Czapek Dox Agar (CDA) pH 7.3±0.2	Sucrose	30.00
	Sodium nitrate	2.00
	Dipotassium phosphate	1.00
	Magnesium sulphate	0.50
	Potassium chloride	0.50
	Ferrous sulphate	0.01 µg/L
	Acetosyringone	200.00 µM
	Agar	15.00

Sterilised the media by autoclave for 15 min at 121 °C and allowed to cool before the addition of acetosyringone

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Table A10: Growth media used for the maintenance of cell lines

Growth medium	Preparation	pH
TNM-FH insect medium (Catalog # T1032)	Suspended 52.4 g powdered media in 900 mL tissue culture grade water and 0.35 g sodium bicarbonate powder was added. Media was supplemented with 10 % (v/v) heat-inactivated fetal bovine serum and 1X antibiotic-antimycotic solution. Make up the final volume to 1000 mL	6.2 (using 1N KOH)
RPMI-1640 (Catalog # R8755)	Suspended 10.4 g powdered media in 900 mL tissue culture grade water and 2.0 g sodium bicarbonate powder was added. Media was supplemented with 10 % (v/v) heat-inactivated fetal bovine serum and 1X antibiotic-antimycotic solution. Make up the final volume to 1000 mL	7.2

Sterilised the media using a membrane filter with a porosity of 0.22 µm and stored the liquid media at 2-8 °C in the dark till use

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Table A11: Composition of artificial diet used for rearing housefly larvae

Component	Ingredients	Quantity
A	Groundnut oil cake	2 g
	Wheat bran	5 g
	Milk powder	2 g
	Honey	1 g
B	Water	10 mL

Constituents A and B were blended for 2 min and poured onto Petri plates

Table A12: Composition of artificial diet used for rearing lepidopteran larvae

Component	Ingredients	Quantity
A	Chickpea floor	105 g
	Methyl para hydroxybenzoate	2 g
	Sorbic acid	1 g
	Yeast tablets	10 g
B	Agar	12.75 g
C	Ascorbic acid	3.25 g
	Multivitaplex	2 capsules
	Vitamin E	2 capsules
	Streptomycin sulphate	0.25 g
D	Formaldehyde solution 0.2 %	5 mL
E	Water	780 mL

390 mL of water was mixed with A and blended for 2 min. Agar was boiled in the remaining 390 mL of water and added to A. Blender was again run for 1 min. C is added to the mixture of A and B and blender run for 1 min. Finally, D is added

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Table A13: List of Antibiotic / selection markers

Antibiotic	Stock conc.	Solvent	Working conc.
Ampicillin	100 mg/mL	ddH_2O	100 µg/mL
Kanamycin	50 mg/mL	ddH_2O	50 µg/mL
Amphotericin B	250 µg/mL	DMSO	0.25 to 2.50 µg/mL
Rifampicin	50 mg/mL	MeOH + drops of 10 N NaOH	50 µg/mL
Cefotaxime	50 mg/mL	ddH_2O	250 µg/mL
Glufosinate-ammonium	100 mg/mL	ddH_2O	250 µg/mL

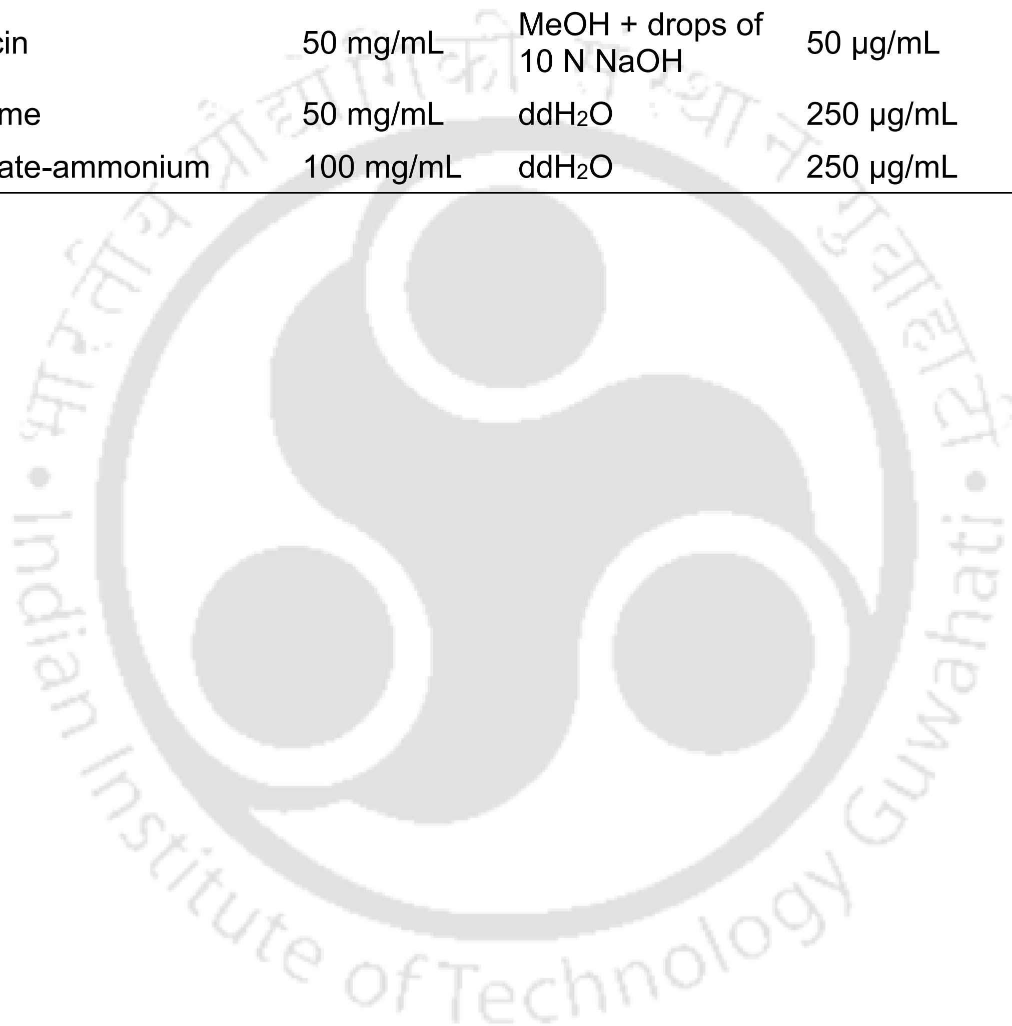
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Table A14: Buffers and solutions

Buffers / Solutions	Composition
Buffers for Agarose Gel electrophoresis	
Tris-**A**cetate-**E**DTA (TAE) buffer, 50x (1 L)	242.0 g Tris base, 57.1 mL CH_3COOH, 100 mL of 0.5 M EDTA (pH 8.0)
Tris-**B**orate-**E**DTA (TBE) buffer, 5X (1 L) pH ~ 8.3	48.4 g Tris base, 11.42 g boric acid, 20 mL of 0.5 M EDTA (pH 8.0)
Buffers/solutions for SDS-PAGE electrophoresis	
30 % acrylamide/bis-acrylamide solution (100 mL)	29.2 g Acrylamide, 0.8 g Bis-acrylamide
Tris-HCl 0.5 M (pH 6.8), (100 ml)	6.06 g of Tris base, pH adjusted to 6.8 with 2 N HCl
Tris-HCl 1.5 M (pH 8.8), (100 ml)	18.18 g of Tris base, pH adjusted to 8.8 with 2 N HCl
10 % **A**mmonium **p**er**s**ulphate (APS) (2 mL)	0.2 g of APS in ddH_2O
10 % **S**odium **d**odecyl **s**ulfate (SDS) (50 mL)	5 g of SDS in ddH_2O
Tris-Glycine Running Buffer, 10X	250 mM Tris base, 1920 mM Glycine, 1 % SDS
Sample Loading Buffer, 2X	0.125 M Tris-HCl (pH 6.8), 4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, 0.004 % bromophenol blue
Staining solution	50 % Methanol, 10 % Acetic acid, 40 % ddH_2O, 0.25 % Coomassie Brilliant Blue R-250
De-staining solution	40 % Methanol, 10 % Acetic acid, 50 % ddH_2O
Buffers/solutions for Western Blot	
TBS, 1X	20 mM Tris base (pH 7.5), 150 mM NaCl
TBST, 1X	TBS containing 0.05 % Tween 20
PBST, 1X	PBS containing 0.05 % Tween 20
Towbin/Transfer buffer, 1X	25 mM Tris base (pH 8.3), 190 mM Glycine, 20 % Methanol
Blocking Solution	1 % Gelatin in TBST
Wash Buffer	TBST or PBST

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Buffers/solutions for Tricine SDS-PAGE gel electrophoresis	
Anode buffer (10X)	1 M Tris-HCl, pH 8.9
Cathode buffer (10X)	1 M Tris-HCl, 1 M Tricine, 1 % SDS, pH 8.25
Gel buffer (3X)	3 M Tris-HCl, 0.3 % SDS, pH 8.45
Acrylamide/**b**is-acrylamide solution (AB-3)	48.0 g of Acrylamide, 1.5 g of Bis-acrylamide
Glycerol 75 % (v/v)	7.5 mL Glycerol, 2.5 mL ddH_2O
Resolving gel (16 %), 14 mL	4.53 mL AB-3, 4.67 mL gel buffer (3X), 1.87 mL Glycerol (75 %), 46.67 µL APS, 4.67 µL TEMED, 2.94 ddH_2O
Resolving gel (10 %), 7 mL	1.41 mL AB-3, 2.33 mL gel buffer (3X), 0.93 mL Glycerol (75 %), 35.0 µL APS, 3.5 µL TEMED, 2.32 ddH_2O
Stacking gel (4 %), 5 mL	0.404 mL AB-3, 1.67 mL gel buffer (3X), 37.5 µL APS, 3.75 µL TEMED and 2.93 mL ddH_2O
Solutions for Silver staining	
Fixing solution	40 % Ethanol, 10 % Acetic acid in ddH_2O
Sensitizing solution	0.2 % Sodium thiosulphate, 6.8 % Sodium acetate, 30 % Ethanol and 0.025 % Glutaraldehyde
Silver nitrate solution	0.1 % Silver nitrate, 0.076 % Formalin
Developing solution	2.5 % Sodium carbonate, 0.05 % Formaldehyde
Stop solution	1.46 % Disodium EDTA
Preserving solution	Preserving solution 30 % Ethanol, 4 % Glycerol
Buffers for rDc1a protein purification	
Lysis buffer	20 mM Tris-Cl (pH 8.0), 200 mM NaCl, 10 % Glycerol, 5 mg/mL Lysozyme
Column buffer	20 mM Tris-Cl (pH 8.0), 200 mM NaCl, 5 % Glycerol, 1 mM sodium azide
Elution buffer	20 mM Tris-Cl (pH 8.0), 200 mM NaCl, 10 mM Maltose, 5 % Glycerol, 1 mM sodium azide
Cleave reaction buffer (Factor Xa protease activity)	20 mM Tris-Cl (pH 8.0), 200 mM NaCl, 2 mM $CaCl_2$, 5 % Glycerol, 1 mM sodium azide, 0.6 mM reduced and 0.4 mM oxidized glutathione

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Solutions for competent cell preparation	
$CaCl_2$ Buffers	100 mM $CaCl_2$, 200 mM $CaCl_2$ + 40 % Glycerol
Buffers for Gibson assembly cloning	
5X isothermal reaction buffer	25 % PEG-8000, 500 mM Tris-HCl pH 7.5, 50 mM MgCl2, 50 mM DTT, 1 mM each of the 4 dNTPs, and 5 mM NAD
Phosphate buffer saline	
Phosphate **b**uffer **s**aline (PBS), 1X	136.7 mM NaCl, 2.6 mM KCl, 8.0 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.2

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Table A15: List of oligonucleotides used

Gene	Sequence
Dc1a (*E. coli* K12 codon optimized)	FP 5'-cgccatatggcgaaagatggtgatgtggaggg-3' RP 5'-cgcggatccttaaacatcacggcacacgcatttgc-3'
pMAL sequencing	FP 5'-ggtcgtcagactgtcgatgaagcc-3' RP 5'-tgtcctactcaggagagcgttcac-3'
MCl1 promoter (PMCl1)	FP 5'-*ggaaacagctatgaccatgattac*gaattcaatcatgcagcgctatgagagc-3' RP 5'-ccgaagaaagttcacgcatgatggtctagggaacgga-3'
MCl1 SP	FP 5'-tccgttccctagaccatcatgcgtgaactttcttcgg-3' RP 5'-caacatcgccatccttagctgccgacgccagggccag-3'
Dc1a (Fungal codon optimized)	FP 5'-ctggccctggcgtcggcagctaaggatggcgatgttg-3' RP 5'-gttgagcagggcggcaggatcctgttaaacatcgcggcagacgcac-3'
MCl1 partial gene	FP 5'-gtgcgtctgccgcgatgtttaacaggatcctgccgccctgctcaac-3' RP 5'-*cgttgtaaaacgacggccagtgcc*aagcttggcatcagagccagcaccggtg-3'
Dc1a RT-PCR	FP 5'-cttcggttctcgccctttc-3' RP 5'-acttcaggcaacggcagtc-3'

FP: Forward primer; RP: Reverse primer; *NdeI* and *BamHI* sites are shown as underlines in FP and RP of *E. coli* K12 codon-optimized Dc1a gene; *EcoRI* and *HindIII* sites are shown as underlines in FP and RP of fungal codon-optimized Dc1a gene; atg and taa: Start and stop codons are highlighted in Blue colour, pCAMBIA3300 vector overlap sequences are shown in italics

Table A16: The codon-optimized gene sequences of spider toxin Dc1a

Gene	Sequence
Dc1a mature toxin gene (*E. coli* K12 codon optimised)	GCG AAA GAT GGT GAT GTG GAG GGT CCG GCG GGT TGC AAG AAA TAC GAC GTT GAG TGC GAT AGC GGC GAA TGC TGC CAG AAG CAA TAC CTG TGG TAT AAA TGG CGT CCG CTG GAC TGC CGT TGC CTG AAG AGC GGT TTC TTT AGC AGC AAA TGC GTG TGC CGT GAT GTT TAA
MCl1$_{SS}$-Dc1a mature toxin gene (Fungal codon-optimised)	*ATG CGT GAA CTT TCT TCG GTT CTC GCC CTT TCG GGC TTG CTG GCC CTG GCG TCG GCA* GCT AAG GAT GGC GAT GTT GAG GGT CCC GCC GGC TGC AAG AAG TAC GAT GTC GAG TGC GAT TCT GGC GAG TGC TGC CAG AAG CAG TAC CTC TGG TAT AAG TGG CGC CCT CTC GAC TGC CGT TGC CTG AAG TCC GGC TTC TTT TCC AGC AAG TGC GTC TGC CGC GAT GTT TAA

MCl1 signal sequence (MCl1$_{SS}$) *is* shown as italics at N-terminus of the codon-optimized gene

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Table A17: Sequencing results of cloned Dc1a gene

Host	Sequence
E. coli	5'-TACAAGCAACAACAATAACAATAACAACAACCTCGGGATCGAGGGAA GGAT TTCACATA ATG GCG AAA GAT GGT GAT GTG GAG GGT CCG GCG GGT TGC AAG AAA TAC GAC GTT GAG TGC GAT AGC GGC GAA TGC TGC CAG AAG CAA TAC CTG TGG TAT AAA TGG CGT CCG CTG GAC TGC CGT TGC CTG AAG AGC GGT TTC TTT AGC AGC AAA TGC GTG TGC CGT GAT GTT TAA GGATCCGAATTCCCT-3'
Metarhizium and *Beauveria*	5'-CAACTGTATTCACTCGCTAATCCGTTCCTGGCTCAAATTCTTTTCCGT TCCCCAGACCATC ATG CGT GAA CTT TCT TCG GTT CTC ACC CTT TCG GGC TTG CTG GCC CTG GCG TCG GCA GCT AAG GAT GGC GAT GTT GAG GGT CCC GCC GGC TGC AAG AAG TAC GAT GTC GAG TGC GAT TCT GGC GAG TGC TGC CAG AAG CAG TAC CTC TGG TAT AAG TGG CGC CCT CTC GAC TGC CGT TGC CTG AAG TCC GGC TTC TTT TCC AGC AAG TGC GTC TGC CGC GAT GTT TAA CAGGATCCTGCCGCCCTGCTCAACTCGGTGTGCTTCCCCAG-3'

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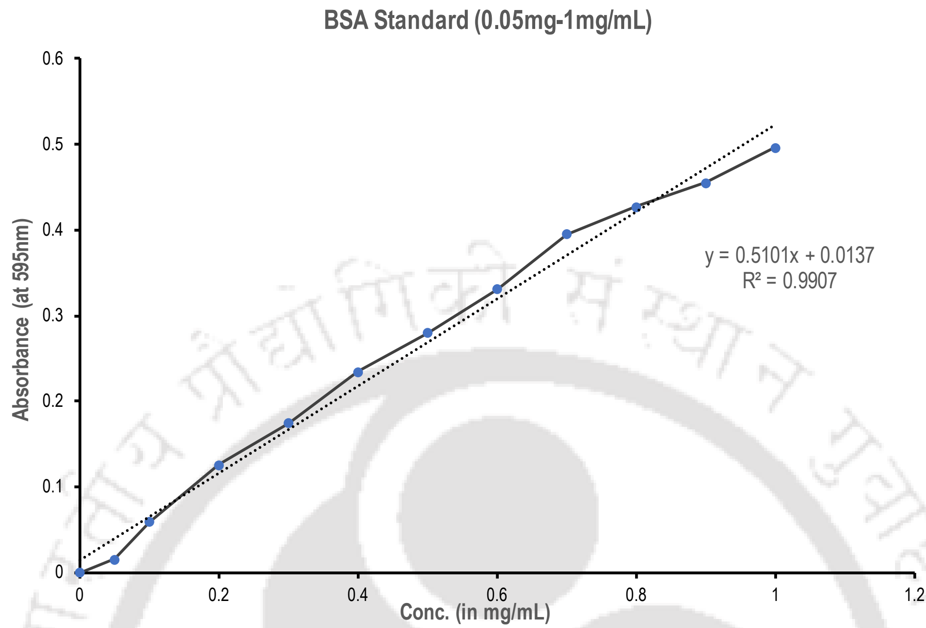

Fig. A1: BSA standard curve.

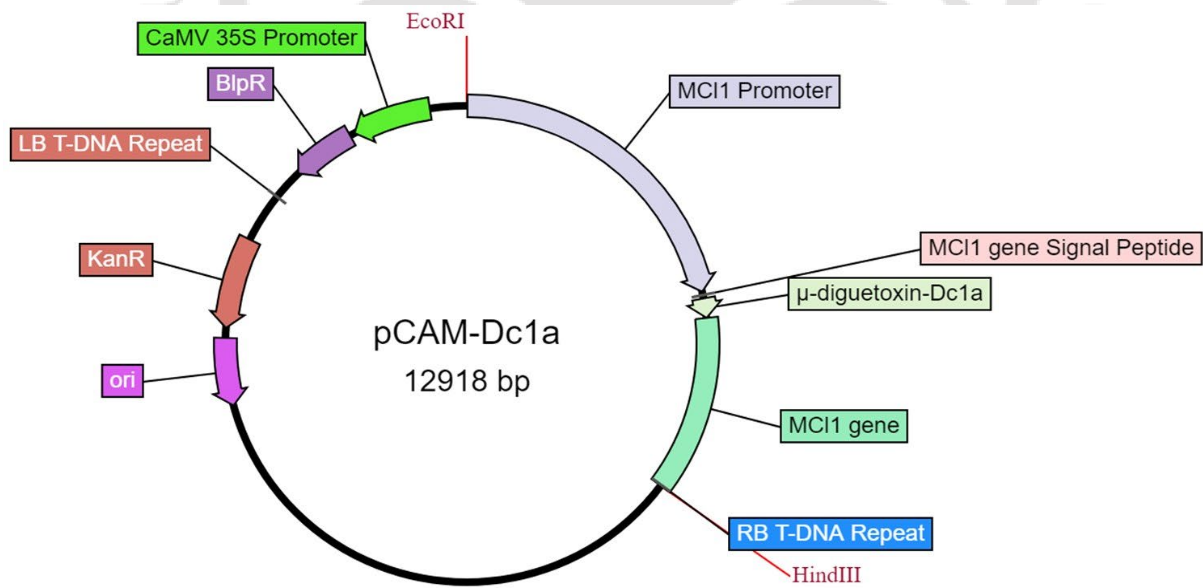

Fig. A2: Pictorial representation of the shuttle vector pCAMBIA3300 showing the diagrammatic representation of cloned gene cassette.

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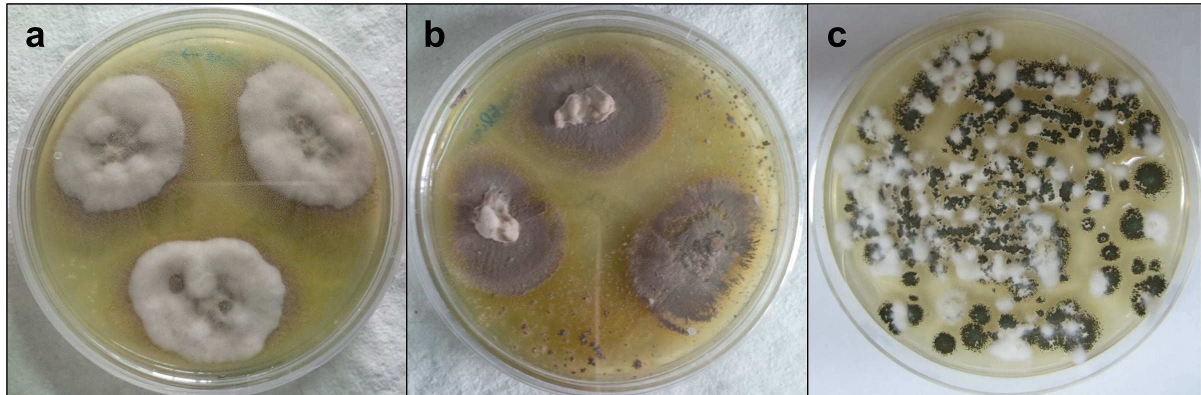
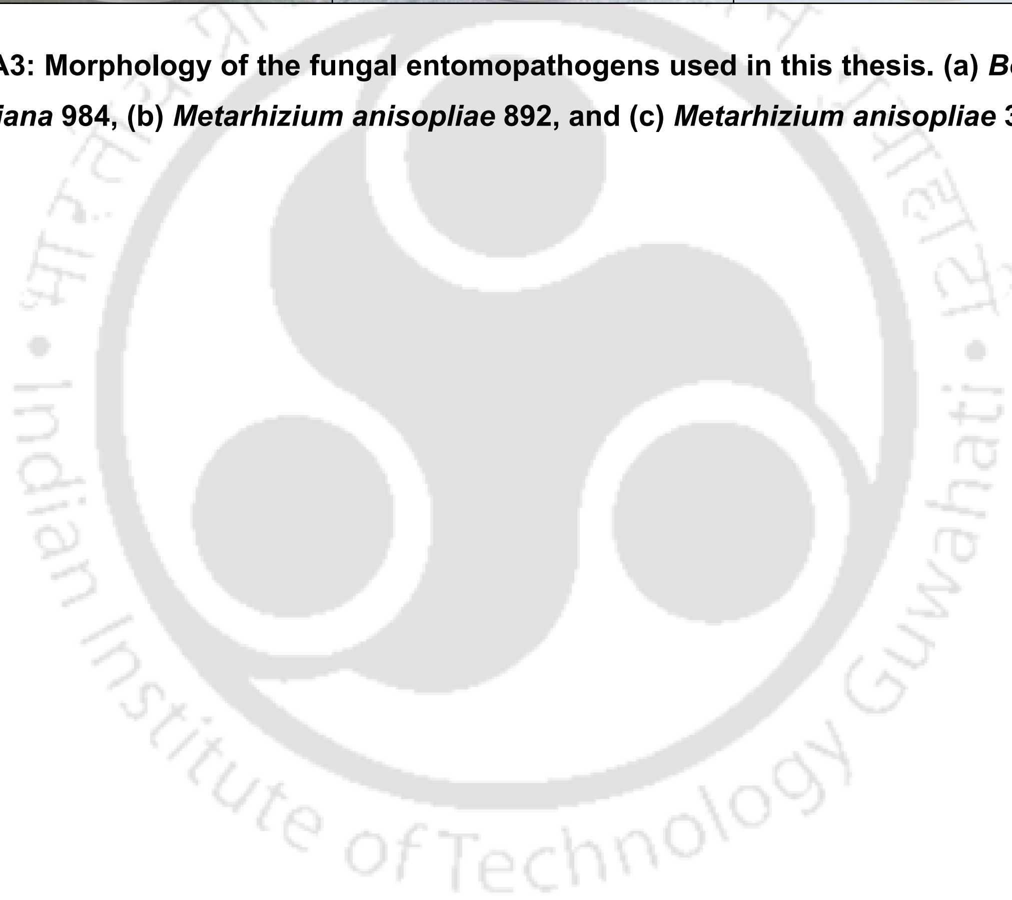


Fig. A3: Morphology of the fungal entomopathogens used in this thesis. (a) *Beauveria bassiana* 984, (b) *Metarhizium anisopliae* 892, and (c) *Metarhizium anisopliae* 3210



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List of Publications and Presentations

Manuscripts under preparation from thesis work

1. Balwant Singh and Gurvinder Kaur. Heterologous expression of a disulfide-rich insecticidal spider peptide under oxidising environment and its functional characterisation against insects of different orders.
2. Balwant Singh and Gurvinder Kaur. Targeted secretion of insecticidal venom peptide from fungal entomopathogen using development stage-specific promoter.

Publications with collaborative work

1. Kawkab Kanjo, Sandeep I. Surin, Tusharika Gupta, M. Dhanasingh, **Balwant Singh** and Gurvinder Kaur Saini (2019). Truncated, strong inducible promoter Pmcl1 from Metarhizium anisopliae., 3 Biotech. 9: 75. doi: 10.1007/s13205-019-1610-2.
2. Pradeep Kumar[1], **Balwant Singh**[1], Vikram Thakur, Abhishek Thakur, Nandita Thakur, Duni Chand. Hyper-production of Taxol from Aspergillus fumigatus, an endophytic fungus isolated from Taxus sp. of the Northern Himalayan region. Biotechnology Reports doi: 10.1016/j.btre.2019.e00395. (**Equal first authors**[1])
3. Duni Chand; **Balwant Singh**[1]; Vikram Thakur; Nandita; Pradeep Kumar[1]; Abhishek Thakur (2019). "Data for: Hyper production of taxol from Aspergillus fumigatus, an endophytic fungus isolated from Taxus sp. of the Himalayan region", Mendeley Data, v2 http://dx.doi.org/10.17632/4wpv2yjk43.2. (**Equal first authors**[1])
4. Rajneesh Kumar, **Balwant Singh**, Gurvinder Kaur Saini, Mohammad Jawed. Impact of copper metal ion on morphology, enzyme activity and changes in the microbial community of aerobic biomass in sequencing batch reactors. (**Under preparation**)

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Poster presentations

1. **Balwant Singh** and Gurvinder Kaur Saini (2016). An investigation on recombinant disulphide-rich insect-selective spider venom peptide, μ-diguetoxin-Dc1a, as an alternative to chemical insecticides. 7th Annual Conference of AMI & International Symposium on "Microbes and Biosphere: What is new what is next, November, 24-27, Gauhati University, Guwahati, Assam. Pp. 311.
2. **Balwant Singh** and Gurvinder Kaur Saini (2017). Engineering a hypervirulent mycoinsecticide by arming fungal entomopathogen, *Metarhizium anisopliae*, with insect selective spider neurotoxin for crop improvement at the International Symposium on Plant Biotechnology for Crop Improvement (ISPBCI) January 20-21, 2017, IIT Guwahati, India.
3. **Balwant Singh** and Gurvinder Kaur Saini (2017). Characterisation and cytotoxicity assays of recombinant spider venom peptide, μ-diguetoxin-Dc1a, on cultured insect and mammalian tissue cells at the International Conference on "Sophisticated Instruments in Modern Research" (ICSIMR) 30th June & 1st July 2017, organised by Central Instrumentation Facility, Indian Institute of Technology Guwahati, Guwahati, Assam.

Oral presentations

1. **Balwant Singh** and Gurvinder Kaur Saini (2018). Evaluating the bio-insecticidal potential of recombinant spider venom peptide, μ-diguetoxin-Dc1a, against two major lepidopteran insect pests of cotton. Presented at first International Conference on Biological Control (ICBC-2018), September 27-29, 2018 at NBAIR, Bangalore, India.

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Awards and Fellowships

1. Qualified Graduate Aptitude Test Examination in Biotechnology (GATE-BT) three times in 2011, 2012 and 2017, conducted by Ministry of Human Resource and Development, Govt. of India.
2. Qualified ICAR-National Eligibility Test (NET) 2016, a national level examination for lectureship, conducted by Agricultural Scientists Recruitment Board, Govt. of India.
3. Oral Presentation Award for presenting a talk in the National Conference on “Recent Advances in Cancer Biology and Therapeutics-2014 (RACBT)” organised by Department of Biotechnology, IIT Guwahati held on 5th December 2014.

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VITAE

BALWANT SINGH
Ph. D. Research Scholar
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati

Contact Details
+91-9085604995
s.balwant@iitg.ac.in
balwant.bsbe.iitg@gmail.com

PROFESSIONAL QUALIFICATIONS

2013 – Present	**Doctor of Philosophy (Ph. D.) in Biosciences and Bioengineering** Indian Institute of Technology, Guwahati, Assam, India • Cumulative Performance Index (Ph.D. Course work) 8.00 out of 10.00
2011 – 2012 One Year Program (Two Semesters)	**Master of Philosophy (M. Phil.) in Biotechnology** Himachal Pradesh University, Shimla, India • Passed with first division (76.33% marks)
2009 – 2011 Two Years Program (Four Semesters)	**Master of Science (M. Sc.) in Biotechnology** Himachal Pradesh University, Shimla, India • Passed with first division (68.92% marks)
2006 – 2009 Three Years Program	**Bachelor of Science Honors (B. Sc. Hons) in Biotechnology** Himachal Pradesh University, Shimla, India • Passed with first division (70.84% marks)

ACADEMIC QUALIFICATIONS

2006 One Year Program	**Senior Secondary (12th standard) Examination** Himachal Pradesh Board of School Education, Dharamshala, India • Passed with first division (69.60%)
2003 One Year Program	**Matriculation (10th standard) Examination** Himachal Pradesh Board of School Education, Dharamshala, India • Passed with first division (67.71%)

NATIONAL LEVEL EXAMINATION QUALIFIED

- Graduate Aptitude Test in Engineering (GATE: BT): Qualified three times in 2011, 2012 and 2017
- ICAR-National Eligibility Test-2016 for lectureship

SKILLS

Molecular Biology & Protein Purification Techniques

Genomic DNA and RNA isolation (Bacteria, Fungus, and Cultured cells); Cloning and expression of proteins in Bacteria, Yeast and Fungus; Primer designing; PCR, RT-PCR, Agarose Gel Electrophoresis, SDS and Native PAGE Gel electrophoresis, Protein purification, Western Blotting Electroporation, etc.

Chromatography Techniques

Gel Filtration Chromatography, Thin Layer Chromatography (TLC), Affinity Chromatography, High Performance Liquid Chromatography (HPLC), Fast Protein Liquid Chromatography (FPLC).

Cell Culture and Microscopic Techniques

Mammalian and insect cell lines maintenance and sub-culturing; in-vitro cell viability and cytotoxicity assays; Cell cycle and apoptosis studies using FACS, Immunofluorescence localization; Bright-Field Microscopy; Inverted Field Microscopy; Field Emission Scanning Electron Microscopy (FESEM); etc.

Instruments Handled

Gel Doc System (Biorad Gel Doc™ EZ System), PCR (Eppendorf Mastercycler, Applied Biosystem), HPLC (Varian ProStar HPLC System and PerkinElmer HPLC), FTIR (PerkinElmer), UV-Vis Spectrophotometer (Agilent Technologies Cary 100 UV-Vis, GeneQuant 1300 from GE Healthcare Life Sciences), Fluorescence Spectrophotometer (Horiba FluoroMax 4), Freeze Dryer (Christ), Constant cell disruptor (TS Series from Constant System), FACS (BD Biosciences), Inverted Fluorescence Microscope (Nikon), Western Blot Transfer apparatus (Thermo Fisher Scientific Power Blotter System), Biosafety cabinet (Thermo Fisher Scientific 1300 Series Class II, Type A2), FPLC AKTA Purifier 100 (GE Healthcare Life Sciences)

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Computational Skills

Biological Software Packages: Modeller 9.19, Gene Designer 2.0, MEGA 10.0.4, Schrödinger Software like PyMOL; GraphPad Prism 6; Wincoot 0.8.9

Software Packages: Sigma Plot 12, IBM SPSS 23, Endnote X9, Mendley, ORIGIN 9.0, Adobe Photoshop and Illustrator CS6, Microsoft Office 2016.

Bioinformatics Tools: BLAST, T-COFFEE, ProtParam, Clustal Omega, SWISS-MODEL, Phyre^2, HADDOCK, etc.

In-vivo Skills

- Handling and maintenance of lepidopteran insect-pest cultures like *Spodoptera litura* (Tobacco cutworm), *Spodoptera frugiperda* (Fall armyworm), *Helicoverpa armigera* (Cotton bollworm), *Chilo partellus* (Spotted stalk borer), *Plutella xylostella* (Diamondback moth).
- Handling and maintenance of dipteran insect cultures like *Aedes aegypti* (Yellow fever mosquito) and *Musca domestica* (Housefly).
- Injection and diet-based bioassays with lepidopteran and dipteran insect-pests.
- Topical application-based bioassays with fungal entomopathogens.

RESEARCH EXPERIENCE

Currently Working on the project **(Ph.D.)**	**TITLE: Studies on the cloning and expression of a spider neurotoxin, Mu-diguetoxin-Dc1a, in entomopathogenic fungi for enhanced bioactivity** **Duration:** 2013 – Present **Supervisor:** Prof. Gurvinder Kaur Saini, Department of Biosciences and Bioengineering, Indian Institute of Technology, Guwahati, INDIA. **Brief Description:** Negative effects of chemical pesticides on the environment and human health emphasize the necessity to develop alternative methods for insect-pest control. The present study involves genetic-engineering of entomopathogenic fungus, *Metarhizium anisopliae* and *Beauveria bassiana*, with insect specific spider neurotoxin. The resultant transgenic fungal entomopathogen will have improved efficacy against insect-pests.
Completed Project **(M.Phil.)**	**TITLE: Taxol (Anticancer Drug) producing endophytic fungus, *Aspergillus fumigatus*, isolated from Northern Himalayas, India** **Duration:** 2011 – 2012 **Supervisor:** Prof. Duni Chand, Department of Biotechnology, Himachal Pradesh University, Shimla, INDIA. **Brief Description:** Since the discovery of Taxol, extensive research has been done to find alternative sources of Taxol to increase its production, but none could meet the high demand for Taxol. In this study, a novel Taxol producing endophytic fungus resembling to *Aspergillus sp*. has been isolated from the stem cuttings of *Taxus wallichiana*, Himalayan yew. Taxol production was characterized by the Spectrophotometric, Thin layer and High-performance liquid chromatography analysis using standard Taxol as reference.

TEACHING EXPERIENCE

July – Nov 2014	Lab demonstrator for M. Tech. students at Indian Institute of Technology Guwahati for the course "Analytical Biotechnology (BT510)".

TRAININGS AND WORKSHOPS ATTENDED

2019	Participated in 20th Indo-Us Flow Cytometry symposium cum workshop on **"Applications of Flow Cytometry in Biotechnology"** organized by International Society for Advancement of Cytometry held on 13th-16th March, 2019 at IIT Guwahati.
2017	Participated in a workshop on **"Advanced Microscopy and Imaging Techniques"** jointly organized by DSS Imagetech Pvt. Ltd, Olympus Systems India Pvt. Ltd and supported by IIT Guwahati from 18th-20th April 2017.

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2015	Participated in Symposium cum Workshop on **"Advances in Computational Biology and Computer Aided Drug Design"** sponsored by DBT-Bioinformatics Infrastructure facility, Department of Biosciences and Bioengineering, IIT Guwahati funded by Government of INDIA & Schrodinger INC. USA
2013	Participated in a workshop on **"Proteomics, Biomarkers and Diagnostics: From Basic Research to Biomedical Applications"** organized at the Regional Centre for Biotechnology, Gurgaon, INDIA.
2013	Participated in DBT sponsored Short-Term Training Program on **"Analytical Instruments & its Applications (Mass Spectrometry-GCMS & LCMS and Spectrophotometry-FTIR, UV-Vis & Fluorescence)"** at the Central Analytical Instrumentation Facility (CAIF) of the Guwahati Biotech Park, Assam, INDIA.
2013	Participated in 8th UGC-NRC-DBS National level Workshop on **"General Biological and Recombinant Techniques"** at Indian Institute of Science, Bangalore, INDIA.
2011	Underwent winter training in different research laboratories of **Central Research Institute (Pasteur Institute),** Kasauli (H.P.), INDIA as a full-time trainee.

PUBLICATIONS IN JOURNALS & CONFERENCE PROCEEDINGS

1. **Balwant Singh**, Pradeep Singh, Gurvinder Kaur Saini and Duni Chand (2014). High Taxol producing *Aspergillus* sp. isolated from Himalayan yew at National Conference on Recent Advances in Cancer Biology and Therapeutics (RACBT)" organized by Department of Biotechnology, IIT Guwahati held on 5th December, 2014 **(Oral Presentation)**
2. **Balwant Singh** and Gurvinder Kaur Saini (2016). An investigation on recombinant disulphide-rich insect-selective spider venom peptide, μ-diguetoxin-Dc1a, as an alternative to chemical insecticides. 7th Annual Conference of AMI & International Symposium on "Microbes and Biosphere: What's new what's next, November, 24-27, Gauhati University, Guwahati, Assam. Pp. 311. **(Poster Presentation)**
3. **Balwant Singh** and Gurvinder Kaur Saini (2017). Engineering a hypervirulent mycoinsecticide by arming fungal entomopathogen, *Metarhizium anisopliae*, with insect selective spider neurotoxin for crop improvement at the International Symposium on Plant Biotechnology for Crop Improvement (ISPBCI) January 20-21, 2017, IIT Guwahati, India. **(Poster Presentation)**
4. **Balwant Singh** and Gurvinder Kaur Saini (2017). Characterization and cytotoxicity assays of recombinant spider venom peptide, μ-diguetoxin-Dc1a, on cultured insect and mammalian tissue cells at the International Conference on "Sophisticated Instruments in Modern Research" (ICSIMR) 30th June & 1st July, 2017, organized by Central Instrumentation Facility, Indian Institute of Technology Guwahati, Guwahati, Assam. **(Poster Presentation)**
5. **Balwant Singh** and Gurvinder Kaur Saini (2018). Evaluating the bio-insecticidal potential of recombinant spider venom peptide, μ-diguetoxin-Dc1a, against two major lepidopteran insect pests of cotton. Presented at first International Conference on Biological Control (ICBC-2018), September 27-29, 2018 at NBAIR, Bangalore, India. **(Oral Presentation)**
6. Kawkab Kanjo, Sandeep I. Surin, Tusharika Gupta, M. Dhanasingh, **Balwant Singh** and Gurvinder Kaur Saini (2019). Truncated, strong inducible promoter Pmcl1 from *Metarhizium anisopliae*., 3 Biotech. 9: 75. doi: 10.1007/s13205-019-1610-2.
7. Pradeep Kumar[1], **Balwant Singh[1]**, Vikram Thakur, Abhishek Thakur, Nandita Thakur, Duni Chand. Hyper-production of Taxol from Aspergillus fumigatus, an endophytic fungus isolated from Taxus sp. of the Northern Himalayan region. *Biotechnology Reports* doi: 10.1016/j.btre.2019.e00395. **(Equal first authors[1])**
8. Duni Chand; **Balwant Singh[1]**; Vikram Thakur; Nandita; Pradeep Kumar[1]; Abhishek Thakur (2019). "Data for: Hyper production of taxol from Aspergillus fumigatus, an endophytic fungus isolated from Taxus sp. of Himalayan region", Mendeley Data, v2 http://dx.doi.org/10.17632/4wpv2yjk43.2. **(Equal first authors[1])**

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AWARDS AND ACHIEVEMENTS

1. **Mukhiya Mantri Protsahan Yojna** award from Himachal Pradesh Government for getting selected into the prestigious Indian Institute of Technology in Guwahati for the year 2013-2014.
2. **Oral Presentation Award** for presenting a talk in the National Conference on "Recent Advances in Cancer Biology and Therapeutics-2014 (RACBT)" organized by Department of Biotechnology, IIT Guwahati held on 5th December, 2014.

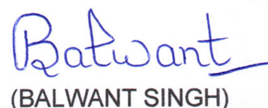
EXTRA CURRICULAR ACTIVITIES

- Worked as a **Research Scholar representative (DPPC member)** in the Department of Biosciences and Bioengineering in IIT Guwahati from year 2015 – 16.
- Worked as **Ph.D. Research Scholar representative** in the **Genesis club** of Department of Biosciences and Bioengineering in IIT Guwahati from year 2014 – 15.
- Served as a **Treasurer and active member** of **Helping Hands Society**, Solan, Himachal Pradesh, INDIA (2007–July 2009) and organized various social events: **Blood Donation and Yoga Camps, Cleanliness Drives, Helping/ Donating for Old Age Homes, Rural education** etc.
- Participated as **volunteer** in **Special Olympics Bharat** (September 4th to 8th 2008) held at Solan, Himachal Pradesh, INDIA.

PERSONAL INFORMATION

Date of Birth	: September 14th, 1987
Sex	: Male
Marital Status	: Single
Languages Known	: English, Hindi, Punjabi
Passport No	: M0560701
Nationality	: INDIAN

I hereby confirm that the information given in this document is accurate and true to the best of my knowledge.



Place: IIT Guwahati
Date: January 2020

(BALWANT SINGH)

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