CHARACTERIZATION OF DICER-LIKE PROTEINS OF *Fusarium oxysporum* f. sp. *cubense* TR4 FOR RNAi-MEDIATED GENE SILENCING ASSESSMENT

By

JAYASEKARA E AMBHAGAHAGE EPITAWATHTHE SAMITHA SAWINDRI

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Chairman : Professor Wong Mui-Yun, PhD
Faculty : Agriculture

Banana fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is a serious epidemic disease worldwide and has recorded significant yield loss in banana farms. The most virulent physiological race of *Foc* is tropical race 4 (TR4). In commercial cultivations, no effective control measure for fusarium wilt has been realized up to now. Ribonucleic acid interference (RNAi) is a post transcriptional gene silencing mechanism, triggered by small double stranded RNA (dsRNA) which leads to sequence specific messenger RNA (mRNA) degradation. It regulates growth and development of organisms. Dicer like (DCL) proteins are responsible for producing small RNAs from long dsRNAs which is the key step in RNAi mechanism. The overall objective of this study was to assess the effect of *Foc* TR4 DCL2 gene silencing on pathogenicity of *Foc* TR4 in banana. The full length DCL1 and DCL2 transcripts were isolated and characterized from *Foc* TR4 in first time in this study. DCL1 and DCL2 proteins include 1501 and 1441 amino acids respectively. In phylogenetic analysis, all *Fusarium* species in both DCL1 and DCL2 phylogenetic trees were closely related to each other and classified under nectriaceae family. *Foc* TR4-DCL2 long dsRNAs were designed *in silico*, based on identified transcript sequence and synthesized using commercially available MEGAscript RNAi kit. Long dsRNA sequence of DCL2 was 736 base pairs (bp) in length. In RNAi silencing efficiency duration of dsRNA, the relative transcript abundance of *Foc* TR4-DCL2 was significantly reduced 72 hours post dsRNA application (HPA) by 44% compared to 0 h and thereafter no significant difference with time until 120 HPA. In effective dose of dsRNAs for RNAi silencing, the relative transcript reduction varied between 22-55% compared to control in all doses except 100 ng/mL. The relative transcript abundance was significantly low at 1000-2000 ng/mL of dsRNA levels. At least 1000 ng/mL was required to elicit a significant reduction of relative transcript abundance compared to control. DCL2 gene silencing affected the conidiation of pathogen. In vitro studies were carried out using complete randomized design (CRD) with three replicates. In glasshouse trials, the used dsRNA dose was 2000 ng per injection at 25 ng/µL dilution rate. In vivo dsRNA assessments were carried out

CRD with five replicates and relative transcript abundance and diease severity were recorded. Area under disease progress curve (AUDPC) values were significantly less in dsRNA treated roots on 7 DPI compared to control. Relative transcript abundance was reduced in 5 and 7 DPI in roots. Disease severity reduction was similar in pre, post and simultaneous application of dsRNA with pathogen infection. Rhizome injection of dsRNA reduced disease severity and relative transcript abundance on day 7. In stability test, the dsRNA was degraded in 8 h after exposure to ultra violet (UV) rays. In conclusion, *in silico* designed DCL2 dsRNA molecules could silence *Foc* TR4 DCL2 gene and it affected conidiation in *Foc* TR4. Direct exogenous application of *Foc* TR4 DCL2 dsRNAs significantly decreased fungal infection and has a potential use to control banana fusarium wilt.

Keywords: RNA interference, dsRNA, Dicer-like gene, Fusarium wilt, Banana

SDG: GOAL 2: Zero Hunger, GOAL 12: Responsible Consumption and Production, GOAL 15: Life on Land

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN PROTEIN SEPERTI DICER *Fusarium oxysporum* f. sp. *cubense* TR4 BAGI PENILAIAN PENYENYAPAN GEN BERPANDUKAN RNAi

Oleh

JAYASEKARA E AMBHAGAHAGE EPITAWATHTHE SAMITHA SAWINDRI

November 2022

Pengerusi : Profesor Wong Mui Yun, PhD
Fakulti : Pertanian

Penyakit layu *Fusarium* pisang yang disebabkan oleh *Fusarium oxysporum* f. sp. *cubense* (*Foc*) merupakan penyakit yang serius dan telah mencatatkan kerugian yang besar di ladang pisang di seluruh dunia. *Race* fisiologi *Foc* yang paling virulen ialah tropika race 4 (TR4). Sehingga kini, tiada langkah kawalan berkesan yang telah direalisasi untuk penyakit layu Fusarium pisang ini. RNA interference (RNAi) ialah mekanisme penyenyapan gen selepas transkrip, yang terhasil oleh RNA bebenang kembar kecil (dsRNA) yang membawa kepada degradasi jujukan spesifik mRNA. RNAi mengawal pertumbuhan dan perkembangan organisma. Protein dicer-like (DCL) bertanggungjawab untuk menghasilkan RNA kecil daripada dsRNA yang panjang. Oleh itu, penyenyapan gen DCL2 akan menyekat pertumbuhan dan perkembangan *Foc* TR4. Transkrip DCL1 dan DCL2 yang panjang diasingkan dan dicirikan daripada *Foc* TR4 pada kali pertama dalam kajian ini. Protein DCL1 dan DCL2 masing-masing mengandungi asid amino 1501 dan 1441. DCL1 mempunyai empat domain manakala DCL2 mempunyai lima domain. Dalam analisis filogenetik, jujukan asid amino protein DCL bagi patogen tumbuhan Ascomycete dikelaskan kepada empat subkumpulan Sodariomycetes, Leotiomycetes, Dothideomycetes dan Eurotiomycetes untuk kedua-dua gen DCL1 dan DCL2. Semua spesis *Fusarium* dalam kedua-dua analisis filogenetik berkait rapat antara satu sama lain dan dikelaskan di bawah keluarga Nectriaceae. dsRNA panjang *Foc* TR4-DCL2 telah direka bentuk secara *in silico*, berdasarkan jujukan transkrip yang dikenalpasti dan disintesis menggunakan kit MEGAscript RNAi yang tersedia secara komersial. Panjang jujukan dsRNA DCL2 adalah 736 bp. Untuk tempoh kecekapan penyenyapan RNAi, tahap transkrip relatif DCL2 telah dikurangkan dengan ketara selepas 72 jam aplikasi dsRNA (hpa) sebanyak 44% berbanding 0 jam, dan tiada perbezaan ketara dicatatkan dengan masa sehingga 120 hpa. Untuk dos berkesan dsRNA, pengurangan transkrip relatif adalah pelbagai dan berada di dalam lingkungan 22-55% berbanding kawalan untuk semua dos kecuali 100 ng/mL. Tahap transkrip relatif pada 1000, 1500 dan 2000 ng/mL dsRNA adalah sangat rendah berbanding tanpa kawalan dsRNA. Dos sekurang-kurangnya 1000 ng/mL diperlukan

untuk mendapatkan penurunan ketara tahap transkrip relatif berbanding kawalan. Pengeluaran konidia mikro menurun selepas penggunaan dsRNA, di mana dos dsRNA yang lebih tinggi adalah lebih efektif berbanding dos dsRNA yang rendah. Dalam percubaan rumah kaca, suntikan dos dsRNA pada 2000 ng pada kadar penggunaan 25 ng/µL telah. Penilaian *in vivo* dsRNA telah dijalankan menggunakan CRD dengan lima replika dan kelimpahan transkrip relatif dan keterukan penyakit telah direkodkan. Nilai kawasan di bawah lengkung kemajuan penyakit (AUDPC) adalah kurang ketara dalam akar yang dirawat dsRNA pada 7 DPI berbanding kawalan. Tahap transkrip relatif telah menurun pada 5 dan 7 DAI di dalam akar. Pengurangan keterukan penyakit adalah sama untuk aplikasi dsRNA secara pra, pasca dan serentak dengan jangkitan patogen. Suntikan dsRNA pada rizom mengurangkan keterukan penyakit dan tahap transkrip relatif pada hari ke-7. Untuk ujian kestabilan, dsRNA telah terdegradasi selepas 8 jam terdedah kepada sinaran UV. Secara kesimpulan, reka bentuk molekul dsRNA DCL2 secara *in silico* boleh mengurangkan tahap transkrip relatif DCL2 dan pengeluaran konidia dalam *Foc* TR4. Aplikasi eksogen DCL2 dsRNA secara langsung telah mengurangkan jangkitan kulat dengan ketara dan berpotensi digunakan untuk mengawal penyakit layu Fusarium pisang.

Kata Kunci: RNA interference, dsRNA, Dicer-like gene, Layu Fusarium, Pisang

SDG: MATLAMAT 2: Kelaparan Sifar, MATLAMAT 12: Penggunaan dan Pengeluaran Bertanggungjawab, MATLAMAT 15: Kehidupan di Darat

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I certify that a Thesis Examination Committee has met on 25 November 2022 to conduct the final examination of Jayasekara E Ambhagahage Epitawaththe Samitha Sawindri on her thesis entitled "Characterization of Dicer-Like Proteins of *Fusarium oxysporum* f. sp. *cubense* TR4 for RNAi-Mediated Gene Silencing Assessment" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Nur Azura binti Adam, PhD
Associate Professor Ts.
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Khairulmazmi bin Ahmad, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Siti Zaharah binti Sakimin, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Hailing Jin, PhD
Professor
College of Natural and Agricultural Sciences
University of California, Riverside
United States
(External Examiner)

RUSMAWATI BINTI SAID, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 27 March 2025

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Wong Mui Yun, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Ganesan a/l Vadamalai, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Norsazilawati binti Saad, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Name and Matric No: Jayasekara E Ambhagahage Epitawaththe Samitha Sawindri GS53705

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Signature: _____

Name of Chairman of Supervisory Committee: Professor Dr. Wong Mui Yun

Signature: _____

Name of Member of Supervisory Committee: Associate Professor Dr. Ganesan a/l Vadamalai

Signature: _____

Name of Member of Supervisory Committee: Dr. Norsazilawati binti Saad

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AUDPC	Area under disease progress curve
%	Percentage
°C	Celcius
µL	Microliter
µm	Micrometer
µg	Microgram
AGO	Argonaute
ATP	Adenosine 5'-triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	Complementary Deoxyribonucleic acid
CDS	Coding sequence
NCBI	National center for biotechnology information
ß	Beta
α	Alpha
kDa	Kilodoltan
TAE	Tri-acetate-EDTA
NaOCl	Sodium hypochloride
rRNA	Ribosomal ribonucleic acid
CRD	Complete randomized design
CTAB	Cetyltrimethylammonium bromide
CHS-A	Chalcone synthase-A
QDE	Quelling deficient

DCL	Dicer like
DCL1/2	DCL1 and DCL2
DNA	Deoxyribonucleic acid
PAZ	Piwi–Argonaute–Zwille
PhD	Doctor of philosophy
dsRBD	dsRNA-binding domain
DPI	Days post infection
DUF	(Domain of unknown function
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
Fg	*Fusarium graminearum*
Fo	*Fusarium oxysporum*
Foc	*Fusarium oxysporum* f. sp. *cubense*
g	Gram
G6DH	Glucose-6-phosphate 1-dehydrogenase
GM	Genetically modified
GSP	Gene specific primer
h	Hours
HIGS	Host induced gene silencing
HPA	Hours post dsRNA application
IDH	Isocitrate dehydrogenase
IGS	Intergenic spacer region
L	Liter
LB	Luria-Bertani

LDH	Layered double hydroxide
mg	Mili gram
min	Mintue
miRNA	MicroRNA
mL	Mililiter
mm	Milimeter
mRNA	Messenger RNA
NFW	Nuclease free water
ng	Nano gram
NGSP	Nested gene specific primer
NTC	Non-template control
ORF	Open reading frame
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
psi	Pounds per square inch
PTGS	Posttranscriptional gene silencing
qPCR	Quantitative polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RdRp	RNA-dependent RNA polymerase
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute

RT-PCR	Real time polymerase chain reaction
sec	Second
SIGS	Spray induced gene silencing
siRNA	Small interference RNA
sRNA	Small RNA
Ta	Annealing temperature
TAE	Tris-acetate-EDTA
TGS	Transcriptional gene silencing
TR4	Tropical race 4
UTR	Untranslated region
UV	Ultra violet
UV-Vis	Ultraviolet–visible
VCG	Vegetative compatible groups
µM	Micro molar
milRNA	microRNA like RNA
ha	hectars
m	meter
spp	Species
SCO	Super optimal broth with catabolite repression
IP	Isoelectric point

CHAPTER 1

INTRODUCTION

Banana is a delicious, widely consumed and nutrient rich fruit crop. It is recorded as the sixth staple crop in the world (Tinzaara et al., 2018). Banana was originated in southeast asia and western pacific regions (Robinson and Saúco, 2010). At present, it is distributed in wet tropical and subtropical regions and staple food for abundant people in that area (Perrier et al., 2011). The total world banana production was 119.8 million tons, with the highest contribution from Asia at 51.9% in 2020 (FAOSTAT, 2020). Out of production limitations, banana fusarium wilt (popular as Panama disease) is a severe threat in most banana growing areas worldwide (Pushpavathi et al., 2018). It is caused by *Fusarium oxysporum* Schlecht f. sp. *cubense* (E.F. Smith) Snyder & H.N. Hansen (Mostert et al., 2017).

Foc was first reported in Australia in 1874, next in Costa Rica and Panama in 1890 and later rapidly invaded in Gros Michel banana fields in other Latin American areas through infected planting materials (Mostert et al., 2017; Ploetz, 2000; Ploetz and Pegg, 1997; Shlvas et al., 1995). The tropical region dispersed TR4 is the most virulent strain out of four races of *Foc* and it was first identified in Taiwan in 1967. *Foc* is currently found in almost all banana growing areas except Mediterranean border, Somalia, and some areas in the South Pacific (Mostert et al., 2017).

Management of this disease is a challenge due to the prolonged survival of chlamydospores in soil, the existence of pathogen in xylem, polycyclic nature of the pathogen and perennial host (Ploetz, 2007; Zhang et al., 2019). Many *in vitro* and *in planta* studies on biological control, chemical control, cultural control and transgenic resistant plants have been carried out to manage banana fusarium wilt. Many of these research outcomes showed the positive effect on suppression of *Foc* growth and disease symptom development in plate cultures and potted banana plants respectively. Fungicide application against *Foc* is ineffective due to difficulty in reaching the pathogen as it is a vascular disease. Also, the development of transgenic banana plants is hindered by controversy over genetically modified crops and lack of suitable genes to develop stable resistance to *Foc* due to evolving pathogen incessantly and conquer the resistant barrier (Ghag et al., 2014a; Ma et al., 2003). On the other hand, the most effective and sustainable approach of resistant cultivars is restricted by low genetic variability, polyploidy of cultivars, long generation times and sterility of cultivars in banana (Elayabalan and Kalaimughilan, 2013). According to that no adequate control measure for effective control of *Foc* TR4 has been realized (Maryani et al., 2019). Therefore, the challenge of developing an effective and environmental-friendly control measure is still in front of us.

In recent past, RNAi has been studied for use in plant disease control which is considered a relatively new era in plant protection. RNAi is a molecular process which negatively regulates the gene expression and it is triggered by small RNAs (sRNAs). There are two distinct group of sRNAs as microRNAs (miRNAs) and small interfering RNAs (siRNAs) based on their biogenesis and precursor structure (Katiyar-Agarwal and Jin,

2010). The small RNA (sRNA) duplexes are incorporated into RNA induced silencing complex (RISC). Then, unwinding antisense sRNA strands bind with target mRNAs in complementarity basis and degrade mRNA molecules which leads to cease the function of gene (Koch et al., 2016; Wang et al., 2016; Zamore et al., 2000; Zhaori, 2017). RNAi has been investigated as a pathogen defence strategy through cease or repression of post-transcriptional gene expression of genes related to pathogen host interactions (Susi, 2007). Further, identification of the 'cross-kingdom RNAi' concept confirms the ability to move siRNAs between kingdoms, inducing gene silencing between host and pathogens (Nowara et al., 2010).

The key step of RNAi process of converting long double stranded RNAs (dsRNAs) into siRNAs is catalysed by a kind of ribonuclease III enzyme called DCL (Catalanotto et al., 2004). Silencing of DCL gene in ascomycete fungal pathogens affect mycelial growth, sporulation and spore germination causing reduced virulence (Wang et al., 2018). Usually there are two dicer proteins in fungi, but in almost all cases DCL2 is needed for effective gene silencing to occur (Wang et al., 2016; Wang et al., 2018; Yin et al., 2020). Silencing of DCL2 gene in *Plasmopara viticola* showed a significant effect on suppression of downy mildew in grapevine (Haile et al., 2021). DCL2 is responsible for the biogenesis of microRNA like RNAs (milRNAs) in *Fusarium graminearum* and *Penicillium italicum* (Yin et al., 2020; Zeng et al., 2018). The ability of *P. italicum* DCL2 RNAi transformants to infect the citrus epidermis was significantly lower compared to DCL1 RNAi transformants (Yin et al., 2020). Therefore, it is hypothesized, silencing of DCL2 transcripts in *Foc* TR4 can be repressed or ceased the sRNA controlled molecular functions responsible for the growth and development of pathogen and ultimately control the fusarium wilt disease development in banana, Berangan. This study is first time attempt to identify effect of RNAi gene silencing through topical application of DCL2 dsRNAs for suppression of *Foc* TR4.

This research was undertaken with the following objectives.

1. To isolate and characterize the full length DCL transcripts in *Foc* TR4.
2. To assess the effect of *Foc* TR4-DCL2 dsRNA on the relative transcript abundance of DCL2 gene and growth and development of *Foc* TR4 under *in vitro* conditions.
3. To assess the effect of *Foc* TR4-DCL2 dsRNA on transcript abundance of DCL2 and pathogenicity of *Foc* TR4 in *Musa* spp. cultivar Berangan.

CHAPTER 2

LITERATURE REVIEW

2.1 Banana

2.1.1 Origin, taxonomy and production

Banana is one of the most popular fruit crop worldwide due to its flavour and nutritional value (Du et al., 2016). It is inferred as indigenous to Southeast Asia and Western Pacific regions, while the centre of diversity is supposed to be in Malaysia or Indonesia (Heslop-Harrison and Schwarzacher 2007; Nayar, 2010; Robinson and Saúco, 2010). Banana is dispersed in tropical and subtropical regions like South Asia, America, Africa, mainland and island Southeast Asia, Melanesia, and the Pacific (De Langhe et. al., 2009).

Angiosperm phylogeny group IV classification system categorizes banana as a monocot under the monophyletic clade of Commelinds, order of Zingiberales, family Musaceae and genus *Musa* (Chase et al., 2016; Heslop-Harrison and Schwarzacher, 2007). Majority of bananas are either *Musa accuminata* or a hybrid of two wild diploid species, *Musa accuminata* Colla (Genome A) and *Musa balbisiana* Colla (Genome B). Polyploidy and hybridization of A and B genomes gives diploid (AA, AB, BB), triploid (AAA, AAB, ABB, BBB) and tetraploid (AAAA, AAAB, ABBB, AABB) bananas (Mohapatra et al., 2010). There are many banana cultivars or landraces around the world, and the majority are triploids, whereas diploids or tetraploids are scarce (D'Hont et al., 2000; Heslop-Harrison and Schwarzacher, 2007; Perrier et al., 2011). Some of AAA cultivars; Berangan, Ambon, Cavendish, Gros Michel, Ibola, Basrai, Lujugira-Mutika, Pisang Masak Hijau and Robusta, AAB cultivars; False horn, Laknau, Maia Maoli, Moongil, Mysore, Nendran, Pisang Raja, Plantain Horn, Pome, Popoulu, Ilohena, Rasthali and Silk and ABB cultivars; Bluggoe, Pisang Awak, Monthan, Peyan, Klue Teparot, Pelipita, Kalapua and Cardaba (Nayar, 2010; Ng, 2015).

The total world banana production was accounted for 119.8 million tons, harvested from an area of 5,203,512 ha, with the highest contribution from Asia as 51.9% in 2020. The world top banana producer was India, next China during 2006-2020 (FAOSTAT, 2020). Banana was considered the most important fresh fruit commodity in terms of the volume of trade around the world (Karamura et al., 2011). Although this crop is famous as a commercial crop, 87% of banana production goes for local consumption in produced countries (De Langhe et al., 2009; Vazhacharickal et al., 2019).

2.1.2 Botany and nutritive value

Banana is a herbaceous perennial plant and cultivated type plants grow up to 2-9 m in height. The true stem is a rhizome that produces suckers, whereas pseudostem consists of compactly rolled leaf sheaths. Apical meristem on rhizome develops the leaves and

flowers. It has an adventitious root system (Karamura et al., 2011). The banana fruit contains 75% moisture, 23% carbohydrates, 1% protein, and 0.5% fat (Singh et al., 2018). It is a source of vitamins like B complex of B1, B2 and niacin and vitamin C and carotenoids including β-carotene, α-carotene, lutein, and zeaxanthin. Also banana rich with minerals such as phosphorus, potassium, calcium, magnesium, sodium, iron, manganese, zinc, copper and boron and digestible food fibers which are essential nutrient components for humans (Ashokkumar et al., 2018; Singh et al., 2018).

2.2 Banana fusarium wilt disease

2.2.1 Causal organism and yield loss

Banana fusarium wilt is a severe threat in most banana growing areas worldwide. It is caused by formae special *Foc* (Mostert et al., 2017). *Foc* is categorized into four physiological races depending on the ability to infect host cultivars as race 1, race 2, race 3 and race 4. Race 1 consists of Gros Michel and cultivars with AAB genome including Lady Finger, Silk, and Pome whereas race 2 includes Bluggoe and cultivars with ABB genome. Race 3 includes *Heliconia* spp while race 4 consists with Cavendish cultivars and cultivars susceptible to race 1 and 2 (Bentley et al., 1998). Further, race 4 is categorized as TR4 and subtropical race 4 based on climatic conditions causing the disease on Cavendish cultivar (Meldrum et al., 2012). Although market demanding race 1 susceptible Gros Michel replaced with race 1 resistant Cavendish, a virulent strain for Cavendish known as *Foc* TR4 was evolved. Cavendish plants infected with *Foc* were first identified in Taiwan in 1967, and the strain was named as TR4 in 1977. Later, it dispersed in countries like China, India, Jordan, Lebanon, Pakistan, Laos, Vietnam, Myanmar, Israel and some other banana growing promising regions of Australia, Caribbean, Africa and Canary islands (Bubici et al., 2019).

The first outbreaks of banana fusarium wilt had been reported in different regions around the world in the early 1900s due to lack of effective control measures against *Foc* (Ploetz, 2015a). Even though no global estimates on banana yield loss are available due to this disease, published figures show a threat to commercial banana production worldwide. About 40,000 ha of banana fields in Central and Southern America were destroyed by fusarium wilt over 50 years (Shlvas et al.,1995). Also, this disease had been wiped out by 30,000 banana plants in Philippines during 1974-1991, 6,700 ha of banana fields in Guangdong province in China in 2006 and 5,000 ha of Cavendish plantations in Lampung district in Sumatra between 1993-2002 (Molina et al., 2009).

2.2.2 Disease symptoms

The characteristic internal symptoms are vascular discoloration from yellow to reddish brown in roots, rhizome, pseudostem and occasionally the bunch stalk. Initial leaf symptoms are water soaked lesions on leaf laminar and followed leaf chlorosis from older leaves to younger leaves (Dong et al., 2012; Moore et al., 1995). Further, leaves slowly collapse at the petiole and droop dead leaves around the pseudostem as a "skirt", while bunched youngest leaves stand erect as a "spiky" appearance (Moore et al., 1995).

This type of droopy and yellowing outer whorl leaves symptoms are called yellow leaf syndrome while droopy outer whorl leaves without yellowing symptoms are known as non-yellow leaf syndrome. Later, splitting of pseudostems vertically near to soil surface can be observed (Pérez-Vicente, 2004). However, there is no symptom development on suckers up to 4 to 5 months old and fruits (Pérez-Vicente, 2004; Ploetz, 2015a).

2.2.3 Morphology and reproduction of *Foc* TR4

Foc TR4 produces three forms of asexual spores as microconidia, macroconidia and chlamydospores. Macroconidia are short to medium in length (average (av.) 67 × 7 µm), straight to slightly curved, slender, thin walled and zero to six celled. Apical cell is papillate, whereas the basal cell is foot shaped. Conidiogenous cells are mono or poly phialidic on sporodochia or developed directly on hyphae. Microconidia are oval to ellipsoid (av. 12 × 5 µm), zero to three septate, borne in false heads on branched conidiophores carried on hyphae (Figure 2.1). Aerial conidiophores branched sparsely or formed laterally. Chlamydospores are roughly walled, globose to subglobose (7-9 to 13-14 x 7-8 to 11-12 µm), usually formed singly or in pairs. They are either terminal or intercalary. Potato dextrose agar (PDA) grown culture colony surfaces are dry, cottony and white in colour with a filamentous margin (Groenewald et al., 2006).

Foc is a sexual reproduction independent group and has the ability for somatic fusion and heterokaryon formation among similar genotypes. This type of heterokaryosis possible strains are known as vegetative compatible groups (VCGs). A total 24 *Foc* VCGs were identified in worldwide and the highest number of VCGs is found in countries like Indonesia and Malaysia (Mostert et al., 2017). Phylogenetic analysis showed that all the isolates in a particular VCG are classified in the same clade and clonal lineage (Koenig et al., 1997). Also, the volatiles producing *Foc* type is known as 'Odoratum' whereas *Foc* type without producing volatiles is 'Inodoratum' (Groenewald et al., 2006). *Fusarium odoratissimum* and all isolates that can infect Cavendish and Gros Michel bananas are classified as *Foc* TR4 (Maryani et al., 2019).

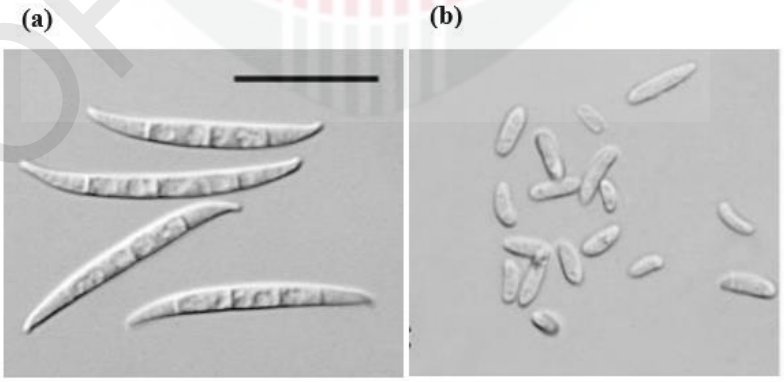

Figure 2.1 : Types of *Fusarium oxysporum* conidia. a: Macroconidia b: Microconidia (Leslie and Summerell, 2008)

2.2.4 Disease infection and progression

Foc is a hemibiotroph organism and has the ability of multiple cycles of infections in a cultivation. The infection process of *Fusarium oxysporum* (*Fo*) consists of several steps: recognition of root, root surface attachment and colonization, penetration and colonization of the root cortex, and hyphal proliferation within the xylem vessel (Dong et al., 2012). The dormant chlamydospores are stimulated for germination by the host or non-host root exudates or by direct contact with susceptible root tissues (Pérez-Vicente, 2004). Pathogen infection occurs through secondary or tertiary feeder roots or when central core of the main roots is directly exposed to pathogen infection bodies. *Fo* enters to roots directly or through wounds, but there are no true appressoria or penetration pegs (Guo et al., 2015). The host barriers are then eliminated to success the infection through digestion of cell wall pectins by pectin methylesterases and polygalacturonases (Guo et al., 2015). The mycelia grow into the epidermal cells and intercellular spaces and form microconidia, macroconidia and chlamydospores. They spread in the cortex tissues, then the endodermis until the xylem vessels intensively. Later, mycelia grow acropetally along the xylem vessels alternating between the sporulating and germinating phases. Tyloses, gums, and gels are formed in xylem vessels in response to pathogen attack to obstruct infection (Ploetz, 2015a). Mycelia and spores block the xylem and phloem vessels resulting in poor water and nutrient uptake. Ultimately host plants are wilted (Ghag et al., 2015; Guo et al., 2015). The fungal hyphae in the phloem grow towards xylem vessels and vice versa (Guo et al., 2015). Later, the conidia and chlamydospores are returned to the soil when the plant dies and chlamydospores may persist dormant or in secondary hosts for many years or twitch to a new disease cycle (Dita et al., 2018; Guo et al., 2015). Chlamydospores can persist in viable in the soil for more than 20 years (Pérez-Vicente, et al., 2004). They germinate poorly in actinomycetes and bacteria rich soils compared to filamentous fungi and yeast rich soils (Ghag et al., 2015).

The hyphal network is appeared covering the root tips when decaying them. Macroconidia are available outside the dying root tips, mainly on the oldest primary roots. At the later stage of disease progression, mycelia are appeared in the gas spaces of the leaf sheaths and coming out from stomata following multiplying on the outside of the leaf sheaths. We can also observe the abundance of macroconidia developing from sporodochia. Also chlamydospores can be observed on inside and outside of the leaf sheath (Warman and Aitken, 2018).

2.2.5 Dissemination of disease and host range

The disease dispersal occurs through passive movement of pathogen propagules due to anthropogenic and natural factors (Dita et al., 2018). Asymptomatic infected suckers are the main carriers for on farm and off farm long distance dispersion. *Foc* contaminated soil disseminates the disease through wind or rain, agricultural machineries, containers, farm equipments, machinery tires, footwear, clothes and nursery media. In addition, fusarium wilt can be dispersed directly through surface runoff, drainage canals, irrigation reservoirs and rivers. Natural disasters like flooding, hurricanes and typhoons may also

contribute to disease dispersal (Dita et al., 2018). Also, vertebrates and invertebrates live in banana plantations can transport contaminated soil particles (Dita et al., 2018). Polymerase chain reaction (PCR) test proved the presence of *Foc* TR4 on exoskeleton of banana weevil borer (*Cosmopolites sordidus*) (Dita et al., 2018). *Foc* can colonize on roots of asymptomatic host plants other than banana. Weed species like *Paspalum fasciculatum*, *Panicum purpurescens*, *Ixophorus unisetus*, *Commelina diffusa*, *Paspalum* spp., *Euphorbia heterophylla*, *Chloris inflate*, *Amaranthus* spp., *Cyanthillium cinereum* and *Tridax procumbens* can be carried *Foc* without noticeable symptoms. However, studies on contribution of cover crops and weeds common in banana plantations for *Foc* dispersal are lack (Dita et al., 2018).

Foc mycelia grow outside from leaf sheaths of infected plants may help to aerial spread the disease with human or animal/insect intervention, but the ability of disease infection through air born conidia is controversial (Warman and Aitken, 2018). In addition, *Foc* infected pseudostems and leaves used as wrapping or packing material during transportation and compost preparation may help to spread the disease (Dita et al., 2018). However, there is no evidence of banana fruits as a *Foc* dispersal agent (Dita et al., 2018).

2.2.6 Management strategies

Management of banana fusarium wilt is an arduous task. Although, physical, chemical, biological, cultural and transgenic approaches have been studied to control this disease, there are some practical difficulties in field application of these findings.

Preplanting soil sterilization methods of heat sterilization, solarization, and composting destroy propagules and are considered residues free methods. These methods destruct pathogens by increasing soil temperature equal or above lethal for the soil organisms. Also pathogen activity can be ceased through antagonism activity of actinomycetes and *Bacillus* spp, volatiles, toxins compounds like ammonia, carbon dioxide, methene and many intermediate organic compounds and alterations in soil gas composition. However, heating limits only to top layer of soil and reduce disease risk only in one cropping cycle. Also these sterilization methods unevenly sterilize the soil and may affect beneficial organisms (Mcgovern, 2015; Katan, 2000; Ploetz, 2015b).

Crop rotation alters the soil microbial population and soil chemical characteristics. It disturbs pathogens through allelochemicals and increases soil microbial activity (Wang et al., 2015). Pineapple-banana and maize-banana rotations effectively suppress the *Foc* population. Soil microbial analysis showed the bacteria community than fungi negatively affect for *Foc* existence. In pineapple-banana rotation, microbial community of acidobacteria, planctomycetes, chloroflexi phyla, Gp1, Gp2 and burkholderia bacterial genera and basidiomycota fungal phyla are increased whereas sordariomycetes are decreased (Wang et al., 2015). Crude extracts of chinese leek (*Allium tuberosum*) totally suppressed the mycelia proliferation and sporulation of *Foc* race 4 under *in vitro*. It repressed the spore production by 91% and spore mortality by 87%. Crop rotation with chinese leek decreased the disease incidence and severity of banana fusarium wilt by 88–97% and 91–96% respectively (Huang et al., 2012).

Biological soil disinfestation by mixing of rice straw, bagasse and pig manure with soil and flooding combined with plastic mulching repress the *Foc* population and thereby low banana fusarium wilt disease incidence (Huang et al., 2015). The incorporation of brassicaceae family plant materials releases glucosinolate hydrolysis products which are toxic to pathogens. Also these decomposing plant materials increase antagonistic organisms in soil. Although the biofumigation is effective against soil pathogens, the disease suppression activity is not consistent and needs high amount of plant materials. Therefore, it is not practical in intensive agricultural systems (Lu et al., 2010).

Application of biofertilizers, silicon, amalgam of iron and boron, copper and zinc improved the resistance to *Foc* infection (Dong et al., 2016; Fortunato et al., 2012; Fu et al., 2017). Abundance of bacteria population with biocontrol agent *Bacillus amyloliquefaciens* NJN-6 was prominently found in biofertilizer amended rhizosphere soils in banana fields (Fu et al., 2017). Endophytes of *Rigidiporus vinctus*, *Trichoderma reesei*, and *Sphingobacterium tabacisoli* are reported as growth promoters while act against *Foc* as growth inhibitor (Savani et al., 2021). Also *Trichoderma harzianum*, *Streptomyces* spp., *Bacillus amyloliquefaciens* and *Bacillus subtilis* repressed the fusarium wilt disease incidence and growth of *Foc* mycelium (Fu et al., 2017; Getha et al., 2005; Thangavelu et al., 2004; Zhang et al., 2011).

Induce resistance compounds of menadione sodiumbisulphite and indoleacetic acid reduce banana fusarium wilt disease development (Borges et al., 2004; Fernández-Falcón et al., 2003). Also, banana fusarium wilt disease can be suppressed by stem injection of mixture of carbendazium, copper oxychloride and boric acid. Likewise. root dipping of benomyl and demethylation-inhibiting fungicides decreased the banana fusarium wilt disease. *In vitro* application of prochloraz, propiconazole, carbendazim, carboxin, benomyl and chitosan have been revealed the negative effect on *Foc* growth (Al-Hetar et al., 2011; Kumar et al., 2019; Nel et al., 2007; Somu et al., 2014). The polydimethylsiloxane-polymethacrylate block copolymers containing quaternary ammonium salts have shown *Foc* TR4 growth inhibition and they are relatively stable in soil (Chang et al., 2021). Low carbon available slurry, compost and dung amended soil reduced disease progression caused by the *Fusarium oxysporum* f. sp. *lini*, while it is negatively correlated with soil pH (Senechkin et al., 2014).

Transgenic banana plants either expressed rice thaumatin-like protein or resistance gene analog 2 exhibited enhanced fusarium wilt disease resistance (Dale et al., 2017; Mahdavi et al., 2012). Transgenic Furenzhi banana plants with chit42 gene from *T. harzianum* showed resistance to *Foc* TR4 (Hu et al., 2013). Transgenic banana plants expressing seed defensin of common chickweed Stellaria media revealed improved resistance to *Foc* TR1 (Ghag et al., 2014b). Although banana plants could achieve a certain level of resistance to fusarium using somaclonal variation technique, fusarium wilt infection to plants depends on the inoculum concentration in soil and adopted cropping systems (Hwang and Ko, 2004; Li et al., 2015).

There are few practical options to avoid spread of banana fusarium wilt disease in banana cultivations. It is recommended for practicing effective quarantine and exclusion of the pathogen to new grounds (Ploetz, 2015b). Also, we can avoid growing susceptible

cultivars in fusarium wilt infected lands and use resistant cultivars and disease free planting materials. Further, production of non-genetically modified banana plants can be explored with novel techniques like RNAi, since GM products are mistrusted by society.

2.3 Gene silencing

2.3.1 Homology dependent gene silencing

Homology dependent gene silencing is an induced inactivation of endogenous nucleic acids by interacting with introduced homologus nucleic acid sequences (Cogoni, 2001; Pickford et al., 2002). It is an epigenetic molecular process of down regulating gene expression of a gene (Parveen et al., 2014). This concept is important for defencing against alien invading nucleic acids like transposons, RNA or DNA (deoxyribonucleic acid) viruses and viroids. It also can be act as a surveillance mechanism to protect the genome from duplications of endogenous DNA sequences in eukaryotic organisms (Cogoni, 2001; Souza et al., 2007). This concept was used further in functional genomics extensively. Functional analysis of calcium signalling-related genes and *Mo*ABC1, *Mo*MAC1 and *Mo*PMK1 in *Magnaporthe oryzae*, MAP kinase signalling genes (Fmk1, Hog1, and Pbs2) in *Fo*, melanin biosynthetic gene in *Rosellinia necatrix*, mitogen-activated protein kinase, a cyclophilin, and a calcineurin regulatory subunit in *Puccinia triticina*, Avra10 in *Blumeria graminis*, INF1 and CDC 14 in *Phytophthora infestans* and chitin synthase in *Sclerotinia sclerotiorum* were performed using this technique (Mark et al., 2005; Nguyen et al., 2008; Panwar et al., 2013; Pareek and Rajam, 2017; Shimizu et al., 2014).

The main homology dependent gene silencing pathways are Transcriptional gene silencing (TGS) and Posttranscriptional gene silencing (PTGS) (Cogoni, 2001). TGS is a stable down regulation of transcription through DNA methylation and chromatin remodelling (Souza et al., 2007). It targets transposons, transgenic inserts, chromosomal repeats and certain protein coding genes (Nishimura et al., 2012). In transgenic organisms, transgenes silencing occurs due to inactivation of promoter sequences which results altered methylation patterns at the transcriptional level. The transgenes may be homologous to other endogenous genomic promoter sequences resulting silencing of respective genes. Chromatin remodelling regulates the transcription of transgenes and the accessibility of target region to the transcription complex of endogenous genes (Meyer, 1999). On the other hand, transcribed transcripts are not translated to proteins due to degradation of transcripts called PTGS.

2.3.2 Discovery of RNAi

RNAi is a naturally occurring endogenous cellular process with complex molecular mechanisms to shut off unwanted or harmful specific nucleic sequences. It down regulates the gene expression before translation by degrading the mRNA, which is triggered by small dsRNAs (Koch et al., 2016; Wang et al., 2016; Zamore et al., 2000). This mechanism presents in almost all eukaryotes (Agrawal et al., 2003; Zamore et al., 2000). RNAi concept was first invented by Andrew Fire and coworkers in 1998. Andrew

Fire and Craig C. Mello were awarded for the nobel prize in physiology and medicine for their work on RNAi in 2006 (Singh et al., 2019).

Although Fire and coworkers introduced this concept first, the phenotypic changes due to RNA silencing were observed earlier in certain eukaryotes. The transgene induced PTGS was first discovered in transgenic petunia plants expressing chalcone synthase gene (Ashfaq et al., 2020). Chalcone synthase-A (CHS-A) is required for the biosynthesis of anthocyanin in flower petals (De Paoli et al., 2009). The transgenic petunias with exogenous transgenes exhibited white and purple variegated pigmentation instead of deepen purple flower colour due to the absence of pigment altogether (Jorgensen et al., 1996). It was suspected as an inactivation of transgenes or suppression of introduced DNA sequences. This phenomenon was named as co-suppression due to greatly expressed single-copy transgenes or poorly expressed transgenes (Hannon, 2002). Later, sRNA analysis showed that two types of 21-mers of sRNAs caused for degradation of CHS-A through mRNA cleavage (De Paoli et al., 2009).

On the other hand, the unc-22 gene encodes nonessential myofilament protein which is responsible for the twitching phenotype in *Caenorhabditis elegans*. The dsRNAs covering a 742 nucleotide segment of unc-22 interfered the activity of unc-22 gene. dsRNAs move into germline and muscle cells from the injection site and they are active via RNA-transport mechanism. It caused the extra appearance of muscle structural defects and weakened motility (Fire et al., 1998). This mechanism was denoted as RNAi.

Neurospora crassa transformants with extra copy number of the gene al-1 involved in the production of carotenoids to appear orange colour phenotype, but they possessed albino phenotype also. The transgenes having transcribed regions of 132 bp could induce gene silencing. It was proved using the reductive al-1 mRNA level in molecular analysis and characteristic orange colour phenotype in quelling-defective mutants and stably quelled albino phenotype in mutagenesis analysis (Cogoni and Macino, 1997). This phenomenon was introduced as quelling in fungi.

These types of RNA mediated gene knockdowns are known as 'RNA silencing' (Baulcombe, 2005). Further, cosuppression in plants, RNAi in animals and quelling in fungi are collectively denoted as RNAi. These three phenomena are phenotypically altered but mechanistically are alike. Since it retards the proliferation and expression of foreign nucleic acids of viruses, viroids, transposons or transgenes, scientists have been explored as a protective tool in many fields like medicine, plant and animal protection and breeding (Chaudhary et al., 2019; Chen et al., 2020; Karmakar et al., 2020; Wei et al., 2020b; Worrall et al., 2019).

2.3.3 RNAi Mechanism

The sRNAs are inducers in RNAi pathway while co-components of DCL, Argonaute (AGO) and RNA-dependent RNA polymerase (RdRp) catalyse the RNAi process. The long dsRNA molecule is broken into siRNA molecules by a ribonuclease III enzyme called DCL (Siomi and Siomi, 2009). It cleaves dsRNA molecules into uniform 3’

overhangs of 2 to 3 nucleotides, 5'-phosphate and 3'-hydroxyl termini called siRNAs (Agrawal et al., 2003; Elbashir et al., 2001). Double stranded molecules with overhang in 3' ends are important for recognizing enzymatic machinery in RNAi pathway (Zotti et al., 2018). These siRNAs are then transferred into a large multiprotein complex of RISC (Geley and Muller, 2004). The passenger strand or sense strand of siRNAs are cleaved by AGO class RNA binding proteins, leaving other strand called guide RNA or antisense strand. Anti-sense siRNA strand bind with RISC ten-fold more compared to sense-strand. It is observed that low thermodynamically stable 5' ends of siRNAs are readily incorporated into RISC (Schwarz et al., 2003). Antisense strands bind with complementary sequences of target mRNA for degradation or the repression of translation (Pak et al., 2012; Vaucheret, 2006). Unwinding of the siRNA duplex and incorporation into the RISC requires adenosine 5'-triphosphate (ATP) for some species while others do not. Production of siRNAs from dsRNA in Drosophila embryo extracts uses ATP, whereas human DCL proteins do not need it (Geley and Muller, 2004). The sequence specific endonucleolytic cleavage site of target mRNA and siRNA is located middle from the 5' end of siRNA (Elbashir et al., 2001). They can bind partial or complete complement mRNA sequences and reduce the expression levels of genes (Zhang, 2009). As a result, these molecules regulate cellular functions like cell differentiation, proliferation, migration, death, metabolism and defence (Zhang, 2009). The sturdiness of this process is maintained through the action of RdRp which amplifies the initial siRNA pool to secondary siRNAs (Pak et al., 2012) (Figure 2.1).

These co-components of DCL, AGO and RdRp are encoded by multigene families, resulting diversification of RNAi pathways in most eukaryotes, (Vaucheret, 2006). Also, these key proteins show higher level sequence conservation among eukaryotes whereas differ from prokaryotes (Shabalina and Koonin, 2008). Single-stranded siRNAs are 10-fold less effective than double stranded siRNAs due to poor stability under *in vitro* and *in vivo* (Schwarz et al., 2003).

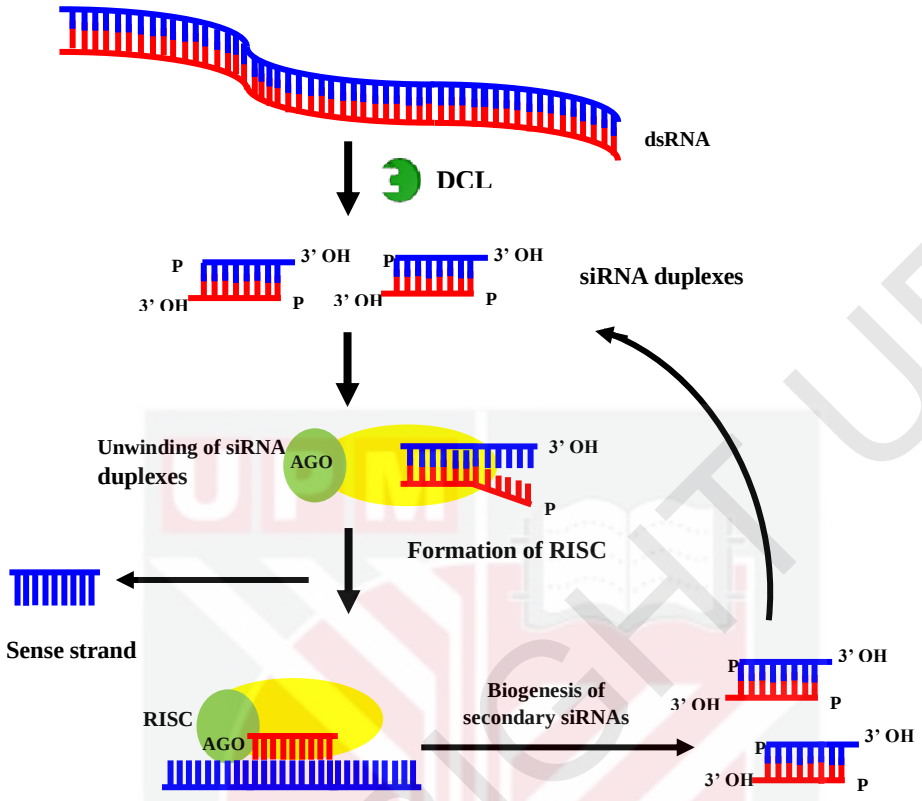

Figure 2.2 : Schematic diagram of siRNA mediated RNAi mechanism. Long dsRNA duplexes are cleaved to siRNAs by the action of DCL. siRNAs bind to the RISC complex and AGO. siRNA and RISC complex then bind to complementary mRNA sequences for degradation of transcripts. Regeneration of siRNAs is done by RdRp

2.3.4 Riggers in RNAi pathway

sRNAs are noncoding, 18 to 30 nucleotides in length and active in both cytoplasm and nucleus (Zhang, 2009). Size and type of sRNAs were varied depending on locus, chromosome and/or cultivar in barley (Hackenberg et al., 2016). Although these noncoding RNAs are not responsible for particular protein, they carry biological information to regulate gene expression. Generally, there are three main groups of sRNAs generated in diverse biochemical pathways in eukaryotes as miRNAs, siRNAs and piwi-interacting RNAs (Zhang, 2009). All these three types of sRNAs are found in germline/animal, miRNAs and siRNAs are in plants and milRNAs and siRNAs are in fungi (Dunoyer et al., 2013; Iwasaki et al., 2015; Mathur et al., 2020; Wang et al., 2019).

miRNAs are 21 to 23 nucleotides in length and derived from genome encoded MIR genes (Quévillon and Simard, 2019). They are single stranded RNA transcripts and fold back themselves as imperfect doublestranded stem loop precursor structures (Liu et al., 2017). In the miRNA synthesis, the initial specific noncoding RNAs called primary microRNAs

are processed into precursor miRNAs by DCL. Then precursor miRNAs are converted to miRNA duplexes in the nucleus. These duplexes move to the cytoplasm and become mature miRNAs. After that, mature miRNAs load into RISC (Kuo and Falk, 2020; Wang et al., 2019). Fungi produce milRNAs instead of miRNAs, which have similar production mechanism to animal and plant miRNAs. However, information on mechanism of gene silencing by fungal milRNAs is lack (Jiang et al., 2017). milRNAs of *Coprinopsis cinerea* and *Fusarium oxysporum* f. sp. *niveum* have been studied by highthroughput sequencing and bioinformatics analysis (Jiang et al., 2017; Lau et al., 2018). A total of 8 milRNA candidate families were identified for *Fo* based on the criteria of miRNA prediction in plants and animals (Chen et al., 2014). Usually, miRNA induced silencing complex binds to 3' UTR of mRNAs (Quévillon and Simard, 2019). They are near-perfect complemented to target mRNA and can impede multiple targets. (Boyd, 2008). The siRNAs are produced either endogenously from RNA transcription in the nucleus, viruses, transposons, transgenes and degraded RNAs or exogenously from viruses and chemical synthesis (Kuo and Falk, 2020; Valencia-Sanchez et al., 2006; Zhang, 2009; Zotti et al., 2018).

There are some similarities and differences between miRNAs and siRNAs. Single miRNA locus generates only one miRNA duplex, while one siRNA locus produces many siRNA duplexes during their biogenesis. Although miRNAs are conserved, the siRNAs are hardly conserved between related plants and animals. Both these rely on dicer enzymes for biogenesis and the AGO associated RISC for function.

2.3.5 DCL proteins

2.3.5.1 Discovery of DCL proteins

Identity of dicer in upstream part of RISC pathway was first discovered by Prof. Hannon and PhD. scholar, Emily Bernstein (Paturi and Deshmukh, 2021). Dicers are core proteins of sRNA synthesis pathways which cleave long dsRNA into siRNAs and pre-miRNAs into microRNAs in the cytoplasm (Kadotani et al., 2004). Genome-wide sequencing analysis in different organisms has discovered that DCL proteins are evolutionarily conserved in a wide range of eukaryotic genomes. DCL proteins of filamentous fungi like *N. crassa*, *M. oryzae*, *V. nonalfalfae*, *B. cinerea* and *Aspergillus nidulans* have been characterized to identify number of encoding genes and evolutionary relationship of them (Catalanotto et al., 2004; Hammond et al., 2008; Jeseničnik et al., 2019; Kadotani et al., 2004; Tauati et al., 2014). A total 442 putative dicer encoding genes were found in 1,440 fungal genomes when predicting putative genes (Choi et al., 2014).

2.3.5.2 Organization of DCL domains

DCL proteins are endonuclease enzymes which are categorized under RNase III family (Meng et al., 2017). In most, multi domain DCL proteins contain N-terminal DEAD box, a RNA helicase domain, a Piwi–Argonaute–Zwille (PAZ) domain, a dicer dimer domain, two ribonuclease (RNase III) domains and an additional dsRNAs-binding domain

(Figure 2.3) (De Jong et al., 2009; Gao et al., 2014; Meng et al., 2017). The lower eukaryotes have lesser number of domains than vertebrate, insect, and plants. Electron microscopic and crystallographic view of dicer showed that it is 'L' shaped and consist with head, body, and base. PAZ domain is located on the head, the RNase IIIb domain on the body and helicase domain on the base (Fukudome and Fukuhara, 2017).

PAZ, RNase III, and dsRBD (dsRNA-binding domain) domains are responsible for dsRNA binding and cleavage (Shi et al., 2006). PAZ domains have a 2 nucleotides 3' overhang binding pocket and a 5' phosphate-binding pocket which are required for initial recognition and anchoring of the substrate. RNase III domains assist for binding sRNAs to AGO and form RISC (Ciechanowska et al., 2021). DExD/H-box helicases are responsible for unwinding RNA or DNA duplexes. The helicase domain serves as a platform for binding of co-factor regulatory proteins (Fukudome and Fukuhara, 2017). Dimerization domain involves binding of dicer partner proteins such as *Arabidopsis thaliana* DCL1 and DCL4 bind hyponastic leaves 1 and dsRNA-binding protein 4 respectively.

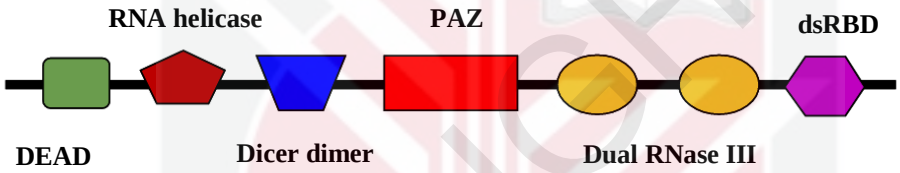

Figure 2.3 : Schematic diagram of the typical domain structure of DCL proteins

The distance between PAZ and RNase III domains decides the length of sRNAs (Fukudome and Fukuhara, 2017). RNase III hydrolyses phosphodiester bonds in dsRNAs to generate sRNAs. RNase III family consists with three subfamilies. The first subfamily represents one RNase III domain and one dsRBD domain. Second subfamily includes two RNase III domains and a single dsRBD. The third subfamily represents two RNase III domains, a dsRBD, helicase domain, a DUF283 (domain of unknown function) and a PAZ domain (Ciechanowska et al., 2021). dsRBDs are responsible for dsRNA-binding function (Fukudome and Fukuhara, 2017).

2.3.6 Cross-kingdom RNAi

Plant endogenous sRNAs and pathogen-derived sRNAs are important for regulating cellular stress responses and plant immunity. Immune-regulatory sRNAs that are differentially regulated upon pathogen attack have been identified. sRNA movement can occur from the host to the pest, pathogen, parasite, symbiont or vice versa. This natural phenomenon which gene silencing is induced between unrelated species from different kingdoms are known as cross-kingdom RNAi. In there, sRNAs move between kingdoms in bi-directionally (Schaefer et al., 2020). These sRNAs are involved in the activation of pathogen-associated molecular pattern-triggered immunity and effector-triggered

immunity in host plants. Further, these sRNAs are either induced or down-regulated upon pathogen attack in order to suppress plant diseases (Weiberg & Jin, 2015).

Although plant sRNAs involved in host defence have been extensively studied, relatively little is known about the function of pathogen-derived sRNAs. Even though sRNA movement mechanism in cross kingdom RNAi is not understood totally, bidirectionally moved sRNAs have been identified and pest and disease suppression was observed after sRNA application to host plants. *B. cinerea* can deliver sRNA effectors into host cells to suppress host immunity. In tomato and Arabidopsis, *B. cinerea* secreted sRNAs silenced the host defence genes of mitogen-activated protein kinases, oxidative stress-related gene peroxiredoxin and cell wall-associated kinase (Weiberg et al., 2013). During *B. cinerea* infection, the expression level of *Bc*-siR37 was induced, and at least eight predicted Arabidopsis target genes were downregulated (Wang et al., 2017). *B. cinerea* possessed double knockout mutants of the DCL genes exhibited poor virulence of pathogen due to cut off plant immune-suppressing siRNAs (Weiberg et al., 2013).

In HIGH, the sRNA movement mechanism between host and fungi is explained theoretically as the hairpin transcripts originated from transgenes are processed to siRNA using host's enzymes. These siRNAs are secreted in exosome-like vesicles for fungal uptake through the haustorial interface (Schaefer et al., 2020).

2.3.7 Fungal sRNA mediated gene silencing

N. crassa uses QDE-1 (Quelling deficient-1), QDE-2, QDE-3 and DCL-1/DCL-2 in quelling process. DCL proteins cut the dsRNA into nearly 25 nucleotide siRNAs and these siRNAs incorporated with RISC containing QDE-2 and QIP. QIP eliminates passenger strand resulting the RISC activation. *N. crassa* has dicer dependent QDE-2-associated sRNAs called milRNAs and dicer-independent siRNAs, reflecting diverse pathways of sRNA generation in the filamentous fungi (Lee et al., 2010). Homologous gene silencing using repetitive DNA sequences (Quelling) at vegetative stage and unpaired DNA gene silencing using repeat-induced point mutation and meiotic silencing at sexual stage are occured in *N. crassa*. It was assumed that aberrant DNA secondary structures are generated by large tandem repeats which identified by QDE-3. The qiRNAs are QDE-2-associated and produced in response to DNA damage (Dang et al., 2011).

RNA silencing was observed in fungal phylums of ascomycota, basidiomycota, zygomycota and oomycetes (Nunes and Dean, 2012). However, RNAi process does not exist in single-celled organisms, *Saccharomyces cerevisiae* (Dang et al., 2011). Fungi have different classes of sRNAs as milRNAs, QDE-2 interacting small RNAs, dicer-independent siRNAs, endogenous short RNAs, LTR retrotransposon-siRNAs and tRFs. However, silencing of individual gene of the pathogens is not assured the deactivation of the protein due to functional redundancy and the partial silencing of mRNA levels (Qi et al., 2019).

2.3.8 Host induced gene silencing

HIGS was developed based on the concept of plants' innate immunity against invading viruses or advancement of virus induced gene silencing. It involves the host expression of siRNA generating constructs directed against genes in associated pathogens, parasites or symbionts (Andrade et al., 2016). Long dsRNAs or long hairpin molecules are generated by host RNAi construct. Then long dsRNAs were converted to artificial sRNAs. These sRNAs translocate into target pathogen cells during infection and downregulate the respective gene (Hou and Ma, 2020). Also, HIGS can generate artificial miRNAs using MIR genes (Hou and Ma, 2020). HIGS mechanism is absent in *S. cerevisiae* and *Ustilago maydis* due to lost the RNAi related proteins with evolution. Success of this technology is determined by the target gene selection, efficiency of target gene silencing and designed RNAi triggers. Also, the efficiency of HIGS depends on adequate supply and efficient transportation of siRNAs between the two organisms (Qi et al., 2019). HIGS technology upgrades not only plant resistance against pest and diseases, but also improves agronomic and breeding traits like high quality of crop products, male sterility varieties, seedless plant varieties, excluding fungal toxins, and improving stress tolerance (Qi et al., 2019). However, the translocation of siRNAs against necrotrophic fungi is limited in HIGS, as they absorb nutrients and metabolites from dead host tissue (Qi et al., 2019). Recent successful HIGS applications on plant pathogens are list in Table 2.1.

2.3.9 Spray induced gene silencing

Spray induced gene silencing (SIGS) is a non-GM alternative to HIGS and explored as a new prospective crop protection tool. It inhibits pathogen infection by topical application of dsRNAs or siRNAs on plant surfaces to silence virulence related genes of pathogen (Wang and Jin, 2017). dsRNAs enter to fungal cells as direct uptake and/or through transferred from plant cells. Absorbed long dsRNAs can be converted to siRNAs by plant or fungal RNAi core components. SIGS has been successfully used to control some fungal diseases (Wang et al., 2016).

The success of SIGS for plant disease control is highly determined by the pathogen's RNA uptake efficiency. dsRNA uptake efficiencies vary across eukaryotic microbe species and cell types. Efficient dsRNA uptake was observed in *B. cinerea*, *S. sclerotiorum*, *Rhizoctonia solani*, *Aspergillus niger* and *Verticillium dahlia* but no uptake in *Colletotrichum gloeosporioides* and weak uptake in *Trichoderma virens* and *P. infestans*. Therefore, pathogens with high RNA uptake significantly inhibited plant disease symptoms while pathogens with low RNA uptake efficiency showed poor disease suppression (Qiao et al., 2021).

Further, effective SIGS depends on processing ability of dsRNAs by fungal RNAi core components (Koch et al., 2016). Also, longer dsRNAs displayed a higher gene silencing efficiency and a stronger disease suppression than shorter dsRNAs, might be due to many derived siRNAs (Werner et al., 2020). Uptake efficiency of sRNA molecules into plants depends on stomata opening but needs to investigate (Koch et al., 2016). The required concentration of siRNAs for the target organism depends on uptake efficiency

from the plant's apoplast and processing by fungal DCLs (Gaffar et al., 2019; Koch et al., 2016). Designing RNAi triggers is important for efficient uptake of dsRNAs and/or siRNAs by the target pathogen in SIGS (Werner et al., 2020). SIGS can target several genes in one pathogen, same pathogen in many crops and essential genes in various pathogens (Wang and Jin, 2017). Successful control of plant pathogens by SIGS is listed in Table 2.2.

Although miRNAs bind to 3' untranslated region of a gene, it is not identified what region is optimal for dsRNA designing in SIGS. The optimal concentration for induce silencing varies with target gene and organism. Multiple dsRNAs will create competition for cellular uptake and can create oversaturation, leading to poor RNAi process (Joga et al., 2016).

Table 2.1 : Successful HIGS applications against plant pathogens

Target species	Gene	Function	Host	References
Soybean mosaic virus	Translation initiation factor 4E	Directing ribosomes to the cap structure of mRNAs for regulating protein synthesis	Soybean	Gao et al., 2020
Phakopsora pachyrhizi	Acetyl-CoA acyltransferase, Three hypothetical proteins, Glycine cleavage system H protein, 40S ribosomal protein S16, Conidiation-related protein 6, Putative 3-hydroxy-3- methylglutaryl-coenzyme A reductase	Regulation of urediniospore germination or appressorium formation	Soybean	Hu et al., 2020
Tomato leaf curl virus (ToLCV)	AC4 genes of ToLCV antisense replicase gene	Suppression of gene silencing	Tomato	Koulagi et al., 2020
Aspergillus flavus	Versicolorin dehydrogenase gene ver-1	Biosynthesis of aflatoxin	Maize	Raruang et al., 2020
F. graminearum (*Fg*)	*Fg*SGE1 *Fg*STE12 *Fg*PP1	Biosynthesis of secondary metabolite, DON Transcriptional factor for penetration structure formation in planta Biosynthesis of phosphatase	Wheat	Wang et al., 2020
Foc TR4	C-24 sterol methyltransferase Cytochrome P450 lanosterol C-14a-demethylase	Biosynthesis of fungal ergosterols	Banana	Dou et al., 2020
Verticillium dahliae	Acetolactate synthase	Biosynthesis of branched-chain amino acids	Cotton	Wei et al., 2020a
C. gloeosporioides	Conidial morphology 1 gene	Reduction of conidial germination, Germ tube development, Appressoria formation and mycelial growth	Chilli and tomato	Mahto et al., 2020
Fusarium oxysporum f. sp. *lycopersici*	Ornithine decarboxylase gene	Biosynthesis of polyamine	Tomato	Singh et al., 2020

Table 2.2 : Successful SIGS applications against fungal pathogens

Target species	Gene	Function	Host	References
P. pachyrhizi,	Acetyl-CoA acyltransferase, 40S ribosomal protein, and glycine cleavage system H protein	Germination of urediniospore or appressorium formation	Soybean	Hu et al., 2020
P. viticola	DCL1 and DCL2	RNAi	Grape	Haile et al., 2021
Podosphaera xanthii	Six conserved and non-annotated proteins	Respiration, glycosylation and efflux transport	Zucchini and Melon	Ruiz-Jiménez et al., 2021
P. infestans	Guanine-nucleotide binding protein b-subunit, haustorial membrane protein, cutinase and endo-1,3(4)-b-glucanase	Signal transduction during pathogenesis, membrane-mediated lipid transport and intercellular distribution of lipid molecules, host tissue degradation	Potato	Ruiz-Jiménez et al., 2021
F. graminearum	AGO1, AGO2, DCL1 and DCL2	RNAi	Barley	Werner et al., 2020
B. cinerea	lanosterol 14α demethylase, chitin synthase 1, elongation factor 2	Biosynthesis of ergosterol, cell wall polymerization, catalyses ribosomal translocation during protein synthesis	Grape	Nerva et al., 2020
P. pachyrhizi	Chitin synthesis gene	Differentiation of appressoria	Soybean	Saito et al., 2022
M. oryzae	Host-defence suppressor	Detoxification of plant driven reactive oxygen species	Rice	Sarkar and Ray-Barman, 2021
S. sclerotiorum and *B. cinerea*	Amino acyl tRNA ligase, Thioredoxin reductase, TIM44	Genes associated with reactive oxygen species responses, transcription, and host colonization	*Brassica napus*	McLoughlin et al., 2018
B. cinerea, S. sclerotiorum, Rhizoctonia solani, Aspergillus niger and *V. dahliae*	Vacuolar protein sorting 51, Dynactin, Suppressor of actin	Vesicle trafficking pathway genes	Tomato, lettuce, rose, grape, collard green, rice	Qiao et al., 2021

2.4 RNAi mediated crop protection

2.4.1 Significance of RNAi mediated crop protection

Agriculture has to respond globally to increase food demand with increasing population growth. Sustainable intensified agricultural systems through mechanization and novel technologies help to achieve that. However, the pest and disease control is a difficult task in intensified agricultural systems. The use of synthetic chemical pesticides has increased due to its efficacy, low cost, and easy application (Aktar et al., 2009). However, it causes for pest and disease resistance development against pesticides and increasing risk to food safety, living organisms and environment (Aktar et al., 2009). The development of RNAi based crop protection has been popularized mainly due to its no effect of resistance development and environmental pollution.

This technology has been explored as a novel platform for molecular crop improvement through silencing of stresses susceptible genes and improving plant disease resistance genes. RNAi may be a promising therapeutic agent to defeat plant invaders in future. Infection of host plant can be controlled through silencing of virulence genes of insects and pathogens. This new approach could be the next generation of pathogen resistant transgenic plants.

The dsRNA molecules are considered environmentally friendly, because it is easily degraded as a natural biological molecule (Mezzetti et al., 2020). In humans, innate immune system recognizes dsRNA via multiple receptors. There is no detectable evidence for effect of dietary consumption of dsRNA from virus infected plant material in humans. Further, no long lasting of non-coding RNAs in biological fluids due to endogenous nucleases, removal of them via the kidneys and failures to cross vascular and cellular barriers. Also, dsRNAs are unstable without protection, but formulation with other additives would impact human and environment (Fletcher et al., 2020). The current information implies that RNAi molecules in human diet is not threat to humans as discussed in the 40th Annual Meeting of The Toxicology Forum (Dalakouras et al., 2020).

The high specificity of siRNAs for particular target gene avoids effect on off target organisms. The foreign dsRNA sequences are cleaved by dicer protein, generating number of siRNAs ranging in size and sequence. Since diversity of siRNAs are high, the possibility of off-target and non-targeted effects are high (Joga et al., 2016). Thus, applying dsRNA on plants, off-target effect on crop plants should be studied, because portion dsRNAs move in to plants. All possible sense and antisense siRNA sequences of a dsRNA fragment and its homology to non-target organisms can be predicted by *in silico* analysis which would help to minimize the effect on environment. OfftargetFinder, BLAST search tool, Genscript siRNA target finder and siRNA finder like computer softwares aid to predict effect on off target organisms. (Fletcher et al., 2020). Lower off target effect can be achieved by using few nucleotides complementarity with its target, including nonconserved regions, applying unique sRNAs and exclude long dsRNAs (Dalakouras et al., 2020). However, chance to influence nontarget is low compared to pesticide usage.

Further, RNAi based fungicides could be effectively controlled multiple pathogens by focusing common target region of several pathogens. Although transgenic plants release more dsRNAs and increase the potency for resistance development, topical application limits the pathogen exposure to dsRNA due to transient character. RNAi resistance may develop due to reduced cellular uptake, mutations in transcript, RNAi suppressors, up regulation of the target gene, down regulation of RNAi machinery genes, nuclease activity and behavioral changes (Cagliari et al., 2019). The monogenic resistance can be avoided alternating 200 bp regions of the target sequence whereas avoidance of polygenic resistance can be achieved using chimeric RNAs or a mixture of different RNAs (Dalakouras et al., 2020). Topical application of dsRNA does not change hereditary in the genome as GM products. Thus, time and complex process are reduced and increased social acceptance (Cagliari et al., 2019).

2.4.2 RNAi mediated crop protection products

The conventional RNAi approaches are based on the recombinant viruses, *Agrobacterium tumefaciens*-mediated transgenes, and stably transformed transgenic plants which need lot of money for development, but RNAi based transgenic crops couldn't be commercialized as expected. As an example, the U.S. Department of Agriculture, U.S. Food and Drug Administration, and U.S. Environmental Protection Agency approved the transgenic maize SmartStax Pro, against corn rootworm is not popularized as expected (Dalakouras et al., 2020).

The efficacy of RNAi depends on the method of RNA application. Chemically synthesized 22 nucleotides sRNAs attached green fluorescent protein which applied on *Nicotiana benthamiana* upon high-pressure spraying induces local and systemic RNAi. But petiole absorption, failed to do so. The petiole absorption and/or trunk injection was confined the dsRNAs to the xylem and apoplast whereas the high-pressure method delivers dsRNAs into plant cells (Dalakouras et al., 2016).

Roots can absorb dsRNA molecules and perform biological activities in plants. dsRNA prepared either silence the functions of shoot meristemless protein expressed in the shoot apical meristem and WEREWOLF transcription factor expressed in the root epidermal cells applied to Arabidopsis seedling roots could display phenotypic defects. Normal root growth was disturbed by application of dsRNAs targeting MOB1A and actin in Arabidopsis and rice roots (Dalakouras et al., 2020).

Since plant cell walls are tough cellulose-rich barrier for biomolecule delivery, RNA molecules are conjugated with carrier compounds like 3D tetrahedron, 1D hairpin tile, and 1D nanostring. However, these carrier compounds quite expensive and/or difficult to synthesize. If plant cell walls are mechanically damaged, carrier molecules are not necessary (Dalakouras et al., 2016, 2020).

Exogenously applied dsRNAs for controlling insects or fungi, should avoid plant DCL processing. Thus, applied dsRNAs will be absorbed by the insects or fungi and be processed by the pathogen/pest dicer proteins, resulting efficient biological activity

against insect or fungal genes. Since, no DCLs in chloroplast, the chloroplast-expressed dsRNAs are greatly uptake by pathogen/pest (Bally et al., 2016; 2018).

Major issue in topical application of dsRNAs is supplying fresh dsRNAs in time to time for sustain the crop protection as dsRNAs are degrading. In fungi, RdRp mediated self-amplification is not available for generation of secondary siRNAs generation contrast to plants. Degradation of dsRNAs under field conditions can be happened by ribonucleases and/or UV radiation. Incorporating nanotechnology with dsRNA deliver system can enhance protective and slow releasing ability of dsRNAs (Fletcher et al., 2020). dsRNA embedded nanoparticle delivery vectors for insect pest have been developed using linear homopolymers, branched polymers, guanidinium-modified polymers, conjugated polymer nanoparticles, dendritic nanocarriers and inorganic nanoparticles (Pugsley et al., 2021). Layered double hydroxide (LDH) is biocompatible and degrade under acidic conditions reducing persistence in the environment. It is also used in human therapeutics. LDH nanosheets coupled with dsRNA (BioClay) were investigated for spray application (Fletcher et al., 2020). Inorganic nanoparticles include clay nanosheets and polymer-coated inorganic nanoparticles while peptide-based nanoparticles are branched amphiphilic peptide capsules, cell-penetrating peptides. Also, chitosan nanoparticles and liposomal encapsulation were used in insect pest dsRNA stabilization (Pugsley et al., 2021).

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF FULL LENGTH DICER LIKE TRANSCRIPTS IN *Fusarium oxysporum* f. sp. *cubense* TROPICAL RACE 4

3.1 Introduction

RNAi is an intrinsic molecular mechanism of down regulating gene expressions in sequence specific manner at transcriptional or post-transcriptional level (Andika et al., 2015; Fukudome and Fukuhara, 2017; Zhang et al., 2017). It plays imperative role in biological processes associated with growth and development, responding biotic and abiotic stresses and defensing against viruses and transposons and ultimately genome stability (Fukudome and Fukuhara, 2017; Seta et al., 2017). Its gene expression down regulation ability can be used as a potential crop protection tool against harmful insects, pathogenic fungi and weeds (Kumaran et al., 2020; Wang et al., 2016; Zhang et al., 2017).

DCL is a multi-domain enzyme belonging to the ribonuclease III (RNase III) family (Siomi and Siomi, 2009). It helps to produce small RNAs from double stranded precursor molecules in RNAi pathway (Fukudome and Fukuhara, 2017; Seta et al., 2017). Thus, it is the key component which regulates the initial step of RNAi. DCL contains six domains namely N-terminal DExD/H-box helicase, a DUF283 domain, a PAZ domain, two RNase III domains, and a dsRNA-binding domain (De Jong et al., 2009; Gao et al., 2014; Meng et al., 2017). DCLs are important proteins for growth, development and pathogenicity in filamentous ascomycota fungi (Wang et al., 2016; Wang et al., 2018; Yin et al., 2020; Zeng et al., 2018). Genome wide sequence analysis has shown that DCLs are evolutionary conserved in a wide range of eukaryotes (Kadotani et al., 2004). The structure and number of DCLs are conserved among different ascomycete pathogens. However, the biological functions are different among species. Investigation of DCLs will be a foundation for exploration of the RNAi mechanism of fungi.

Foc TR4-DCL amino acid sequences which were deposited in UniProt data base are recorded as predicted proteins in *Foc* genome analysis (unreviewed) and they are partial transcript sequences. No studies have been done for characterizing their protein coding sequences. The objectives of this study were to identify the transcript sequences responsible for encoding of DCL1/2 proteins in *Foc* TR4 and predict and analyse homology, protein domains, *in silico* protein structures and phylogenetic evolution based on the amino acid sequences.

3.2 Materials and methods

3.2.1 Fungal culture

Foc TR4 culture preserved in Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia was used in this study. Fungal cultures were maintained in PDA and potato dextrose broth (PDB) media. PDA and PDB media were prepared by dissolving each 39 g (OXOID) and 24 g (OXOID) of media per 1 L of water respectively. Media were sterilized at 121˚C and 15 psi for 20 min in an autoclave (STURDY, SA-300H, Switzerland). Antibiotic of streptomycine sulphate was added to PDA at 0.02 g/L (Nacalai tesque) concentration after cooling about 40-45˚C. About 10 mL of PDA media was poured per petri plate, 90 mm × 15 mm in size while 50 mL of PDB was poured to 250 mL size conical flasks. Mycelial plugs of 5 mm in diameter were punched from actively growing culture edges using cork borer and transferred aseptically to petri dishes and conical flasks containing PDA and PDB (Dash et al., 2020; Teixeira et al., 2017). All cultures were incubated at 25-28˚C and 12 h lighting.

3.2.2 Identification of *Foc* TR4 using Koch's postulate

3.2.2.1 Preparation of fungal spore suspension

Foc TR4 liquid cultures were maintained at 25-28˚C and 12 h lighting in a growth chamber for five days (Al-Hetar et al., 2011). Sterilized distilled water was added and flooded the mycelia for releasing spores. Then conidia suspension was filtered through a double layer muslin cloth and the conidia density in the suspension was measured using a haemacytometer (Neubauer-Improved bright line hemacytometer, Marien feld Germany). The microconidia density was adjusted 1×10^6 conidia per mL (Al-Hetar et al., 2011). Few drops of Tween-20 were mixed with conidia suspension.

3.2.2.2 Pathogen inoculation and detection of symptoms in planta

Two months old tissue culture banana plants of cultivar Berangan were inoculated with fungal isolate following root dipping method. Uprooted banana plants were dipped in 10^6 spores/mL solution for 30 min and transferred to plastic pots filled with sterilized coir dust: vermiculite in 1: 0.5 ratio. After inoculation plants were maintained in a glasshouse at 28–35˚C, 75% relativity humidity and 16 h light (Widinugraheni et al., 2018). Another set of Berangan banana plants were maintained under similar conditions as untreated controls after dipping in sterilized distilled water instead of spore suspension. Rhizome and pseudostem samples were collected 35 days after inoculation and cut into two halves in vertical direction. External and internal disease symptoms were recorded.

3.2.3 Identification of *Foc* TR4 using morphological characteristics

Morphological and cultural characteristics of cultures grown in PDA were studied and compared with those mentioned in the published reports (Bechem and Afanga, 2018; Gaddeya et al., 2012; Leslie and Summerell, 2008; Maryani et al., 2019). Morphological features of spores and mycelia were observed using a compound microscope attached a digital camera. Slide mounted cultures stained with lactophenol cotton blue were used for microscopic view.

3.2.4 Molecular identification of *Foc* TR4

3.2.4.1 Extraction of fungal genomic DNA

PDB grown culture was filtered through a double layer of muslin cloth and washed repeatedly with sterile distilled water to remove excess salts adhering to it. Mycelia were kept on sterilized filter paper for drying inside the laminar flow for 15-20 min. The genomic DNA was extracted using Cetyl trimethylammonium bromide (CTAB) method according to Sahu et al., (2012) with slight modifications (Sahu et al., 2012).

Genomic DNA was extracted from 100 mg of air-dried mycelia. Air dried mycelia was ground into fine powder in liquid nitrogen using pre chilled mortar and pestle. The cells were lysed in 700 µL of lysis buffer (20 mM EDTA, 100 mM Tris-HCl, 1.5 M NaCl, 2% CTAB, at pH-8.0). Lysis buffer was preheated at 65˚C for 15 min before adding to cells. *β*-mercaptoethanol (20 µL) was added to sample and mixed thoroughly using a vortex (ZX3, Advanced vortex mixer - VELP Scientifica, Italy). The sample was incubated in a water bath at 65˚C for 40 min by inverting the centrifuge tube gently for mixing in every 10 min. The sample was kept to cool to room temperature and 700 µL of chloroform: Isoamyl alcohol (24:1 V/V) was added. Then the sample was mixed thoroughly. The mixture was centrifuged at 12,000 rpm for 10 min and supernatant was transferred into a new 1.5 mL microcentrifuge tube. Chloroform: Isoamyl alcohol (24:1 V/V) extraction was repeated and supernatant was decanted to new centrifuge tube.

DNA was precipitated with two third volume of chilled isopropanol following incubation for overnight at 4˚C. The sample was centrifuged at 9,000 rpm for 10 min and decanted supernatant. DNA pellet was washed two times with 1 mL of chilled 70% ethanol at 9,000 rpm for 10 min. Pellet was air dried at room temperature for 15 min. DNA pellet was then resuspended in 50 µL of nuclease free water (NFW) and stored in -20˚C until use (Aboul-Maaty and Oraby, 2019; Aitchitt et al., 1993).

3.2.4.2 DNA quantification and assessment of quality

DNA was quantified using a NanoDrop UV-Vis spectrophotometer (NanoDrop™ 2000C, Thermo Scientific, USA) while integrity was assessed on 1.5% (w/v) agarose gel using gel electrophoresis (Major science, Mini Pro 300, Taiwan). The DNA purity was

determined by 260/280 absorbance ratio of 1.8-2.0 and a single absorbance peak at 260 nm. The polysaccharide contamination was determined by 260/230 absorbance ratio of > 2.0. Integrity of DNA sample was assessed by DNA degradation smears or contamination on agarose gel by visualizing UV gel documentation imaging system (Gel Doc XR+ system - Bio rad, USA) (Healey et al., 2014; Sahu et al., 2012).

3.2.4.3 Optimization of primer annealing temperature

Foc TR4 specific primer pair which developed for single nucleotide polymorphisms in intergenic spacer region was amplified (*Foc* TR4-F: 5'-CACGTTTAAGGTGCCATGAGAG-3' and *Foc* TR4-R: 5'-CGCACGCCAGGACTGCCTCGTGA-3') a single 463 bp product (Dita et al., 2010). Gradient PCR was performed with an increasing annealing temperature (Ta) from 56°C to 67°C depending on the melting temperature of each primer (Eppendorf Mastercycler Pro, Vapo protect). The PCR conditions were set according to the protocol of ExTEN 2× mater mix (1st Base) provided by manufacturer's recommendations.

3.2.4.4 PCR, gel electrophoresis and sequence analysis of *Foc* TR4

The PCR reaction consisted with 12.5 µL of ExTEN 2× mater mix (1st Base), 0.3 µM of each primer, 100 ng of DNA template and 8.5 µL of NFW. The PCR cycling conditions consisted of initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR product was separated by gel electrophoresis on 1.5% agarose gel stained with Fluoro safe (1st Base) in Tris-acetate-EDTA buffer (1st Base). DNA loading dye (1st Base) and 100 bp ladder (1st Base) were used as an indicator of PCR product migration and molecular weight size maker respectively. Each well in agarose gel was set containing 1 µL of DNA loading dye (1st Base) and 5 µL of PCR product. PCR products were separated using gel electrophoresis (Major science, Mini Pro 300, Taiwan) and visualized the fluorescent band using UV gel documentation imaging system. The sequence was verified by Sangar sequencing in Apical Scientific Pvt Ltd, Malaysia. Sequence homology search was performed using Basic Local Alignment Search Tool (BLAST) in NCBI.

3.2.5 Preparation of cDNA for isolation of DCL transcripts

3.2.5.1 Total RNA extraction

Total RNA extraction using CTAB method was conducted according to Chang et al. (2016). *Foc* TR4 fungal mass (100 mg) was ground to fine powder in liquid nitrogen using pre chilled mortar and pestle and quickly transferred to 2 mL microcentrifuge tube. The cells were lysed in preheated (for 5 min at 65˚C) 700 µL of lysis buffer (20 mM EDTA, 100 mM Tris-HCl, 1.5 M NaCl, 2% CTAB, at pH-8.0). *β*-mercaptoethanol (20 µL) was added and thoroughly mixed using a vortex for 30 sec. The lysate was incubated in water bath at 65˚C for 40 min by inverting the centrifuged tube gently for mixing in

every 10 min and later kept the sample to cool to room temperature. All sample preparation steps were carried out on ice in following extraction steps. An equal volume (700 µL) of Chloroform: Isoamyl alcohol (24:1 V/V) was added and mixed vigorously in a vortex for 30 sec. Then mixture was centrifuged at 14,000 g and 4°C for 10 min. Upper most aqueous phase was transferred into a new centrifuge tube and Chloroform: Isoamyl alcohol (24:1 V/V) extraction was repeated. Then the upper phase was adjusted to 3 M LiCl for RNA precipitation. Sample was mixed gently by inverting and then incubated at -20°C for overnight. RNA was pelleted by centrifuging at 12,000 g for 20 min at 4°C and discarded supernatant. RNA pellet was washed two times with 1 mL chilled 70% ethanol for 10 min at 12,000 rpm. Pellet was air dried at room temperature for 10 min followed by dissolving in 30 µL of NFW and stored in -80˚C until use.

3.2.5.2 Removal of DNA contamination

DNA contamination in the total RNA sample was degraded with DNase 1 (Thermo scientific, Califonia, USA) treatment. Total RNA sample was incubated with 1 µg of DNA/RNA, 1 µl of 10× reaction buffer with $MgCl_2$, 1 µL of DNase I and NFW at 37°C for 30 min. Then the mixture was again incubated with1 µL of 50 mM EDTA at 65°C for 10 min. Sodium acetate (3M) and absolute ethanol were added to mixture in $1/10^{th}$ volume and 2.5 volume respectively after cooling to room temperature. Sample was incubated for overnight at -20°C and centrifuged at 13,500 rpm for 10 min at 4°C. Supernatant was decanted and washed the RNA pellet twice with chilled 70% ethanol for 10 min at 12,000 rpm. Finally, RNA was resuspended in 20 µL of NFW.

3.2.5.3 Assessment of RNA quantity and quality

RNA was quantified using a NanoDrop UV/Vis spectrophotometer (NanoDrop™ 2000C, Thermo Scientific, USA) while integrity was assessed on 1.0% (w/v) agarose gel using gel electrophoresis. The RNA purity was assessed by 260/280 absorbance ratio of 1.8-2.2 and a single absorbance peak at 260 nm while polysaccharide contamination was determined by 260/230 absorbance ratio of > 1.7. Integrity of RNA sample was assessed by 28S:18S rRNA ratio of 2:1 and no RNA degradation smears or contamination on agarose gel (Schroeder et al., 2006; Wieczorek et al., 2012).

3.2.5.4 First-Strand cDNA Synthesis

First strand of cDNA was synthesized using GoScript Reverse Transcription System (Promega, Madison, WI, USA) following the manufacturer's instructions. Total RNA up to 5 µg was incubated with 1 µL of Oligo (dT) in thermocycler (Biometra's T-personal thermal cycler, Germany) at 70°C for 5 min and immediately chilled in ice-water for 5 min. The Oligo (dT) adaptor primer was used as reverse transcription primer. The PCR tube with RNA reaction mix was centrifuged for 10 sec to collect the condensate (Sprout mini centrifuge) and placed on ice until the reverse transcription reaction mix was ready. Reverse transcription reaction mix was prepared on ice mixing NFW (6 µL), GoScript™ 5× reaction buffer (4 µL), $MgCl_2$ (2.5 µL), PCR Nucleotide Mix (1 µL), Recombinant RNasin® ribonuclease inhibitor (0.5 µL) and GoScript™

reverse transcriptase (1.0 µL) in given order and combined with RNA reaction mix. First strand cDNA synthesis conditions were consisted of annealing at 25°C for 5 min, synthesis of cDNA at 42°C for 60 min and inactivation the reverse transcriptase at 70°C for 15 min. The generated cDNA was stored at -20°C until use.

3.2.6 Sequence retrieval and primer designing

The amino acid sequences of DCL proteins of *Foc* TR4 were downloaded from UniProt database as query sequences. These sequences were *in silico* predicted proteins based on the genomic sequence of *Foc* TR4. The retrieved sequences were searched in tBLASTn in NCBI and top most three homology sequences were retrieved. The primers were designed to amplify retrieved DCL1/2 sequences based on conserved regions of selected partial mRNA sequences of uncharacterized/hypothetical proteins in different *Fo* species. Retrieved DCL1 sequences for primer designing were *Fusarium odoratissimum*: XM_031203178 (99.89%); *Fusarium oxysporum* f. sp. *lycopersici*: XM_018388129 (97.98%); *Fusarium oxysporum*: XM_031188969 (97.84%). DCL2 sequences used for primer designing were *Fusarium odoratissimum*: XM_031202747 (99.52%); *Fusarium oxysporum* f. sp. *lycopersici*: XM_018393845 (98.27%); *Fusarium oxysporum*: XM_031189728 (98.14%). The primers were designed using Primer3 software (http://primer3.ut.ee).

3.2.7 Confirmation of retrieved partial DCL sequences

3.2.7.1 PCR amplification

The PCR amplifications of DCL1/2 were performed in PCR reactions containing 12.5 µL of high fidelity master mix, 0.3 µM of each primer, 2 µL of cDNA template (500 ng of total RNA) and 8.5 µL of NFW. PCR amplification carried out as initial denaturation at 98°C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec, and final extension at 72°C for 2 min (Biometra's T-personal Thermal cycler). The cDNA amplicons of DCL1/2 in *Foc* TR4 were separated on 1.5% agarose gel.

3.2.7.2 Gel extraction of PCR products

Desired DCL1/2 amplicons were purified from agarose gel using GeneJET gel extraction kit according to the manufacturer's instructions. PCR amplicons with expected sizes were excised from agorose gel using a scalpel under visualizing UV light. Gel slices were weighted and one volume of binding buffer was added to one volume of gel. The mixture was incubated at 55°C for 10 min while inverting the tube in every few min until gel slices completely dissolved. The mixture was briefly mixed using a vortex and poured on the purification column. The column was centrifuged at 13,000 rpm for 1 min. The flow through was then discarded. The purification column was washed twice with 700 µL of wash buffer followed by centrifugation at 13,000 rpm for 1 min. Residual buffer was removed by additional centrifugation at 13,000 rpm for 1 min. DNA fragment

was eluted to 1.5 mL collection tube after adding 30 µL of elution buffer to centre of purification column followed by centrifugation at 13,000 rpm for 1 min. The purified DNA fragment was stored at -20°C until use.

3.2.7.3 Cloning of PCR products

Gel extracted PCR products were ligated to p.JET1.2 vector using GeneJET PCR cloning kit following manufacture's protocol (Thermo scientific). Blunt-end ligation reaction was set up on ice combining 2× Reaction Buffer (10 µL), purified PCR product (2 µL), pJET1.2/blunt Cloning Vector (1 µL), T4 DNA Ligase (1 µL) and NFW up to 20 µL. Ligation mixture was briefly vortexed and centrifuged for 3-5 sec. Then ligation mixture was incubated at 22°C for 5 min. The transformation was done using DH5α competent cells. According to protocol, 50 µL of competent cells and 3 µL of ligation reaction mixture were added to 1.5 mL centrifuge tube and flicked 4-5 times. Mixture was then placed on ice for 30 min. The cells were subjected to heat shock keeping at 42°C for exactly 30 sec. Cells were transferred to ice for 2 min. Pre-warmed (37˚C) LB broth media was added to transformed cells up to 250 µL. Then cells were incubated at 37˚C for 1 hr by shaking at 225 rpm. After that, transformed cells were diluted to1:5 with LB broth and spread on LB agar plates containing ampicillin (50 µg/mL). Following day, half of single whitish colony was used for PCR screening while remaining half was used for streaking on LB agar plates.

3.2.7.4 Colony PCR for verification of positive transformants

PCR reaction mixture was prepared adding 12.5 µL of high fidelity master mix, 0.3 µM of gene specific forward and reverse primers and 10.0 µL of NFW. Half of the whitish colony from plasmid-streaked plate was picked using toothpick and dipped in PCR mixture aseptically. PCR amplification carried out as initial denaturation at 98˚C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec, and final extension at 72°C for 2 min. The cDNA amplicons of DCL1/2 in *Foc* TR4 were separated on 1.5% agarose gel.

3.2.7.5 Multiplication of positive transformants and isolation of plasmid DNA

Colony that had been streaked to singles and PCR confirmed was picked and inoculated to 5 mL of LB broth mixed with ampicillin (50 µg/mL). Then liquid cultures were kept in a shaker at 37˚C while shaking at 225 rpm for 18-20 h. Bacterial cultures were pipetted to 1.5 mL centrifuged tubes and pelleted at 13,000 rpm for 5 min. Then pellets were subjected to plasmid extraction using Miniprep (Thermoscientific) plasmid extraction kit following manufacturer protocol.

Pelleted cells were resuspended in 250 µL of resuspension solution until completely mixed without cell clumps remaining by pipetting up and down. Lysis Solution (250 µL) was added and mixed by inverting the tube 4-6 times until becoming viscous and slightly clear. Then the neutralization solution (350 µL) was added and mixed quickly and

thoroughly by inverting the tube 4-6 times. The cell debris and chromosomal DNA were pelleted by centrifuging at 13,500 rpm for 5 min. The supernatant was transferred to the GeneJET spin column.

The spin column was centrifuged at 13,500 rpm for 1 min and discarded the flow-through. Isolated plasmid in GeneJET spin column was washed two times with wash solution (500 µL) followed centrifugation at 13,500 rpm for 1 min. Flow-through was discarded and centrifuged the spin column for additional 1 min at 13,500 rpm to remove residual wash solution. The GeneJET spin column was transferred to new 1.5 mL microcentrifuge tube and added 50 µL of the elution buffer to the center of GeneJET spin column membrane. The plasmid DNA sample was incubated for 2 min at room temperature and centrifuged for 2 min at 13,500 rpm. Then, purified plasmid DNA was sent for sequencing.

3.2.8 5' and 3' rapid amplification of cDNA ends (RACE) PCR

3.2.8.1 Primer designing and RACE PCR

The retrieved incomplete 5' and 3' ends of DCL sequences were determined using RACE PCR (SMARTer®, Takara Bio USA, Inc) following manufacturer protocol. Inner and outer gene specific primers (GSPs) were designed to amplify 5' and 3' ends of identified partial transcript sequences. GSPs were between 23 to 28 bp in length with GC content of 50-70% and melting temperature ≥ 65°C. Also, they were not complementary to 3' end of the universal primer mix and have 15 bp overlap region (GATTACGCCAAGCTT) with the pUC19 vector at their 5' ends. The primer specificity was ensured using NCBI BLAST tool. In first strand cDNA synthesis, RACE Ready 5' and 3' cDNA mixes were prepared in separate reaction tubes using 5'-CDS primer A and 3' CDS primer A respectively. cDNA mix of 5' and 3' were incubated at 72°C for 3 min followed at 42°C for 2 min. The master mix was then prepared mixing buffer mix (5× first strand buffer, Dithiothreitol, dNTPs), RNase inhibitor and SMART Scribe reverse transcriptase and cDNA mix. After that, first strand cDNA was synthesized incubating the mixture at 42°C for 90 min followed at 70°C for 10 min. Prepared cDNA was diluted in 10 µL of Tricine-EDTA buffer and stored in -20°C until use.

The 5' and 3' end amplifications were performed using nested PCR approach to reduce non-specific amplification. In the first round PCR of both 5' and 3' ends amplifications, the universal primer mix supplied in the RACR kit and outer GSPs were used as primers. Conditions for first round PCR in 5' ends were: 94°C for 3 min, 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min and final extension at 72°C for 10 min. First round PCR products were diluted to 1:5 for 5' end and 1: 20 for 3' end with Tricine-EDTA buffer and used as templates for second round PCRs. In second round PCR amplifications, the universal primer short supplied in kit and inner GSPs were used as primers. The same conditions of first round PCR except 35 cycles were used for the second round PCR in 5' end amplification. On the other hand, conditions for 3' end first round PCR were: 94°C for 3 min, 25 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C

for 1 min and final extension at 72°C for 10 min. The same conditions except 35 cycles and Ta 68°C were used for the second round PCR in 3' end.

3.2.8.2 Gel extraction of RACE PCR products

RACE PCR products were separated on 1.5% agarose gel using gel electrophoresis. The 5' and 3' RACE PCR amplicons were purified from agarose gel using GeneJET gel extraction kit according to the manufacturer's instructions.

3.2.8.3 Cloning of RACE PCR products

PCR products were ligated into the pUC19 vector provided in the using In-Fusion HD cloning kit by following manufacturer instructions. Gel purified RACE product and linearized pUC19 vector in 1:3 ratio, In-Fusion HD master mix and NFW in 10 µL of total reaction volume were combined. The reaction mixture was incubated at 50°C for 15 min and transferred to ice.

The transformation was performed using stellar competent cells given in the Takara RACE kit. According to protocol, 50 µL of competent cells and 2.5 µL of In-Fusion reaction mixture were added to 1.5 mL centrifuge tube and placed on ice for 30 min. Then the cells were subjected to heat shock keeping at 42°C for exactly 60 sec. Pre-warmed (37°C) Super optimal broth with catabolite repression (SOC) media was added to transformed cells up to 500 µL. The cells were then incubated at 37°C for 1 hr keeping in a shaker at 225 rpm. After that, 1:5 SOC media diluted transformed cells were spread on LB agar plates containing Ampicillin (100 µg/mL) and X gal. Following day, half of single whitish colony was used for PCR screening while remaining half was streaked on LB agar plates for plasmid isolation. Randomly selected three clones from each 5' and 3' RACE PCR were chosen for isolation of plasmid DNA. Colony PCR was performed using inner GSPs and universal primer short.

3.2.8.4 Plasmid DNA purification

Single colony was picked from plasmid bacteria *E. coli*-streaked plate and inoculated to 5 mL of LB broth mixed with ampicillin (100 µg/mL) aseptically. Then liquid media samples were kept in a shaker at 37°C while shaking at 225 rpm for 12-16 h and subjected to plasmid extraction using Miniprep plasmid extraction kit (Thermoscientific) following manufacturer protocol. Then, purified plasmid DNA was sent for sangar sequencing.

3.2.9 Isolation of full length transcripts

The full length coding sequences of DCL1/2 transcripts were cloned to single fragments for sequence analysis. GSPs and nested gene specific primers (NGSPs) were designed

based on the 5' and 3' UTR sequences. The PCR amplifications were performed in PCR reactions containing 500 ng of total RNA included cDNA, 25.0 µL of high fidelity mater mix, 0.3 µM of forward and reverse primers and 17.0 µL of NFW in 50 µL total reaction volume. Nested PCR amplification was performed in same total volume with 20 times diluted PCR products and NGSPs. Both PCR amplifications of each transcript were performed as initial denaturation at 98˚C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec, and final extension at 72˚C for 2 min (Biometra's T-personal Thermal cycler). The PCR products with expected sizes were analyzed on 1.5% agarose using gel electrophoresis. Desired DCL1/2 amplicons were excised using scalpel blade from agarose gel and purified using GeneJET gel extraction kit. The purified PCR products were ligated to p.JET1.2 vector using GeneJET PCR cloning kit and cloned into DH5α competent cells. Plasmid DNA was isolated using Miniprep (Thermoscientific) plasmid extraction kit. The digested DNA products were verified by gel electrophoresis and sent for sangar sequencing.

3.2.10 Analysis of sequences

Vector sequences were excluded from plasmid sequences before analyzing DCL1/2 transcripts. Nucleotides were verified from chromatogram using sequence scanner 2 software and ambiguous nucleotides were replaced. The pairwise alignment was performed using Bioedit 7.2 to align forward and reverse sequences to get consensus sequences. The putative identity of the edited sequences was assessed using BlastX in NCBI. The partial sequences and 3' and 5' UTRs were joined to get full lengths of DCL1/2 using contig assembly program in Bioedit 7.2.

Amino acid sequences were deduced from the complete coding transcript sequences of DCLs using ExPaSy translate tool (https://web.expasy.org/translate/). BLASTp was performed for the deduced amino acid sequences at Gene bank in NCBI, in order to compare DCL1/2 in *Foc* TR4 among different closely related homologs (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequences were analyzed for similarity with the retrieved sequences in the GenBank database. The protein domain features of DCLs were predicted using SMART programme (http://smart.embl-heidelberg.de/) and visualized with Illustrator for biological sequences (http://ibs.biocuckoo.org/online.php). Molecular weight and isoelectric point of the proteins were predicted using ProtParam program (https://web.expasy.org/protparam/).

3.2.11 Secondary and tertiary structure Modelling

The secondary structures of DCL protein sequences were generated using PSIPRED programe (http://bioinf.cs.ucl.ac.uk). Further, modelling the 3D structures of DCL proteins were executed using Phyre2 structure prediction server in the normal mode analysis (http://www.sbg.bio.ic.ac.uk). Derived PDB templates from Phyre2 were visualized using EzMOL (http://www.sbg.bio.ic.ac.uk/ezmol/).

3.2.12 Phylogenetic tree analysis

Phylogenetic trees were constructed to study evolutionary relationship between *Foc* TR4 DCL1 and DCL2 deduced amino acid sequences with DCL1/2 amino acid sequences from other plant pathogenic ascomycete fungi. A total 24 species of DCL1 and 21 species of DCL2 from Ascomycota fungi were retrieved from NCBI to construct phylogenetic trees. Retrieved organisms were listed in Table 3.1 and 3.2 Multiple sequence alignments were executed using the MUSCLE programme for selected sequences. The neighbour-joining trees were constructed using MEGA X and its nodes reliability was tested with 1000 bootstrap replicates.

Table 3.1 : Species used for phylogenetic analysis of DCL1 protein

Accession number	Species
ENH70439	*Foc* race 1
EXM26363	*Fusarium oxysporum* f. sp. *vasinfectum*
EXL73917	*Fusarium oxysporum* f. sp. *conglutinans* race 2
XP 018246138	*Fusarium oxysporum* f. sp. *lycopersici*
EXK37014	*Fusarium oxysporum* f. sp. *melonis*
EMT69905	*Fusarium odoratissimum*
CVL00677	*Fusarium mangiferae*
XP 028491576	*V. nonalfalfae*
KAF4487078	*Colletotrichum fructicola*
KAF3806497	*C. gloeosporioides*
Q7S8J7	*N. crassa*
A4RKC3	*Pyricularia oryzae*
KLU86274	*Magnaporthiopsis poae*
RKF76912	*Golovinomyces cichoracearum*
PBP21904	*Diplocarpon rosae*
EMR90866	*B. cinerea*
PSK38057	*Elsinoe australis*
XP 023449244	*Cercospora beticola*
KAB2581198	*Lasiodiplodia theobromae*
RYN75217	*Alternaria alternata*
QIA50577	*P. italicum*
Q2U6C4	*Aspergillus oryzae*
A2RAF3	*Aspergillus niger*
KAA1139065	*Puccinia graminis* f. sp. *tritici*
MW367432	*Foc* TR4

Table 3.2 : Species used for phylogenetic analysis of DCL2 protein

Accession number	Species
EXA37371	*Fusarium oxysporum* f. sp. *pisi*
ENH74054	*Foc race 1*
EXM25129	*Fusarium oxysporum* f. sp. *vasinfectum*
XP 018253198	*Fusarium oxysporum* f. sp. *lycopersici*
RYC87782	*Fusarium oxysporum* f. sp. *narcissi*
EXK34908	*Fusarium oxysporum* f. sp. *melonis*
EMT62501	*Fusarium odoratissimum*
QGI68953	*Fusarium fujikuroi*
KAF4441946	*Fusarium acutatum*
KAF3799482	*C. gloeosporioides*
KAF4418260	*Colletotrichum fructicola*
EAA34302	*N. crassa*
XP 003715365	*Pyricularia oryzae*
EMR89149	*B. cinerea*
RKF75262	*Golovinomyces cichoracearum*
XP 023460000	*Cercospora beticola*
RYN73636	*Alternaria alternata*
KAB2572547	*Lasiodiplodia theobromae*
A2QX45	*Aspergillus niger*
KDE75911	*Aspergillus oryzae*
TKA57964	*Rhodotorula* sp.
MW344087	*Foc* TR4

3.3 Results

3.3.1 Identification of *Foc* TR4 isolate

3.3.1.1 Morphological characters

Isolate of *Foc* TR4 developed cultural and morphological traits typical of those described for *Fo* and *Foc* TR4 (Bechem and Afanga, 2018; Gaddeya et al., 2012; Leslie and Summerell, 2008; Maryani et al., 2019). White colour cottony growth appearance with filamentous margin was observed on PDA media. Underside of colony was observed orange pinkish (pale orange) colour (Figure 3.1). Aseptate oval to ellipsoid shape microconidia was abundant on PDA. Chlamydospores were globes in shape and formed singly. Septate mycelia and branched conidiophores were observed (Figure 3.2).

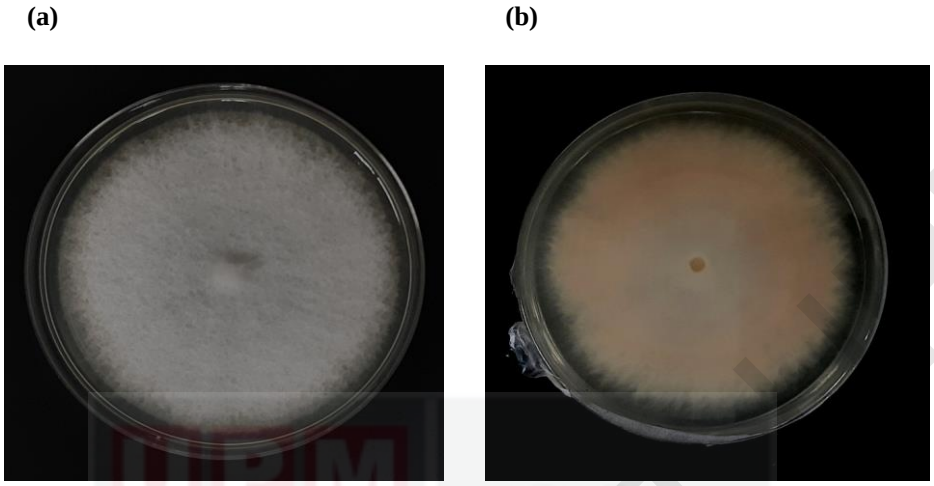

Figure 3.1 : *Foc* TR4 colony on PDA media (a) Front view of culture plate (b) Back view of culture plate

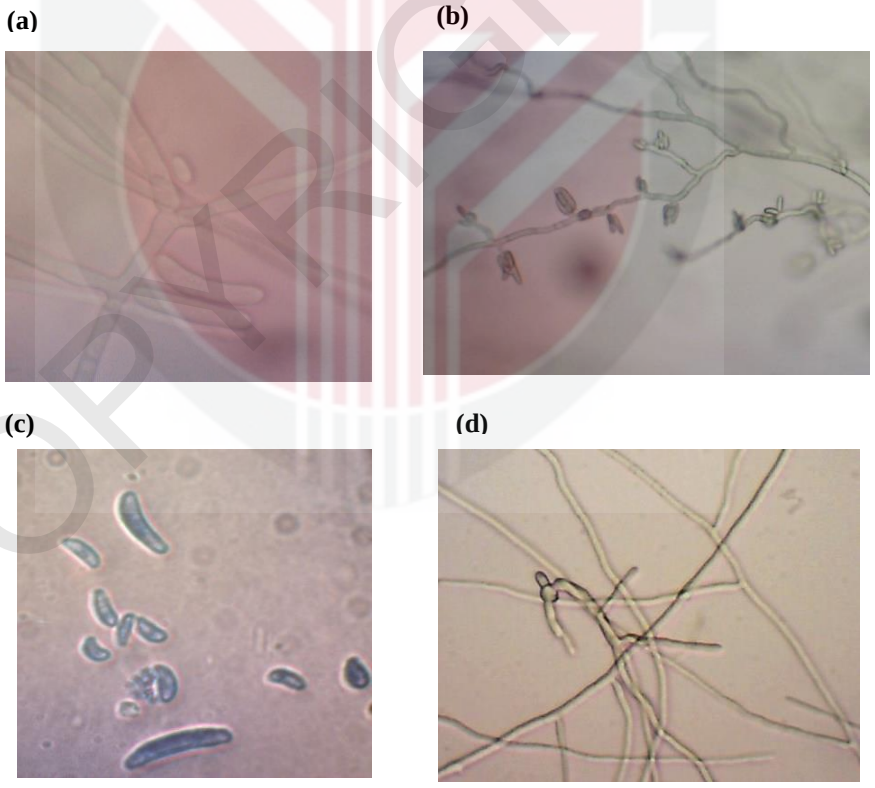

Figure 3.2 : Microscopic view of *Foc* TR4 morphological characters (a) Mycelia (b) Conidiophores (c) Microconidia (d) Chlamydospore

3.3.1.2 Pathogenicity test

Foc TR4 caused disease symptoms typical of fusarium wilt on banana plantlets. Inoculated plants showed external disease symptoms of yellowing and initial leaf marginal drying at 21 days after inoculation. Yellowing of lower leaves had been increased thereafter. Also plant internal symptom of reddish brown discolouration of rhizome was observed. No symptoms were appeared in non-inoculated control plants (Figure 3.3) (Dong et al., 2012; Moore et al., 1995). *Foc* TR4 isolate was successfully recovered from rhizome showing disease symptoms on PDA medium.

Figure 3.3 : Pathogenicity assay of *Foc* TR4 according to Koch's postulate (a) - (e) plant external symptoms; (f) - (j) plant internal symptoms at 7, 14, 21, 28, and 35 days after *Foc* TR4 inoculation respectively

3.3.1.3 Molecular confirmation of *Foc* TR4

The purities of genomic DNA samples measured at 260/280 and 260/230 absorbance were ranged from 1.8 - 2.0 and >2.0 respectively. A high molecular mass band with minimum shearing was observed for DNA. The conditions of the PCR were carefully standardized and all the parameters were established. The optimum Ta was found to be 57°C. The PCR amplification using genomic DNA (50 ng) extracted from fungal mycelia generated a 463 bp fragment (Figure 3.4). The isolate was diagnosed by comparing with the copies at the gene bank, NCBI. It showed 100% identity with isolation MN830364.1. The query cover of the sequence was 100% with error value 0.0. The amplified sequence was deposited in NCBI under the accession number MW122510 (Appendix A).

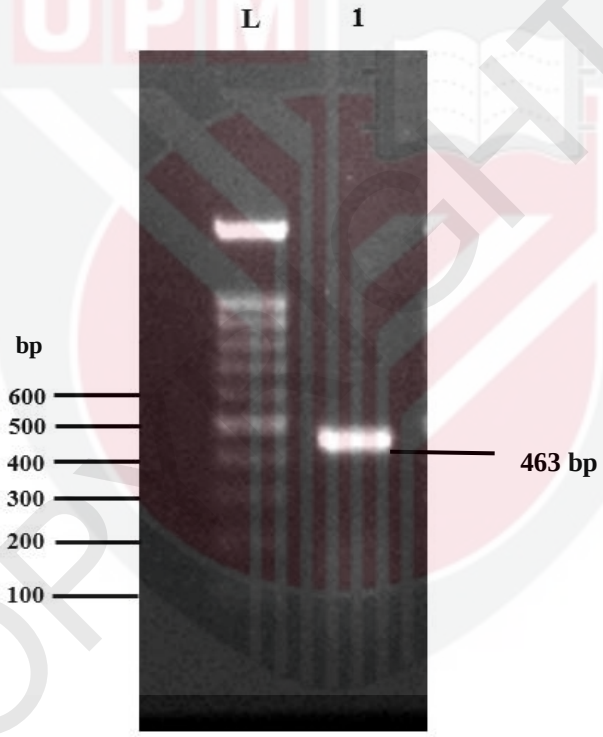

Figure 3.4 : Amplification of PCR product using DNA from pure culture of *Foc* TR4. Run on 1.5% agarose gel. L- 100 bp DNA ladder, 1- PCR product

3.3.2 Quality of RNA samples

The purities of RNA samples measured at 260/280 and 260/230 absorbance were ranged from 1.8 to 2.0. The integrity of RNA samples was confirmed by visualizing clear 28 S and 18 S bands with 2:1 ration intensity on 1% agarose gel (Figure 3.5).

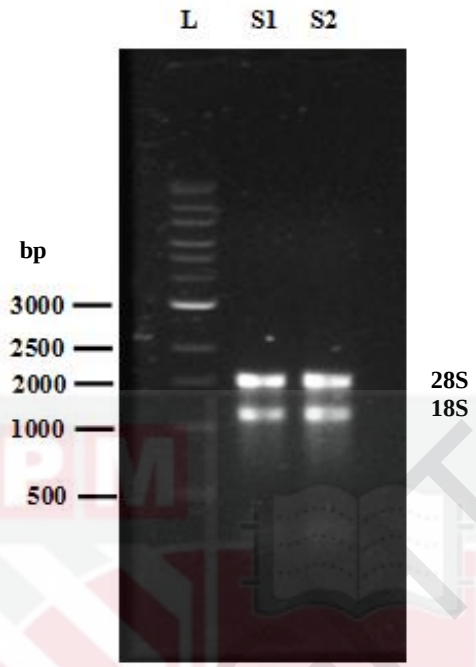

Figure 3.5 : Total RNA bands on the 1% agarose gel. L: ladder, S1 and S2: RNA extracted from pure culture of *Foc* TR4

3.3.3 Primer distribution

The primer distribution along *Foc* TR4-DCL1 and *Foc* TR4-DCL2 partial transcript sequences with overlapping regions between adjacent amplicons were shown in figure 3.6 and primer sequences were mentioned in Table 3.1.

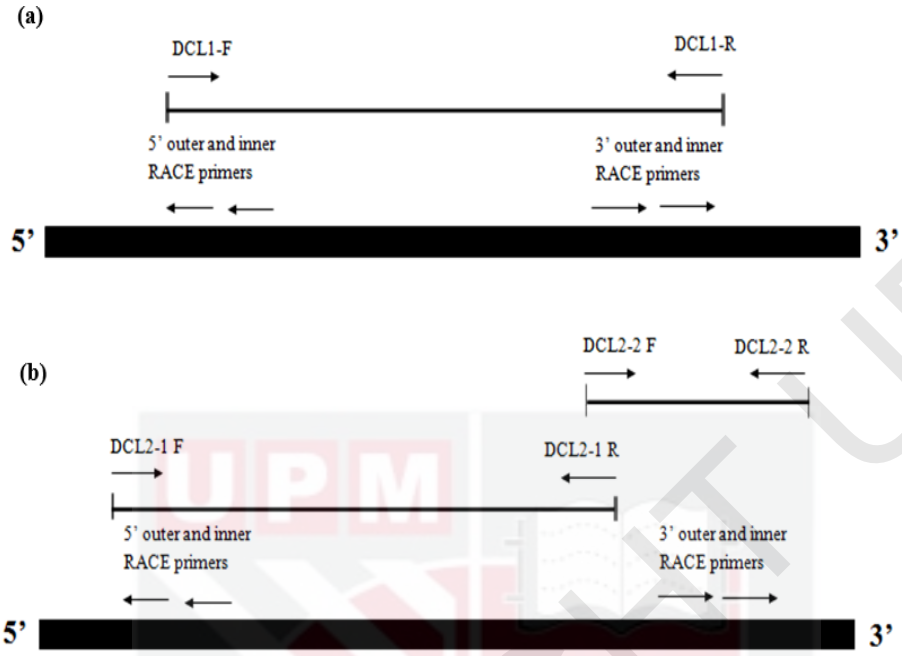

Figure 3.6 : Schematic diagram of distribution of primers and identified partial sequences along DCL transcripts (a) *Foc* TR4-DCL1 (b) *Foc* TR4-DCL2, Black lines- partial sequences, Arrows- primers

3.3.4 Analysis of partial transcript sequences and 5' and 3' cDNA ends

The designed primers were able to generate a PCR product of the expected size. The partial sequence of *Foc* TR4-DCL1 consisted with 4003 bp (Figure 3.7a) length whereas *Foc* TR4-DCL2 was identified in 4036 bp (Figure 3.7b) and 600 bp (Figure 3.7c) sizes. The isolation of 5' and 3' ends of DCL1 and DCL2 (DCL1/2) were achieved through nested 5' and 3' RACE PCR. The DCL1 5' nested RACE PCR produced a single band around 300 bp (Figure 3.8a) while DCL2-5' nested RACE PCR generated multiple bands of approximately 650 bp (Figure 3.8b). Multiple bands were due to non-specific primer binding. DCL1/2 3' nested RACE PCRs formed single bands around 550 bp (Figure 3.8c) and 850 bp (Figure 3.8d) respectively. The specificity of the amplified PCR product was determined by sequence analysis. 5' end of DCL1/2 had 63 bp and 162 bp 5' UTRs at the upstream of the predicted initiation codon (ATG) respectively. 3' ends of DCL1/2 consisted with 110 bp and 595 bp 3' UTRs at the downstream of the translation stop codon (TGA) respectively. DCL1 consisted with 73 bp poly A tail while DCL2 had 26 bp poly A tail.

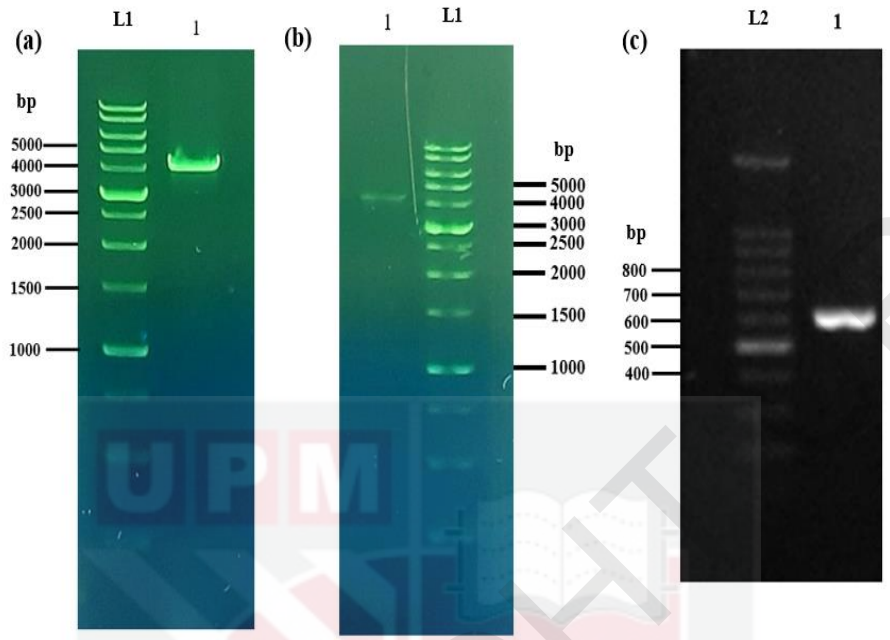

Figure 3.7 : PCR products of NCBI retrieved partial sequences. PCR products were run on 1.5% TAE agarose gel. (a) *Foc* TR4-DCL1 (b) (c) *Foc* TR4-DCL2, 1- PCR products, L1- 1 kb DNA ladder, L2- 100 bp DNA ladder

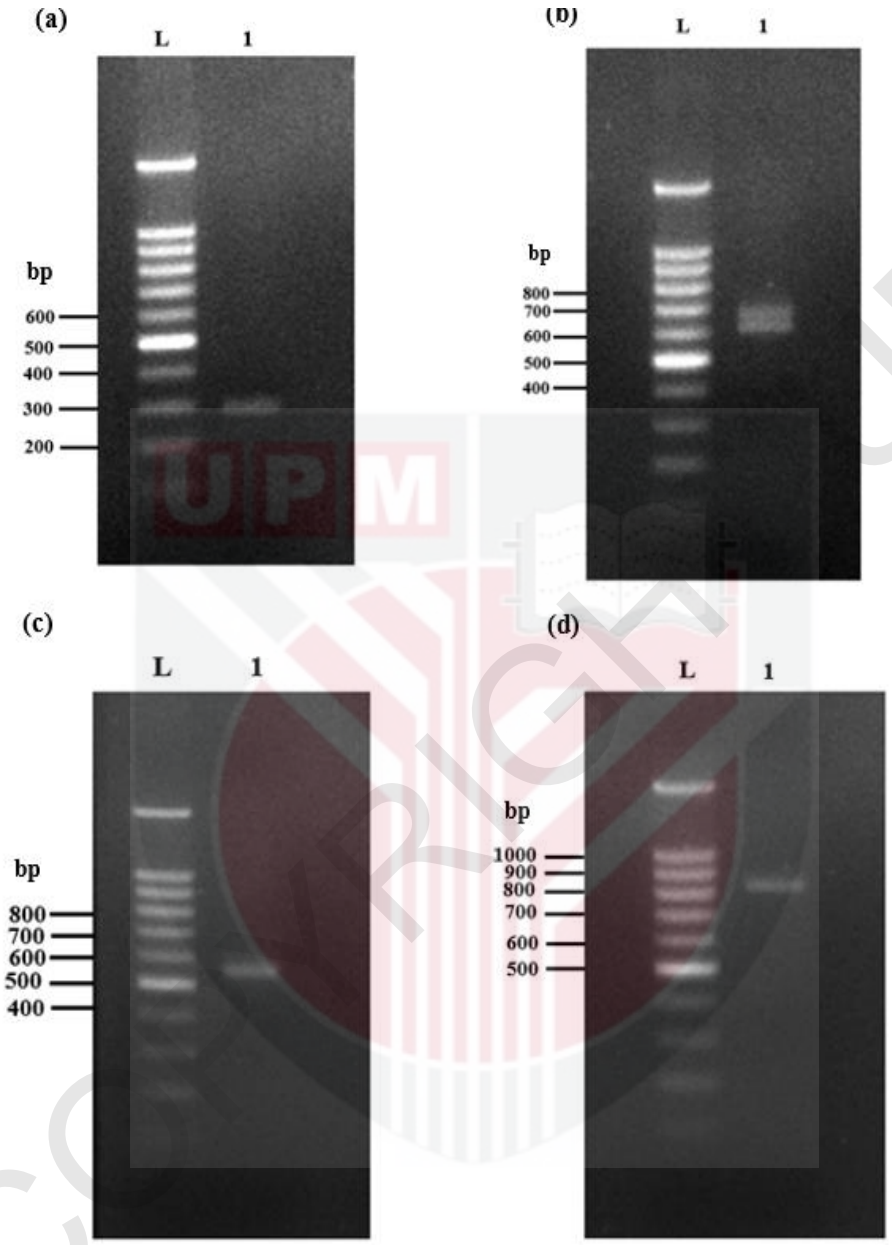

Figure 3.8 : RACE PCR products of 5' and 3' ends. PCR products were run on 1.5% TAE agarose gel. (a) 5' end of *Foc* TR4-DCL1 (b) 5' end of *Foc* TR4-DCL2 (c) 3' end of *Foc* TR4-DCL1 (d) 3' end of *Foc* TR4-DCL2, 1- PCR products, L- 100 bp DNA ladder

3.3.5 Analysis of full length transcript sequences

Full length transcripts encoding DCL1 and DCL2 proteins were isolated using PCR amplification. Isolated full length sequences were 4622 bp and 4430 bp in DCL1/2 respectively (Figure 3.3). The used primers to amplify ORFs of both genes were listed in Table 3.1 The cDNA amplicon of *Foc* TR4-DCL1 consisted with nucleotide sequences of 4749 bp whereas *Foc* TR4-DCL2 had 5106 bp. The longest OFRs of DCL1 and DCL2 were 4503 bp and 4323 bp respectively. Of these sequences, *in silico* translation of cDNA sequences suggested that *Foc* TR4-DCL1 had 1501 a.a whereas *Foc* TR4-DCL2 had 1441 a.a (Figure 3.10 and 3.11).

The deduced a.a. sequences showed high degree of similarity to other fusarium DCL orthologues. DCL1 was displayed high identity with XP_031066757.1, uncharacterized protein *Fusarium odoratissimum* (99.93% identity and E-value: 0.0) whereas DCL2 showed high similarity with XP_031068194.1, uncharacterized protein *Fusarium odoratissimum* (100% identity and E-value: 0.0).

The obtained transcript sequences and deduced amino acid sequences were deposited in the NCBI GenBank in accession numbers of MW367432 (*Foc* TR4-DCL1) and MW344087 (*Foc* TR4-DCL2). These two DCL genes shared nucleotide and amino acid sequence identities of 58.66% and 27.11% respectively. The predicted molecular weight and PI of *Foc* TR4-DCL1 were 170.04 kDa and 6.57 whereas *Foc* TR4-DCL2 were 163.26 kDa and 5.98 respectively.

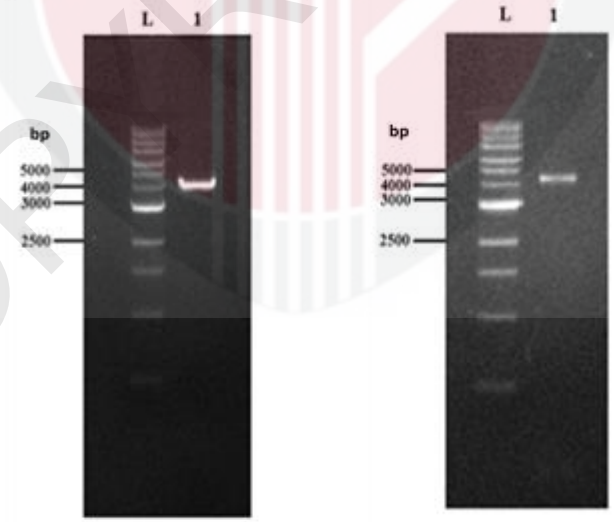

Figure 3.9 : PCR products of full length transcripts. PCR products were run on 1.5% TAE agarose gel. (a) *Foc* TR4-DCL1 (b) *Foc* TR4-DCL2, 1- PCR products, L- 100 bp DNA ladder

Table 3.3 : List of transcript and RACE gene specific primers

Primer name	Primer sequence	Transcript length (bp)
DCL1-F-long	AGACACAGAGAGATGCAGCA	4003
DCL1-R-long	TTGGAGAGGAACGTGACTGG	
DCL2-F-long	ACCTCGACACCATCACCAAT	4036
DCL2-R-long	CCTCTTTGGGATGCTGAACG	
DCL2-2-2-F	ACACGTTCAGCATCCCAAAG	627
DCL2-2-2-R	TCGTCTGAGAACCCGATCAA	
DCL1-5'-outer	TTGTGAATCCGAGCGACAAC	-
DCL1-5'-inner	GATTACGCCAAGCTTGGCTCTCGTTCTGGTTCATG	-
DCL2-5'-outer	CCCGATGAGTGGTTGGAGTA	-
DCL2-5'-inner	GATTACGCCAAGCTTGTAAGCAGTCTCATGGGCAC	-
DCL1-3'-outer	ACACGTTCAGCATCCCAAAG	-
DCL1-3'-inner	GATTACGCCAAGCTTGAACAAGCACCCAGTCACGTT CCTCTCC	-
DCL2-3'-outer	GGCCAATGTCATGTTAGAAACCGAGGAG	-
DCL2-3'-inner	GATTACGCCAAGCTTTGGAGAGCGGTTGGTAGGAGA AGTCG	-
DCL1-Full-F	TTGATCGCTCTCTCAATGC	4620
DCL1-Full-R	TGAAGCGCTTTGTGTCTCTG	
DCL2-Full-F	CGCAAACCTTCTTTCGTTCT	4430
DCL2-Full-F	CGCATTCATCTCCTCTGTCA	

1								TTGATCGCTCTCTCAATGCAAATGTATCTTCATCCTGAGCTTCATCCTGAGCAGTATACTAATCCACGCTCCAAC													
76		ATG	TCG	GAT	CAC	GAG	TAC	ATC	GAC	ACT	GAT	GAC	GAA	GAC	GAT	CGA	TAT	CGC	CTC	ATC	
1		**M**	**S**	**D**	**H**	**E**	**Y**	**I**	**D**	**T**	**D**	**D**	**E**	**D**	**D**	**R**	**Y**	**R**	**L**	**I**	
136	GTG	AAC	CCG	TCG	AAG	CCT	CGA	AAG	ATC	ACG	GAA	AAG	AAG	CTC	CGA	GAC	CAA	GCT	GCT	CTA	
20	**V**	**N**	**P**	**S**	**K**	**P**	**R**	**K**	**I**	**T**	**E**	**K**	**K**	**L**	**R**	**D**	**Q**	**A**	**A**	**L**	
196	CAA	GCG	CAC	ATA	GAA	AAG	ACA	CAG	AGA	GAT	GCA	GCA	AGG	AGT	GCC	GCT	CCT	TTC	AGC	GAT	
40	**Q**	**A**	**H**	**I**	**E**	**K**	**T**	**Q**	**R**	**D**	**A**	**A**	**R**	**S**	**A**	**A**	**P**	**F**	**S**	**D**	
256	CCT	AGT	GAA	CAG	TCC	CTC	GCC	CAG	CTC	ATG	AAC	CAG	AAC	GAG	AGC	CAC	AAG	ATC	ATC	GCC	
60	**P**	**S**	**E**	**Q**	**S**	**L**	**A**	**Q**	**L**	**M**	**N**	**Q**	**N**	**E**	**S**	**H**	**K**	**I**	**I**	**A**	
316	TCG	CCT	CGA	GAA	TAC	CAG	ATT	GAA	CTG	TTC	GAG	CGC	GCT	AAA	GAG	AGA	AAC	CTC	CTT	GTG	
80	**S**	**P**	**R**	**E**	**Y**	**Q**	**I**	**E**	**L**	**F**	**E**	**R**	**A**	**K**	**E**	**R**	**N**	**L**	**L**	**V**	
376	GTG	TTG	CCT	ACA	GGT	ACC	GGC	AAA	ACA	CTC	ATC	TCC	GCC	TTG	CTG	CTA	CGA	CAT	CAC	CTC	
100	**V**	**L**	**P**	**T**	**G**	**T**	**G**	**K**	**T**	**L**	**I**	**S**	**A**	**L**	**L**	**L**	**R**	**H**	**H**	**L**	
436	GAA	CAG	GAG	CTT	GAA	GCT	CGA	GCC	ATC	GGA	AAA	CTC	AAG	AAG	GTT	GCC	TTC	TTT	CTG	GTA	
120	**E**	**Q**	**E**	**L**	**E**	**A**	**R**	**A**	**I**	**G**	**K**	**L**	**K**	**K**	**V**	**A**	**F**	**F**	**L**	**V**	
496	GAA	AAG	GTC	GCC	CTC	TGT	GTT	CAG	CAA	CAT	GCT	GTC	CTC	AGC	TGC	AAC	CTT	GGA	GCC	CAC	
140	**E**	**K**	**V**	**A**	**L**	**C**	**V**	**Q**	**Q**	**H**	**A**	**V**	**L**	**S**	**C**	**N**	**L**	**G**	**A**	**H**	
556	CCA	GTT	ACC	AAG	TTC	GTG	GGC	CAT	ATG	AAA	GGA	ATG	ACA	AAG	ACT	AAA	GAG	TAC	TGG	GAT	
160	**P**	**V**	**T**	**K**	**F**	**V**	**G**	**H**	**M**	**K**	**G**	**M**	**T**	**K**	**T**	**K**	**E**	**Y**	**W**	**D**	
616	GAG	AAG	TTT	GGC	GAA	AAC	ATG	GTT	GTG	GTC	TGC	ACT	GCT	CAG	ATT	CTC	CTC	GAC	TGC	CTC	
180	**E**	**K**	**F**	**G**	**E**	**N**	**M**	**V**	**V**	**V**	**C**	**T**	**A**	**Q**	**I**	**L**	**L**	**D**	**C**	**L**	
676	ACT	TGT	GGC	TTC	ATC	ACC	ATG	TCC	CGG	ATA	AAT	CTT	CTC	ATA	TTC	GAC	GAG	GCA	CAC	CAC	
200	**T**	**C**	**G**	**F**	**I**	**T**	**M**	**S**	**R**	**I**	**N**	**L**	**L**	**I**	**F**	**D**	**E**	**A**	**H**	**H**	
736	ACA	AAG	AAG	AAG	CAC	CCG	TAC	GCT	CGA	ATT	ATG	GAT	GGG	TAT	TAT	TGC	AAA	CAC	GAA	GGC	
220	**T**	**K**	**K**	**K**	**H**	**P**	**Y**	**A**	**R**	**I**	**M**	**D**	**G**	**Y**	**Y**	**C**	**K**	**H**	**E**	**G**	
796	GAA	AAA	CCT	CGC	ATA	CTG	GGA	ATG	ACT	GCT	TCG	CCT	GTG	GAC	GCG	CGC	ACC	AAG	GAC	GTG	
240	**E**	**K**	**P**	**R**	**I**	**L**	**G**	**M**	**T**	**A**	**S**	**P**	**V**	**D**	**A**	**R**	**T**	**K**	**D**	**V**	
856	AGA	GAC	ACT	GCC	CTG	GAG	CTT	GAG	AGG	TCT	CTT	GAC	AGC	CAG	ATT	GCA	ACG	ATT	TCA	GAT	
260	**R**	**D**	**T**	**A**	**L**	**E**	**L**	**E**	**R**	**S**	**L**	**D**	**S**	**Q**	**I**	**A**	**T**	**I**	**S**	**D**	
916	GAG	GTG	CTG	ATG	GAA	ACC	ATG	CAC	CGA	AAG	AAG	CAA	ACC	GAA	GAA	ACT	GTC	AAC	TAC	AGC	
280	**E**	**V**	**L**	**M**	**E**	**T**	**M**	**H**	**R**	**K**	**K**	**Q**	**T**	**E**	**E**	**T**	**V**	**N**	**Y**	**S**	
976	AGG	CTT	GAA	GCT	CCC	GAT	GAT	ACA	AAG	ACG	CAT	CTA	TGG	GAG	CAG	ATC	TTT	GCC	CTA	GTT	
300	**R**	**L**	**E**	**A**	**P**	**D**	**D**	**T**	**K**	**T**	**H**	**L**	**W**	**E**	**Q**	**I**	**F**	**A**	**L**	**V**	
1036	TCT	CGC	AAC	GAA	CAG	TTC	AAG	TTG	TCG	CTC	GGA	TTC	ACA	AAA	GAG	GCC	TCG	TCA	GTC	CTC	
320	**S**	**R**	**N**	**E**	**Q**	**F**	**K**	**L**	**S**	**L**	**G**	**F**	**T**	**K**	**E**	**A**	**S**	**S**	**V**	**L**	
1096	GGT	ACT	TGG	TGT	GCG	GAC	CGA	TAT	TGG	GAG	CTC	GCA	ATG	ACA	GAT	CTC	GAG	ACT	GAA	CGG	
340	**G**	**T**	**W**	**C**	**A**	**D**	**R**	**Y**	**W**	**E**	**L**	**A**	**M**	**T**	**D**	**L**	**E**	**T**	**E**	**R**	
1156	CTC	GCA	GCC	AGA	ACT	AGC	CGG	GAA	TTT	ATT	GGA	GAC	ATT	GCA	GTA	ACG	AGA	GCA	GAT	CTA	
360	**L**	**A**	**A**	**R**	**T**	**S**	**R**	**E**	**F**	**I**	**G**	**D**	**I**	**A**	**V**	**T**	**R**	**A**	**D**	**L**	
1216	GCA	ACA	GAG	GCC	GTC	CGG	CGT	GTC	CAG	GAA	ATT	GTT	CAG	GCT	CAC	AAG	TTC	GGT	GCA	ATC	
380	**A**	**T**	**E**	**A**	**V**	**R**	**R**	**V**	**Q**	**E**	**I**	**V**	**Q**	**A**	**H**	**K**	**F**	**G**	**A**	**I**	
1276	GAC	CCA	AAG	GCC	GAA	GGG	CTC	TCG	GCC	AAG	GTG	AAA	AGT	CTC	CAT	GAG	ATC	TTG	TCG	CAC	
400	**D**	**P**	**K**	**A**	**E**	**G**	**L**	**S**	**A**	**K**	**V**	**K**	**S**	**L**	**H**	**E**	**I**	**L**	**S**	**H**	
1336	GCT	TTC	ACA	ATG	GAT	GGC	ACC	AAG	CGT	TGC	ATT	GTT	TTT	GTC	GAG	AAG	CGA	TAC	ACA	GCC	
420	**A**	**F**	**T**	**M**	**D**	**G**	**T**	**K**	**R**	**C**	**I**	**V**	**F**	**V**	**E**	**K**	**R**	**Y**	**T**	**A**	
1396	CGT	TTA	CTT	TCG	GAT	CTC	TAT	AGC	CAA	CCT	TCC	ATG	AGG	ATC	CCA	GGA	ATT	TCT	GCT	TCA	
440	**R**	**L**	**L**	**S**	**D**	**L**	**Y**	**S**	**Q**	**P**	**S**	**M**	**R**	**I**	**P**	**G**	**I**	**S**	**A**	**S**	
1456	TAC	ATG	GTT	GGC	TGT	CAA	TCA	ACC	AAT	TCG	AGC	TTC	GGC	ACC	ATG	TCG	TTT	CGA	GAA	CAA	
460	**Y**	**M**	**V**	**G**	**C**	**Q**	**S**	**T**	**N**	**S**	**S**	**F**	**G**	**T**	**M**	**S**	**F**	**R**	**E**	**Q**	
1516	GTC	CTG	GCT	CTC	CAC	GAA	TTC	AAG	CGC	GGC	AAT	ACC	AAC	TGC	CTT	TTC	ACA	ACA	CCT	GTT	
480	**V**	**L**	**A**	**L**	**H**	**E**	**F**	**K**	**R**	**G**	**N**	**T**	**N**	**C**	**L**	**F**	**T**	**T**	**P**	**V**	
1576	GCA	GAA	GAG	GGC	ATC	GAC	GTT	CCA	GAT	TGC	GAC	CTT	GTT	ATT	CGT	TTC	GAT	CTC	TAC	AAC	
500	**A**	**E**	**F**	**G**	**I**	**D**	**V**	**P**	**D**	**C**	**D**	**L**	**V**	**I**	**R**	**F**	**D**	**L**	**Y**	**N**	
1636	TCC	GTC	ATC	CAA	TAT	TTG	CAA	TCC	AAA	GGA	CGC	GCT	CGA	CAA	GCA	CGC	TCT	CGA	TAT	ATC	
520	**S**	**V**	**I**	**Q**	**Y**	**L**	**Q**	**S**	**K**	**G**	**R**	**A**	**R**	**Q**	**A**	**R**	**S**	**R**	**Y**	**I**	
1696	ACC	ATG	ATG	GAG	GAG	GGA	AAT	TTG	ACA	CAT	ATT	CGA	AGT	GCG	AAA	CAA	GCA	TTG	AGA	GAT	
540	**T**	**M**	**M**	**E**	**F**	**G**	**N**	**L**	**T**	**H**	**I**	**R**	**S**	**A**	**K**	**Q**	**A**	**L**	**R**	**D**	
1756	TCA	CAG	GCT	CTG	GAA	AAG	TTC	TGT	CTC	TCT	CTT	TCT	GCT	GAC	CGA	AAA	CTC	CAT	GAT	GAT	
560	**S**	**Q**	**A**	**L**	**E**	**K**	**F**	**C**	**L**	**S**	**L**	**S**	**A**	**D**	**R**	**K**	**L**	**H**	**D**	**D**	
1816	TTG	ATA	GAC	GAG	GAC	ATG	GAG	ATG	ATG	GTT	GAG	CGC	ATG	CAG	CAT	CAA	GTG	TAC	GAG	ATA	
580	**L**	**I**	**D**	**E**	**D**	**M**	**E**	**M**	**M**	**V**	**E**	**R**	**M**	**Q**	**H**	**Q**	**V**	**Y**	**E**	**I**	
1876	CCT	TCG	ACA	GGC	GCA	CGC	CTT	ACG	TTT	CCC	GCC	AGC	CTT	GAA	ATC	CTG	GCC	AGA	TTT	GTT	
600	**P**	**S**	**T**	**G**	**A**	**R**	**L**	**T**	**F**	**P**	**A**	**S**	**L**	**E**	**I**	**L**	**A**	**R**	**F**	**V**	
1936	TCT	CAC	TTG	TCA	ACT	GAC	AGC	TAC	GCC	AAA	GTT	GAA	TAT	CAA	GTA	CAG	AAG	GTT	GGC	GAT	
620	**S**	**H**	**L**	**S**	**T**	**D**	**S**	**Y**	**A**	**K**	**V**	**E**	**Y**	**Q**	**V**	**Q**	**K**	**V**	**G**	**D**	
1996	CGA	TAT	CTA	GCT	GAC	GTG	GTC	ATG	CCA	TTA	CAG	TCC	CCG	GTC	AAC	TTT	GTG	AAG	GGT	ACC	
640	**R**	**Y**	**L**	**A**	**D**	**V**	**V**	**M**	**P**	**L**	**Q**	**S**	**P**	**V**	**N**	**F**	**V**	**K**	**G**	**T**	
2056	CAA	CAA	CGT	AGC	AAA	TTG	CTG	GCC	AAG	TGT	TCT	GCA	GCA	TTT	GAA	GCC	TGC	AAG	ATA	CTG	
660	**Q**	**Q**	**R**	**S**	**K**	**L**	**L**	**A**	**K**	**C**	**S**	**A**	**A**	**F**	**E**	**A**	**C**	**K**	**I**	**L**	
2116	ATC	AGA	GAC	AAA	CAT	GTT	GAC	GGC	AAT	CTT	CAG	CCG	ACA	TTC	AAG	AAA	AAG	GTT	CAC	GCT	
680	**I**	**R**	**D**	**K**	**H**	**V**	**D**	**G**	**N**	**L**	**Q**	**P**	**T**	**F**	**K**	**K**	**K**	**V**	**H**	**A**	
2176	ATG	CGG	AAC	GCA	CGG	CTA	GCT	GTG	AGT	TCA	AAC	AAG	AAG	GCT	GAC	TAC	GAC	ATG	CGA	TTG	
700	**M**	**R**	**N**	**A**	**R**	**L**	**A**	**V**	**S**	**S**	**N**	**K**	**K**	**A**	**D**	**Y**	**D**	**M**	**R**	**L**	
2236	CGG	CCA	GAG	ATT	TGG	GCT	GAA	CGA	GGG	GAG	TGG	ACT	GAA	TGT	TTT	GCG	ACT	ACA	ATC	ACC	
720	**R**	**P**	**E**	**I**	**W**	**A**	**E**	**R**	**G**	**E**	**W**	**T**	**E**	**C**	**F**	**A**	**T**	**T**	**I**	**T**	
2296	ATC	AAA	GAC	GGG	GGC	GCC	CTT	AGG	AAG	GCA	ATA	GCC	GGA	AAG	TCG	TTA	ATA	CTT	CTC	TCG	
740	**I**	**K**	**D**	**G**	**G**	**A**	**L**	**R**	**K**	**A**	**I**	**A**	**G**	**K**	**S**	**L**	**I**	**L**	**L**	**S**	
2356	CGC	AAG	CCG	ATC	CCG	AAA	CTG	CCA	GGT	GTT	CCT	CTA	TTC	TTT	GGC	AAT	GGA	CCT	TCC	TCT	
760	**R**	**K**	**P**	**I**	**P**	**K**	**L**	**P**	**G**	**V**	**P**	**L**	**F**	**F**	**G**	**N**	**G**	**P**	**S**	**S**	
2416	ATC	GCT	GAA	TTG	ACG	CCT	AAT	CAG	GAT	GCT	CTT	AAA	CTC	AAC	CCT	GAA	GAA	GCT	GAC	GGA	

780	**I**	**A**	**E**	**L**	**T**	**P**	**N**	**Q**	**D**	**A**	**L**	**K**	**L**	**N**	**P**	**E**	**F**	**A**	**D**	**G**
2476	TTG	GCC	AAA	TTC	ACT	CAT	CGG	GTA	TTC	GCC	GAT	GTG	TTC	AGC	AAA	GAA	TAC	GAT	ACC	ACG
800	**L**	**A**	**K**	**F**	**T**	**H**	**R**	**V**	**F**	**A**	**D**	**V**	**F**	**S**	**K**	**E**	**Y**	**D**	**T**	**T**
2536	TCC	GAC	CAA	TAT	CCA	TAC	CTC	CTC	GCA	CCT	TAT	GCC	GAC	AGT	CCC	CAA	TCA	GAC	ACT	CGA
820	**S**	**D**	**Q**	**Y**	**P**	**Y**	**L**	**L**	**A**	**P**	**Y**	**A**	**D**	**S**	**P**	**Q**	**S**	**D**	**T**	**R**
2596	TCA	CAT	ATT	GAT	TGG	GGT	GTT	GTC	AAC	CTT	GTG	AGC	AAG	AAC	GAG	GTG	CTC	GAA	TGG	GAG
840	**S**	**H**	**I**	**D**	**W**	**G**	**V**	**V**	**N**	**L**	**V**	**S**	**K**	**N**	**E**	**V**	**L**	**E**	**W**	**E**
2656	AAT	GCG	CCA	GAA	GAC	TTC	TTC	GTC	AAC	AAG	CTT	GTG	ACT	GAT	CCA	TAT	GAT	GGA	GGA	CGC
860	**N**	**A**	**P**	**E**	**D**	**F**	**F**	**V**	**N**	**K**	**L**	**V**	**T**	**D**	**P**	**Y**	**D**	**G**	**G**	**R**
2716	AAG	CTT	GTC	ATC	AAA	GGT	ATC	GAC	AAA	ACG	AAG	AAG	CCA	TCC	GAC	CCA	ACA	CCC	GAA	GGA
880	**K**	**L**	**V**	**I**	**K**	**G**	**I**	**D**	**K**	**T**	**K**	**K**	**P**	**S**	**D**	**P**	**T**	**P**	**E**	**G**
2776	GTC	CCC	GAA	TCA	AGA	AGC	AGA	GCT	TAT	AGG	TCT	GTG	GAA	CCG	ACA	ATA	ATG	CAA	TAC	AGT
900	**V**	**P**	**E**	**S**	**R**	**S**	**R**	**A**	**Y**	**R**	**S**	**V**	**E**	**P**	**T**	**I**	**M**	**Q**	**Y**	**S**
2836	AAC	AGT	CTG	TAT	TAT	AAA	TCT	CGC	CAA	AGG	GTG	AAG	TGG	CGA	GAT	GAT	CAG	CCT	GTG	GTC
920	**N**	**S**	**L**	**Y**	**Y**	**K**	**S**	**R**	**Q**	**R**	**V**	**K**	**W**	**R**	**D**	**D**	**Q**	**P**	**V**	**V**
2896	AAG	GCA	GAG	CTT	CTT	TCC	TTG	AGA	CGA	AAC	CTT	CTG	GAT	GAG	TTT	GAA	GTT	GAC	GAG	AAG
940	**K**	**A**	**E**	**L**	**L**	**S**	**L**	**R**	**R**	**N**	**L**	**L**	**D**	**E**	**F**	**E**	**V**	**D**	**E**	**K**
2956	GTC	AAC	ATG	GAG	TGT	TTC	ATC	ATT	CTG	GAG	CCT	CTC	CAT	GTG	TCT	CCT	CTA	CCT	ATC	AAT
960	**V**	**N**	**M**	**E**	**C**	**F**	**I**	**I**	**L**	**E**	**P**	**L**	**H**	**V**	**S**	**P**	**L**	**P**	**I**	**N**
3016	GTG	GTC	TCT	ATG	GCC	ATG	GCA	TTT	CCA	GCA	ATC	ATC	CAT	CGA	ATA	GAT	TCC	GTC	CTG	ATT
980	**V**	**V**	**S**	**M**	**A**	**M**	**A**	**F**	**P**	**A**	**I**	**I**	**H**	**R**	**I**	**D**	**S**	**V**	**L**	**I**
3076	GTG	CTG	GAT	GCA	TGT	AAT	CTC	CTT	GAC	CTT	GGA	ATT	CCA	CCG	GAA	CTC	GCA	CTG	GAG	GCC
1000	**V**	**L**	**D**	**A**	**C**	**N**	**L**	**L**	**D**	**L**	**G**	**I**	**P**	**P**	**E**	**L**	**A**	**L**	**E**	**A**
3136	ATG	ACC	AAA	GAC	AGC	GAC	AAC	TCT	GGG	GAG	CAT	GAC	ACA	GAA	CAG	ATC	AAT	TTC	CAA	ACA
1020	**M**	**T**	**K**	**D**	**S**	**D**	**N**	**S**	**G**	**E**	**H**	**D**	**T**	**E**	**Q**	**I**	**N**	**F**	**Q**	**T**
3196	GGC	ATG	GGC	GAC	AAC	TAT	GAA	CGG	CTA	GAG	TTC	CTC	GGC	GAC	TGT	TTT	CTC	AAG	ATG	GCC
1040	**G**	**M**	**G**	**D**	**N**	**Y**	**E**	**R**	**L**	**E**	**F**	**L**	**G**	**D**	**C**	**F**	**L**	**K**	**M**	**A**
3256	ACC	ACG	ATC	TCA	ATC	TAC	ACA	CTG	ATG	CCT	GAC	GGA	AAT	GAG	TGC	AAA	TAC	CAC	GTT	GAG
1060	**T**	**T**	**I**	**S**	**I**	**Y**	**T**	**L**	**M**	**P**	**D**	**G**	**N**	**E**	**C**	**K**	**Y**	**H**	**V**	**E**
3316	CGC	ATG	CTT	CTC	ATT	TGC	AAC	CAA	AAC	CTC	TTC	AAT	CAT	GCG	GTT	GAT	CGC	AAA	CTC	CAA
1080	**R**	**M**	**L**	**L**	**I**	**C**	**N**	**Q**	**N**	**L**	**F**	**N**	**H**	**A**	**V**	**D**	**R**	**K**	**L**	**Q**
3376	GAG	TTC	GTC	CGA	TCC	AAG	GCT	TTT	GAC	AGA	CGA	ACA	TGG	TAC	CCC	GAT	CTT	CCA	CTC	AAG
1100	**E**	**F**	**V**	**R**	**S**	**K**	**A**	**F**	**D**	**R**	**R**	**T**	**W**	**Y**	**P**	**D**	**L**	**P**	**L**	**K**
3436	AAA	GGA	AAG	GCA	CCG	AAA	ACT	TCG	ATA	AAG	CAT	AAT	CTG	GCC	GAC	AAG	ACG	ATT	GCA	GAC
1120	**K**	**G**	**K**	**A**	**P**	**K**	**T**	**S**	**I**	**K**	**H**	**N**	**L**	**A**	**D**	**K**	**T**	**I**	**A**	**D**
3496	GTA	TGT	GAA	GCT	CTC	ATT	GGG	GCA	GCA	TAC	CTC	ACA	AGC	AAG	GCC	AGC	AAC	ATG	GAT	CTG
1140	**V**	**C**	**E**	**A**	**L**	**I**	**G**	**A**	**A**	**Y**	**L**	**T**	**S**	**K**	**A**	**S**	**N**	**M**	**D**	**L**
3556	GCT	GTC	AAA	GCC	GTG	ACG	CGA	ATG	ACC	AAG	TCA	AAG	AAC	CAC	AGG	ATG	AAG	GCT	TTC	AAA
1160	**A**	**V**	**K**	**A**	**V**	**T**	**R**	**M**	**T**	**K**	**S**	**K**	**N**	**H**	**R**	**M**	**K**	**A**	**F**	**K**
3616	GAT	TAC	TAT	AAA	GCC	TAC	AAG	GTG	CCT	GAG	TGG	CAG	GAA	GGT	AAT	GCG	TCC	GCC	TCT	CAA
1180	**D**	**Y**	**Y**	**K**	**A**	**Y**	**K**	**V**	**P**	**E**	**W**	**Q**	**F**	**G**	**N**	**A**	**S**	**A**	**S**	**Q**
3676	CGC	AGC	CTT	GTG	CAA	AAG	GTG	GCA	CAA	GCT	ACA	GGA	TAT	CGC	TTC	AAG	TCT	GCA	CAG	CTT
1200	**R**	**S**	**L**	**V**	**Q**	**K**	**V**	**A**	**Q**	**A**	**T**	**G**	**Y**	**R**	**F**	**K**	**S**	**A**	**Q**	**L**
3736	GTC	CAG	AGT	GCA	TTC	AAA	CAT	CCT	TCG	TGG	CCA	TAT	GAG	TCT	GTC	CCA	GAT	TAT	CAG	CGC
1220	**V**	**Q**	**S**	**A**	**F**	**K**	**H**	**P**	**S**	**W**	**P**	**Y**	**E**	**S**	**V**	**P**	**D**	**Y**	**Q**	**R**
3796	CTC	GAA	TTT	CTG	GGC	GAT	TCG	CTT	CTC	GAC	ATG	GCC	ATC	GTC	GAT	TAT	CTC	TAT	CAA	AAC
1240	**L**	**E**	**F**	**L**	**G**	**D**	**S**	**L**	**L**	**D**	**M**	**A**	**I**	**V**	**D**	**Y**	**L**	**Y**	**Q**	**N**
3856	TTC	CCC	AGA	GCA	GAC	CCT	CAG	TGG	CTG	ACG	GAA	CAC	AAG	ATG	GCC	ATG	GTA	TCG	AAT	CAG
1260	**F**	**P**	**R**	**A**	**D**	**P**	**Q**	**W**	**L**	**T**	**E**	**H**	**K**	**M**	**A**	**M**	**V**	**S**	**N**	**Q**
3916	TTC	CTT	GGA	TGC	CTC	TGC	GTT	AAG	CTT	GGT	CTG	CAC	CAA	CAC	CTA	CTC	TCC	AGC	ACA	TCG
1280	**F**	**L**	**G**	**C**	**L**	**C**	**V**	**K**	**L**	**G**	**L**	**H**	**Q**	**H**	**L**	**L**	**S**	**S**	**T**	**S**
3976	AGT	CTT	CTC	AGC	CAA	ATC	CGC	GAT	TAT	GTC	GTC	GAA	CTC	GAA	GTT	GCT	GAA	GAA	AAT	GCT
1300	**S**	**L**	**L**	**S**	**Q**	**I**	**R**	**D**	**Y**	**V**	**V**	**E**	**L**	**E**	**V**	**A**	**E**	**E**	**N**	**A**
4036	CGC	AAG	GAA	GCG	AAA	GAA	GAT	GGG	ACT	CCC	ATG	CGC	AAG	GAC	TTC	TGG	CTG	AAG	GTG	GAT
1320	**R**	**K**	**E**	**A**	**K**	**E**	**D**	**G**	**T**	**P**	**M**	**R**	**K**	**D**	**F**	**W**	**L**	**K**	**V**	**D**
4096	TCG	GCA	CCC	AAG	GTT	TAC	GCC	GAT	GTG	ATT	GAA	GCT	CTT	GTT	GGA	GCC	ATG	TTT	GTC	GAT
1340	**S**	**A**	**P**	**K**	**V**	**Y**	**A**	**D**	**V**	**I**	**E**	**A**	**L**	**V**	**G**	**A**	**M**	**F**	**V**	**D**
4156	TCC	AAG	TAC	AAA	TAT	TCG	GTT	GTC	GAG	AAT	TTC	TTT	ACC	AAG	TTC	ATC	CAG	CCA	TAT	TTC
1360	**S**	**K**	**Y**	**K**	**Y**	**S**	**V**	**V**	**E**	**N**	**F**	**F**	**T**	**K**	**F**	**I**	**Q**	**P**	**Y**	**F**
4216	CAA	GAC	ATG	AGA	CTT	TAC	GAT	ACC	TTT	GCG	AAC	AAG	CAC	CCA	GTC	ACG	TTC	CTC	TCC	AAG
1380	**Q**	**D**	**M**	**R**	**L**	**Y**	**D**	**T**	**F**	**A**	**N**	**K**	**H**	**P**	**V**	**T**	**F**	**L**	**S**	**K**
4276	AAG	ATG	CAT	CAG	GAA	TTT	GGT	TGC	ACA	AAC	TGG	CGC	ATC	AGC	GCA	GAT	GCT	GTA	CCA	TGC
1400	**K**	**M**	**H**	**Q**	**E**	**F**	**G**	**C**	**T**	**N**	**W**	**R**	**I**	**S**	**A**	**D**	**A**	**V**	**P**	**C**
4336	AGT	GCA	GAT	CAA	GGC	ATG	GCG	GCG	CTG	AAA	GAT	ACC	GAA	ATG	TGC	GCT	GTT	TTC	ATG	GTG
1420	**S**	**A**	**D**	**Q**	**G**	**M**	**A**	**A**	**L**	**K**	**D**	**T**	**E**	**M**	**C**	**A**	**V**	**F**	**M**	**V**
4396	CAT	CAA	AAG	GTT	ATC	GCG	GAT	GAC	ACA	TCT	GAG	AGT	GGG	AGG	TAT	TCT	AAG	CAG	CGT	GCC
1440	**H**	**Q**	**K**	**V**	**I**	**A**	**D**	**D**	**T**	**S**	**E**	**S**	**G**	**R**	**Y**	**S**	**K**	**Q**	**R**	**A**
4456	GCG	ACG	AAA	GCG	CTC	GAG	ATA	CTT	GAT	TCG	TTT	GGT	GAG	GAT	GTT	GAG	GCG	GCG	AAG	AGG
1460	**A**	**T**	**K**	**A**	**L**	**E**	**I**	**L**	**D**	**S**	**F**	**G**	**E**	**D**	**V**	**E**	**A**	**A**	**K**	**R**
4516	TTT	TTG	GGG	TGT	GAT	TGT	CAG	CTT	GGA	GGA	GGG	GAT	GTT	GAG	GTT	GAT	CAT	GGA	ACA	GCT
1480	**F**	**L**	**G**	**C**	**D**	**C**	**Q**	**L**	**G**	**G**	**G**	**D**	**V**	**E**	**V**	**D**	**H**	**G**	**T**	**A**
4576	GTT	TGA	GCTGAAGTCGGCACTAGA																	
1500	**V**	*																		

Figure 3.10 : Nucleotide and deduced amino acid sequences of DCL1 ORF. Nucleotide (upper line) and amino acid (lower line). Stop codon is marked with an asterisk (GenBank accession number: MW367432)

1	T	T	G	A	G	A	G	C	A	A	A	A	C	A	C	T	A	C	C	C	G
61	T	C	C	T	G	C	G	T	G	A	A	G	A	T	A	A	C	C	T	C	A
120	A	G	A	C	A	T	C	A	A	A	A	C	T	A	C	T	A	T	T	C	A

																	ATG	GAA	ATC	GAT	GAA
1																	**M**	**E**	**I**	**D**	**E**
180	GAG	AAG	TCT	CCC	AAC	AGT	GGG	AGT	GCA	GAC	TCC	TCA	CGG	TCG	GAG	AGT	CCT	GCC	ACC	ACC	
6	**E**	**K**	**S**	**P**	**N**	**S**	**G**	**S**	**A**	**D**	**S**	**S**	**R**	**S**	**E**	**S**	**P**	**A**	**T**	**T**	
240	TCG	ACA	CCA	TCA	CCA	ATG	ATC	GAT	GTC	GAA	GAA	ACA	GTG	ACC	TTA	ATC	CCT	ACC	GAC	AAG	
26	**S**	**T**	**P**	**S**	**P**	**M**	**I**	**D**	**V**	**E**	**E**	**T**	**V**	**T**	**L**	**I**	**P**	**T**	**D**	**K**	
300	GCC	CTA	AAT	GGA	AAA	CAA	AAG	GCC	GCA	AAC	AAG	TCC	AAA	GAT	GCA	TCT	CCA	GTC	CCG	GAC	
46	**A**	**L**	**N**	**G**	**K**	**Q**	**K**	**A**	**A**	**N**	**K**	**S**	**K**	**D**	**A**	**S**	**P**	**V**	**P**	**D**	
360	ATC	ATC	AGG	CCT	CGT	GCC	TAC	CAG	CGA	GAG	ATG	TTG	GAG	CAG	AGC	CTG	AAG	AGA	AAC	GTG	
66	**I**	**I**	**R**	**P**	**R**	**A**	**Y**	**Q**	**R**	**E**	**M**	**L**	**E**	**Q**	**S**	**L**	**K**	**R**	**N**	**V**	
420	ATT	GTT	GCG	ATG	GAT	ACA	GGC	AGC	GGA	AAG	ACA	CAA	GTC	GCC	GTT	CTT	CGC	ATT	CAA	GCG	
86	**I**	**V**	**A**	**M**	**D**	**T**	**G**	**S**	**G**	**K**	**T**	**Q**	**V**	**A**	**V**	**L**	**R**	**I**	**Q**	**A**	
480	GAA	CTC	GAG	AGA	AGT	TCA	CCC	AAC	AAG	CTT	GTG	TGG	TTC	TTA	GGA	AAG	ACT	GTT	AGC	CTC	
106	**E**	**L**	**E**	**R**	**S**	**S**	**P**	**N**	**K**	**L**	**V**	**W**	**F**	**L**	**G**	**K**	**T**	**V**	**S**	**L**	
540	TGC	GAA	CAA	CAG	TTT	AAT	GTT	ATC	AAG	AGA	CAA	ATG	CCA	TCG	GTG	CCC	ATG	AGA	CTG	CTT	
126	**C**	**E**	**Q**	**Q**	**F**	**N**	**V**	**I**	**K**	**R**	**Q**	**M**	**P**	**S**	**V**	**P**	**M**	**R**	**L**	**L**	
600	ACG	GGG	CAG	CTC	AAC	ATC	GAT	GCA	TGG	TCG	CCA	GAA	GTT	TGG	CCT	CGA	ATT	CTC	AAG	GGC	
146	**T**	**G**	**Q**	**L**	**N**	**I**	**D**	**A**	**W**	**S**	**P**	**E**	**V**	**W**	**P**	**R**	**I**	**L**	**K**	**G**	
660	ACT	CGT	ATC	ATC	GTA	TCT	ACA	TTC	TCC	ATA	TTG	CGT	GAT	GCT	CTT	GAC	CAC	GCC	TTT	GTC	
166	**T**	**R**	**I**	**I**	**V**	**S**	**T**	**F**	**S**	**I**	**L**	**R**	**D**	**A**	**L**	**D**	**H**	**A**	**F**	**V**	
720	ACA	ATG	GAC	ATG	CTC	GCT	TTA	ATA	GTA	TTT	GAC	GAA	GCC	CAT	AAT	TGC	GTT	AAG	AAC	AGC	
186	**T**	**M**	**D**	**M**	**L**	**A**	**L**	**I**	**V**	**F**	**D**	**E**	**A**	**H**	**N**	**C**	**V**	**K**	**N**	**S**	
780	TCT	GGG	CGG	AAG	ATC	ATG	ATG	AAC	TTC	TAC	CAT	CGA	CAC	AAG	AAC	GTG	GGT	ATG	CCA	GTA	
206	**S**	**G**	**R**	**K**	**I**	**M**	**M**	**N**	**F**	**Y**	**H**	**R**	**H**	**K**	**N**	**V**	**G**	**M**	**P**	**V**	
840	CCT	GCC	ATA	CTC	GGC	TTG	ACT	GCC	AGT	CCC	ATT	ATC	TCA	TCA	GAT	CTT	AAG	GAG	ATC	GAA	
226	**P**	**A**	**I**	**L**	**G**	**L**	**T**	**A**	**S**	**P**	**I**	**I**	**S**	**S**	**D**	**L**	**K**	**E**	**I**	**E**	
900	AAA	CTA	GAG	AAT	ACA	ATG	GAC	GCT	ATC	TGC	GTT	ACT	CCA	ACC	ACT	CAT	CGG	GAG	CAA	CTA	
246	**K**	**L**	**E**	**N**	**T**	**M**	**D**	**A**	**I**	**C**	**V**	**T**	**P**	**T**	**T**	**H**	**R**	**E**	**Q**	**L**	
960	CTC	AAG	CAT	GTC	AAC	AAG	CCT	CAC	CTC	ATT	CGC	TCA	CTC	TAC	GAT	GCT	ACG	AAG	ATC	CCT	
266	**L**	**K**	**H**	**V**	**N**	**K**	**P**	**H**	**L**	**I**	**R**	**S**	**L**	**Y**	**D**	**A**	**T**	**K**	**I**	**P**	
1020	CCC	CGT	ACG	CCA	CTG	ATG	CAA	CAA	CTG	CAA	TCA	GAA	TAC	TCG	AAC	ATG	GAT	ATC	GCA	AAG	
286	**P**	**R**	**T**	**P**	**L**	**M**	**Q**	**Q**	**L**	**Q**	**S**	**E**	**Y**	**S**	**N**	**M**	**D**	**I**	**A**	**K**	
1080	GAT	CCT	GAG	GTT	CTT	AAG	TTA	AAG	GAT	CTT	GTC	GCT	AAT	GAT	GAA	AAA	AAG	CGG	GAA	AAG	
306	**D**	**P**	**E**	**V**	**L**	**K**	**L**	**K**	**D**	**L**	**V**	**A**	**N**	**D**	**E**	**K**	**K**	**R**	**E**	**K**	
1140	TTA	ATG	AAC	GTG	ATT	ATG	AAG	CAC	GAC	ACG	TTC	TCG	CAA	CAG	CAA	ATC	AGA	GGC	CTC	TGG	
326	**L**	**M**	**N**	**V**	**I**	**M**	**K**	**H**	**D**	**T**	**F**	**S**	**Q**	**Q**	**Q**	**I**	**R**	**G**	**L**	**W**	
1200	AAC	AAG	AGT	AGA	GAA	ATC	CTT	GCC	GAA	CTT	GGC	AGC	TGG	GCA	GTA	GAC	CAT	TAC	ATT	TCA	
346	**N**	**K**	**S**	**R**	**E**	**I**	**L**	**A**	**E**	**L**	**G**	**S**	**W**	**A**	**V**	**D**	**H**	**Y**	**I**	**S**	
1260	CAG	ATG	ATC	AAG	ACC	TTC	CTT	GAC	AGA	ATC	GAC	GCA	TAC	AGT	ACT	TTC	ACC	GAT	GCA	TGG	
366	**Q**	**M**	**I**	**K**	**T**	**F**	**L**	**D**	**R**	**I**	**D**	**A**	**Y**	**S**	**T**	**F**	**T**	**D**	**A**	**W**	
1320	TGT	AAC	GAG	GAC	AGG	ACC	TAT	CTT	GCA	GGA	CAC	TTG	CGC	CGA	ATC	AAC	ATC	GGT	GTC	GTT	
386	**C**	**N**	**E**	**D**	**R**	**T**	**Y**	**L**	**A**	**G**	**H**	**L**	**R**	**R**	**I**	**N**	**I**	**G**	**V**	**V**	
1380	GAC	AAC	TCA	CCA	CCA	ACC	GCT	CAA	ACC	GTC	TCA	CAC	AAA	ACT	AGC	CGA	CTG	ATC	CAT	GAA	
406	**D**	**N**	**S**	**P**	**P**	**T**	**A**	**Q**	**T**	**V**	**S**	**H**	**K**	**T**	**S**	**R**	**L**	**I**	**H**	**E**	
1440	CTT	CTT	GCG	GCC	GAG	AAC	CAT	GTA	GTT	GGA	ATC	ATA	TTC	GTC	AAG	GAA	CGC	ACT	GCA	GCG	
426	**L**	**L**	**A**	**A**	**E**	**N**	**H**	**V**	**V**	**G**	**I**	**I**	**F**	**V**	**K**	**E**	**R**	**T**	**A**	**A**	
1500	CAT	ACA	CTT	TGC	GAG	CTT	CTT	AAC	AAC	TAC	CCC	CCT	ATT	CGA	GAT	CGT	TAC	CGC	GTC	GGT	
446	**H**	**T**	**L**	**C**	**E**	**L**	**L**	**N**	**N**	**Y**	**P**	**P**	**I**	**R**	**D**	**R**	**Y**	**R**	**V**	**G**	
1560	GCA	ATA	GTC	GGG	GCT	TCG	AGC	TAC	GGA	CTT	CAG	AAA	CAA	AAA	GTC	TAT	GAG	TAC	TCC	AGT	
466	**A**	**I**	**V**	**G**	**A**	**S**	**S**	**Y**	**G**	**L**	**Q**	**K**	**Q**	**K**	**V**	**Y**	**E**	**Y**	**S**	**S**	
1620	GGC	CCT	GGC	CCT	CAA	GTC	CTC	GAT	GAT	TTC	AGA	TCA	GGG	ACT	ATC	AAT	CTC	CTG	GTG	GCT	
486	**G**	**P**	**G**	**P**	**Q**	**V**	**L**	**D**	**D**	**F**	**R**	**S**	**G**	**T**	**I**	**N**	**L**	**L**	**V**	**A**	
1680	ACC	AGT	GTC	TTA	GAA	GAA	GGC	ATT	GAT	GTT	CCC	GCA	TGC	AAC	CTA	GTT	GTG	TCC	TTC	GAC	
506	**T**	**S**	**V**	**L**	**E**	**E**	**G**	**I**	**D**	**V**	**P**	**A**	**C**	**N**	**L**	**V**	**V**	**S**	**F**	**D**	
1740	GAG	ATC	GCT	ACC	CTC	AAG	TCA	TTC	ATC	CAG	CGC	CGT	GGA	CGA	GCT	CGC	ATG	CAG	GAA	TCC	
526	**E**	**I**	**A**	**T**	**L**	**K**	**S**	**F**	**I**	**Q**	**R**	**R**	**G**	**R**	**A**	**R**	**M**	**Q**	**E**	**S**	
1800	AAA	ATG	ATT	GCC	TTG	CTG	AGC	TCA	AGC	ACA	GAC	ATC	CGC	GCC	TGG	GAA	GAA	CTG	GAG	AAA	
546	**K**	**M**	**I**	**A**	**L**	**L**	**S**	**S**	**S**	**T**	**D**	**I**	**R**	**A**	**W**	**E**	**E**	**L**	**E**	**K**	
1860	GGC	ATG	AAG	GAA	CGT	TAC	GAA	GAT	GAC	CAA	CGA	GAA	TTG	GCT	CGA	TTG	GAT	GAA	CAG	GCA	
566	**G**	**M**	**K**	**E**	**R**	**Y**	**E**	**D**	**D**	**Q**	**R**	**E**	**L**	**A**	**R**	**L**	**D**	**E**	**Q**	**A**	
1920	CGG	GCA	GAG	GAG	ATT	GAT	TCC	ACC	TAC	TAT	ATC	GTA	AAG	AAC	ACA	GGA	GCA	CGG	CTC	GAC	
586	**R**	**A**	**E**	**E**	**I**	**D**	**S**	**T**	**Y**	**Y**	**I**	**V**	**K**	**N**	**T**	**G**	**A**	**R**	**L**	**D**	
1980	CTC	GAC	AAT	GCT	CGA	CAA	CAC	CTG	GAT	CGC	TTT	TGT	CAA	ATT	GTT	TTC	CGC	TCA	GAT	TAT	
606	**L**	**D**	**N**	**A**	**R**	**Q**	**H**	**L**	**D**	**R**	**F**	**C**	**Q**	**I**	**V**	**F**	**R**	**S**	**D**	**Y**	
2040	GTC	GGT	CAA	CTC	CCC	GAC	TAC	GTC	TTC	CAT	AAA	GAA	GAG	ACT	CCA	TAC	GGC	GGA	CCG	ATC	
626	**V**	**G**	**Q**	**L**	**P**	**D**	**Y**	**V**	**F**	**H**	**K**	**E**	**E**	**T**	**P**	**Y**	**G**	**G**	**P**	**I**	
2100	TTG	AGA	GCT	ACG	GTT	ACT	TTG	CCA	GGA	GGG	TTG	CCA	CTC	AAT	CTT	CGT	ACG	TTC	AAA	AGT	
646	**L**	**R**	**A**	**T**	**V**	**T**	**L**	**P**	**G**	**G**	**L**	**P**	**L**	**N**	**L**	**R**	**T**	**F**	**K**	**S**	
2160	GCT	TGC	GGG	TGG	AAG	TCT	GAA	CGG	AAT	GCC	ATG	AGA	GAT	GCC	GCA	TTT	CAG	GCA	TAC	GTC	
666	**A**	**C**	**G**	**W**	**K**	**S**	**E**	**R**	**N**	**A**	**M**	**R**	**D**	**A**	**A**	**F**	**Q**	**A**	**Y**	**V**	
2220	GCT	TTG	TAC	GAA	GCA	GGG	CTG	GTC	ACA	GAT	AAC	CTG	GCC	CCT	CTC	AAT	GCT	AAG	TCA	GAG	
686	**A**	**L**	**Y**	**E**	**A**	**G**	**L**	**V**	**T**	**D**	**N**	**L**	**A**	**P**	**L**	**N**	**A**	**K**	**S**	**E**	
2280	AAG	GTG	GAA	GAA	AAG	ACC	GAC	CTA	ATG	CCG	GAG	CAC	CTA	TTC	GAC	CCC	TGG	ATC	CAG	ATC	
706	**K**	**V**	**E**	**E**	**K**	**T**	**D**	**L**	**M**	**P**	**E**	**H**	**L**	**F**	**D**	**P**	**W**	**I**	**Q**	**I**	
2340	GCC	CAA	CGC	TGG	AAG	TCG	GGT	TGT	GAT	AAG	CAA	TTC	TAC	TTG	TTC	GAG	TTT	ACC	GAT	GAC	
726	**A**	**Q**	**R**	**W**	**K**	**S**	**G**	**C**	**D**	**K**	**Q**	**F**	**Y**	**L**	**F**	**E**	**F**	**T**	**D**	**D**	
2400	GAG	TTT	CCC	GGC	CCT	TGT	TTA	TTT	GAC	GTC	ATG	TTA	CCT	GCT	CTA	ATC	GAC	CTG	CCT	CGA	
746	**E**	**F**	**P**	**G**	**P**	**C**	**L**	**F**	**D**	**V**	**M**	**L**	**P**	**A**	**L**	**I**	**D**	**L**	**P**	**R**	

2460	AAG	ATC	ACG	TTA	TAC	CCT	GGG	AAT	GGC	TCC	AAG	TGG	TCC	ATC	GTA	TGC	GAT	TCG	TTT	GTC
766	**K**	**I**	**T**	**L**	**Y**	**P**	**G**	**N**	**G**	**S**	**K**	**W**	**S**	**I**	**V**	**C**	**D**	**S**	**F**	**V**
2520	GAT	ATA	CCC	GAT	GAG	GTA	TCT	TCA	AAA	GAA	CCA	GAT	CAA	ACT	TCG	GCT	CTC	TTG	GCT	ATG
786	**D**	**I**	**P**	**D**	**E**	**V**	**S**	**S**	**K**	**E**	**P**	**D**	**Q**	**T**	**S**	**A**	**L**	**L**	**A**	**M**
2580	AAC	TTT	CAG	CAC	CGC	TGG	CCG	GTC	GAA	GAT	CGC	GAG	CAT	GTT	ATC	AAG	ATG	TCC	ATA	GCC
806	**N**	**F**	**Q**	**H**	**R**	**W**	**P**	**V**	**E**	**D**	**R**	**E**	**H**	**V**	**I**	**K**	**M**	**S**	**I**	**A**
2640	AGT	ACG	ACC	ATC	TTC	CCT	GCC	CAC	CAC	GTC	ACC	CGG	GAT	GAC	ATG	GGT	GCA	TTA	CCC	TTC
826	**S**	**T**	**T**	**I**	**F**	**P**	**A**	**H**	**H**	**V**	**T**	**R**	**D**	**D**	**M**	**G**	**A**	**L**	**P**	**F**
2700	CGC	GGA	GAC	GGG	GTG	GAA	GAG	GCG	AAG	AAA	CAC	AGA	GTT	CTT	GTG	CGA	GAC	CAA	TAC	AAA
846	**R**	**G**	**D**	**G**	**V**	**E**	**E**	**A**	**K**	**K**	**H**	**R**	**V**	**L**	**V**	**R**	**D**	**Q**	**Y**	**K**
2760	ACG	CCG	TTT	CAC	TAC	GTC	GGC	ACG	ATT	CCC	TCA	AAA	CCT	GAT	TTT	TAT	GAT	GTG	CAA	CGC
866	**T**	**P**	**F**	**H**	**Y**	**V**	**G**	**T**	**I**	**P**	**S**	**K**	**P**	**D**	**F**	**Y**	**D**	**V**	**Q**	**R**
2820	CCA	TTC	TAT	GAA	TAT	GAC	AAA	GGT	CCA	TTG	AAT	GAA	GAG	TAT	CTA	GTT	CTT	GAA	AGA	TGG
886	**P**	**F**	**Y**	**E**	**Y**	**D**	**K**	**G**	**P**	**L**	**N**	**E**	**E**	**Y**	**L**	**V**	**L**	**E**	**R**	**W**
2880	GGT	CTT	CGA	GTC	GAC	TTA	CTT	CAT	GAT	TTG	AAG	GAT	GGT	CAG	GCC	AAG	TCA	AGC	ACT	AAA
906	**G**	**L**	**R**	**V**	**D**	**L**	**L**	**H**	**D**	**L**	**K**	**D**	**G**	**Q**	**A**	**K**	**S**	**S**	**T**	**K**
2940	CCT	TAC	GAG	TGT	GTG	CTT	CCC	ATT	TCT	TAT	GCC	CGT	GTT	GAT	AGG	GTC	AAG	AGA	CGA	GTC
926	**P**	**Y**	**E**	**C**	**V**	**L**	**P**	**I**	**S**	**Y**	**A**	**R**	**V**	**D**	**R**	**V**	**K**	**R**	**R**	**V**
3000	GTC	AAG	TGC	GGT	CTG	TTC	ATT	CCA	CAT	ATC	ATA	CAT	GAG	CTA	GAG	GTG	CAG	CTT	ATT	GCA
946	**V**	**K**	**C**	**G**	**L**	**F**	**I**	**P**	**H**	**I**	**I**	**H**	**E**	**L**	**E**	**V**	**Q**	**L**	**I**	**A**
3060	TCT	GAA	TTA	TCT	TCT	ACA	CTT	CTG	AAG	CCA	GTT	GGC	ATC	AAG	AAT	TTG	CAG	TTG	GTG	CTT
966	**S**	**E**	**L**	**S**	**S**	**T**	**L**	**L**	**K**	**P**	**V**	**G**	**I**	**K**	**N**	**L**	**Q**	**L**	**V**	**L**
3120	GAG	GCC	GTA	AGC	TCG	CCT	AGC	GCT	CGT	GAA	CCT	GTC	GAC	TAC	CAG	AGA	CTT	GAG	TTT	TTG
986	**E**	**A**	**V**	**S**	**S**	**P**	**S**	**A**	**R**	**E**	**P**	**V**	**D**	**Y**	**Q**	**R**	**L**	**E**	**F**	**L**
3180	GGC	GAT	TCT	ATT	TTG	AAG	TTT	TGC	ACC	GTC	ACT	CAG	GCT	TAT	TCT	GAA	CAC	CCG	TCA	TGG
1006	**G**	**D**	**S**	**I**	**L**	**K**	**F**	**C**	**T**	**V**	**T**	**Q**	**A**	**Y**	**S**	**E**	**H**	**P**	**S**	**W**
3240	CCC	GAA	GGC	CTT	CTG	AAT	CAC	TTT	AAA	GAT	CGA	CTC	GTA	TCC	AAC	GAA	CGA	CTC	CAA	CGA
1026	**P**	**E**	**G**	**L**	**L**	**N**	**H**	**F**	**K**	**D**	**R**	**L**	**V**	**S**	**N**	**E**	**R**	**L**	**Q**	**R**
3300	GTG	TGT	CTT	GAG	AAA	GGG	CTC	TCA	AAG	TTC	ATT	CTT	TCG	AAG	CCT	TTC	ACC	GCC	TTG	AAA
1046	**V**	**C**	**L**	**E**	**K**	**G**	**L**	**S**	**K**	**F**	**I**	**L**	**S**	**K**	**P**	**F**	**T**	**A**	**L**	**K**
3360	TGG	AGG	CCA	CGC	TAT	CTT	GAT	GAC	ATT	CTT	AAA	GAA	AAA	CCT	GCC	CCT	CGA	CCC	GCG	CCA
1066	**W**	**R**	**P**	**R**	**Y**	**L**	**D**	**D**	**I**	**L**	**K**	**E**	**K**	**P**	**A**	**P**	**R**	**P**	**A**	**P**
3420	GTG	GGT	GGA	TCG	AAG	ACC	TTG	GCC	GAT	GTT	GTA	GAG	GCA	CTT	ATC	GGA	GCC	TCT	TAC	CAA
1086	**V**	**G**	**G**	**S**	**K**	**T**	**L**	**A**	**D**	**V**	**V**	**E**	**A**	**L**	**I**	**G**	**A**	**S**	**Y**	**Q**
3480	GAC	GGA	GGG	CTG	ACC	AAG	GCC	CTT	AAA	TGC	ATT	TCA	GTC	TTT	TTG	GGC	GAT	AGC	TGC	AAC
1106	**D**	**G**	**G**	**L**	**T**	**K**	**A**	**L**	**K**	**C**	**I**	**S**	**V**	**F**	**L**	**G**	**D**	**S**	**C**	**N**
3540	TGG	CAT	CAA	GAG	GGT	GTG	GCT	AGA	GAC	ATT	CTC	TTT	GCT	GCA	GCT	CCT	AGC	GAC	GTT	CAG
1126	**W**	**H**	**Q**	**E**	**G**	**V**	**A**	**R**	**D**	**I**	**L**	**F**	**A**	**A**	**A**	**P**	**S**	**D**	**V**	**Q**
3600	CTT	CCC	AGC	AAC	CTT	GAA	CCC	CTT	GAA	GAC	CTA	ATC	AAG	TAT	AAA	TTC	AAG	AAG	AAG	TCT
1146	**L**	**P**	**S**	**N**	**L**	**E**	**P**	**L**	**E**	**D**	**L**	**I**	**K**	**Y**	**K**	**F**	**K**	**K**	**K**	**S**
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1186	**E**	**Q**	**L**	**E**	**F**	**L**	**G**	**D**	**A**	**V**	**L**	**D**	**Y**	**I**	**V**	**V**	**T**	**K**	**M**	**F**
3780	CAG	CAC	TAT	CCG	CCA	ATT	CCT	ACT	GGT	CGC	CTT	CAT	ATG	ATC	AAA	ACG	GCC	ATT	GTG	AAT
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1226	**G**	**E**	**F**	**L**	**S**	**L**	**V**	**N**	**L**	**V**	**N**	**S**	**I**	**Q**	**R**	**K**	**D**	**L**	**E**	**V**
3900	AGG	TCC	GAT	GGT	GAG	CTT	GTA	ACC	AAA	ACT	ACT	GCA	CTT	CCA	CTT	TGG	AAG	TTC	ATG	AGG
1246	**R**	**S**	**D**	**G**	**E**	**L**	**V**	**T**	**K**	**T**	**T**	**A**	**L**	**P**	**L**	**W**	**K**	**F**	**M**	**R**
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1266	**F**	**N**	**S**	**S**	**E**	**M**	**A**	**N**	**V**	**M**	**L**	**E**	**T**	**E**	**E**	**K**	**F**	**E**	**S**	**M**
4020	CGA	CGG	GAG	CTT	GTC	ATA	GCT	TTG	TGG	GGT	GAT	GGT	CAC	TAC	CCC	TGG	GCG	CTC	CTC	GCA
1286	**R**	**R**	**E**	**L**	**V**	**I**	**A**	**L**	**W**	**G**	**D**	**G**	**H**	**Y**	**P**	**W**	**A**	**L**	**L**	**A**
4080	CGC	CTT	CAC	CCC	CAA	AAG	TTT	TAT	TCG	GAT	ATG	TTT	GAA	GCT	CTT	CTC	GGT	GCC	GTC	TGG
1306	**R**	**L**	**H**	**P**	**Q**	**K**	**F**	**Y**	**S**	**D**	**M**	**F**	**E**	**A**	**L**	**L**	**G**	**A**	**V**	**W**
4140	GTC	GAC	TCG	GGA	TCT	ATC	GAA	GAA	TGC	ACG	CGT	GTT	CTT	GAA	ACA	TTT	CAG	GTG	ATG	GAC
1326	**V**	**D**	**S**	**G**	**S**	**I**	**E**	**E**	**C**	**T**	**R**	**V**	**L**	**E**	**T**	**F**	**Q**	**V**	**M**	**D**
4200	TAT	CTG	GAA	TTT	GTT	ATC	AAG	GAG	GAC	ATA	CAC	GTT	CAG	CAT	CCC	AAA	GAG	GAA	CTT	CCT
1346	**Y**	**L**	**E**	**F**	**V**	**I**	**K**	**E**	**D**	**I**	**H**	**V**	**Q**	**H**	**P**	**K**	**E**	**E**	**L**	**P**
4260	AAG	TTC	GCT	GAT	AGA	CAA	AAG	ATG	AAA	TAC	ACT	ACA	ACC	CTG	ATC	GAA	GAA	CCC	GAG	CCT
1366	**K**	**F**	**A**	**D**	**R**	**Q**	**K**	**M**	**K**	**Y**	**T**	**T**	**T**	**L**	**I**	**E**	**F**	**P**	**E**	**P**
4320	CGA	TGG	AGT	TGT	GGA	GTC	ACA	ATT	GGA	GAG	CGG	TTG	GTA	GGA	GAA	GTC	GAA	GGT	TCT	CTC
1386	**R**	**W**	**S**	**C**	**G**	**V**	**T**	**I**	**G**	**E**	**R**	**L**	**V**	**G**	**E**	**V**	**E**	**G**	**S**	**L**
4380	AAC	AAG	GTT	GAG	GCT	ATG	ACC	AAA	GCT	GCG	GAG	AAT	GCG	ATT	CGA	CTT	TTG	ACA	GAG	GAG
1406	**N**	**K**	**V**	**E**	**A**	**M**	**T**	**K**	**A**	**A**	**E**	**N**	**A**	**I**	**R**	**L**	**L**	**T**	**E**	**E**
4440	ATG	AAT	GCG	GTA	AAT	CAG	CAA	CAA	GAT	GCC	ATG	GAC	GCC	TCC	TAG	GAA	GGG	GAT	CAG	GAA
1426	**M**	**N**	**A**	**V**	**N**	**Q**	**Q**	**Q**	**D**	**A**	**M**	**D**	**A**	**S**	*					
4500	CAC	GCC	TCG	CAG	AGA	GAT	GAC	CTT	GCA	CAG	AAA	CAG	GAC	GAG	GTC	ACG	GCC	GAT	ACT	TCG
4560	CCA	GAG	AAG	GAG	TCC	ACT	GAT	CGT	TTA	ATG	GAA	GGC	ATT	CTA	ACC	GAT	AAT	CCG	GAG	GAC
4620	AAG	GAG	CCG	GTT	GTT	CCT	CCA	AAG	AAA	GAG										

Figure 3.11 : Nucleotide and deduced amino acid sequences of DCL2 ORF. Nucleotide (upper line) and amino acid (lower line). Stop codon is marked with an asterisk (GenBank accession number: MW344087)

3.3.6 Domain analysis

SMART analysis revealed that the deduced *Foc* TR4-DCL1 protein comprising four domains of DEAD-like helicase super family domain (DExDc, residues 77-292, 7.11e-18), Helicase super family c-terminal domain (HELICc, residues 436-534, 9.61e-15) and dual Ribonuclease III family domains (RIBOc; residues 1014-1176, 4.29e-9 and 1218-1384, 4.98e-28). DCL2 transcript encoded deduced protein has five domains of DExDc (residues 65-269, 1.85e-16), HELICc (residues 446–543, 2.35e-9), dual RIBOc (residues 982-1126; 1.04e-16 and 1165-1352; 2.96e-21) and Double stranded RNA binding motif (DSRM; residues 1360-1423; 0.0168) (Figure 3.12).

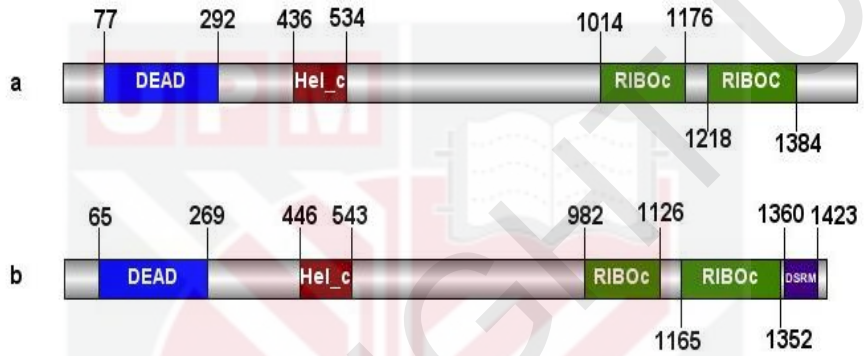
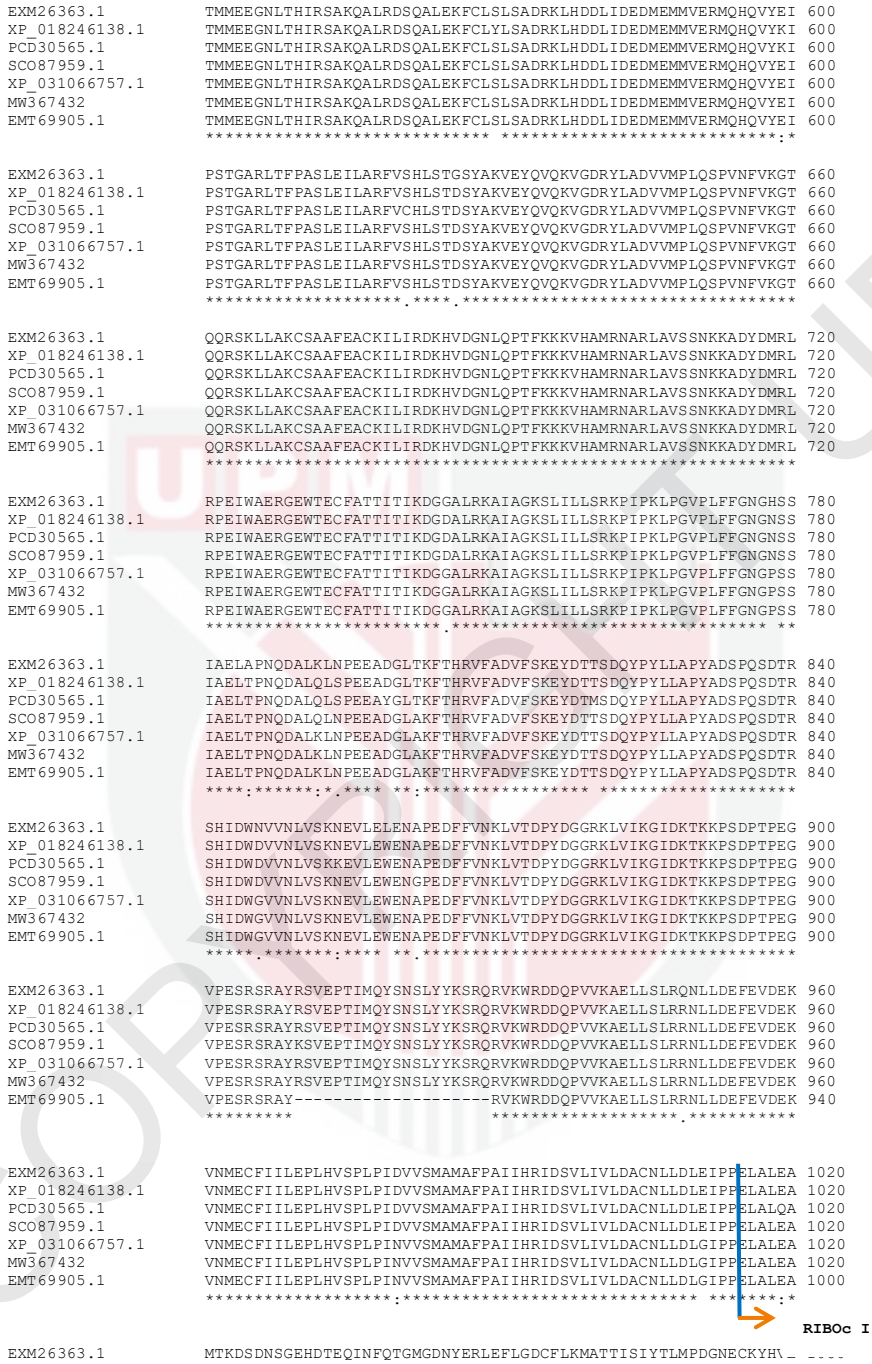


Figure 3.12 : Domain distribution in (a) *Foc* TR4 DCL1 and (b) *Foc* TR4 DCL2

3.3.7 Homology analysis of DCL protein sequences

BLAST homology analysis yielded close matches of *Foc* TR4 DCL1/2 with many other fusarium physiological races. These deduced amino acid sequences shared as high as 98.13 – 99.93% and 98.05 – 100% sequence similarity with other DCL1 and DCL2 fusarium physiological races respectively. Multiple sequences alignment of selected highly homologous species revealed that all domains were conserved among them. The common amino acids were shown in asterisk mark and the domains common to all DCLs were shown between arrows in the vertical column (Figure 3.13 and 3.14). This is further confirmed identified DCL proteins are orthologs of fusarium species DCL proteins.


DExDc
HELICc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PCD30565.1 VPESRSRAYRSVEPTIMQYSNSLYYKSRQRVKWRDDQPVVKAELLSLRRNLLDEFEVDEK 960
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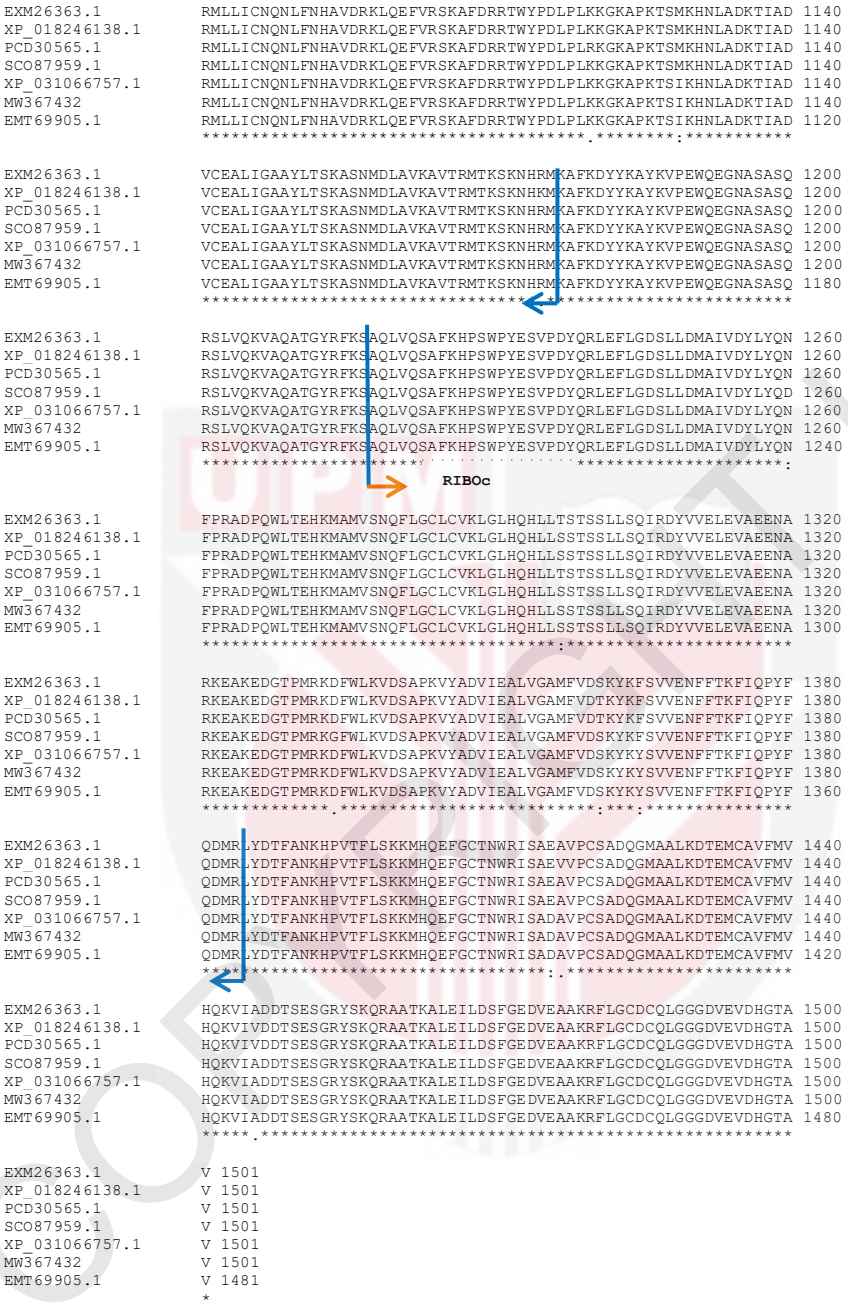

Figure 3.13 : Amino acid sequence alignment of the predicted *Foc* TR4 DCL1 with homologs of other Fusarium formae specials. Conserved residues among species are indicated in stars, Gray colour shade residues are not conserved. EXM26363.1: *Fusarium oxysporum* f.sp. *vasinfectum*, XP_018246138.1: *Fusarium oxysporum* f.sp. *lycopersici*, PCD30565.1: *Fusarium oxysporum* f.sp. *radices-cucumerinum*, SCO87959.1: *Fusarium oxysporum*, XP_031066757.1: *Fusarium odoratissium*, MW367432: *Fusarium odoratissium* (UPM), EMT69905.1: *Fusarium odoratissium*

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EXM25129.1          TYYIVKNTGARLDLDNARQHLVRFCQIVFRSDYVGQLPDYVFHKEETPYGGPILRATVTL 654
RYC87782.1          TYYIVKNTGARLDLDNARQHLDRFCQIVFRSDYVGQLPDYVFHKEETPYGGPILRATVTL 654
XP_018253198.1      TYYIVKNTGARLDLDNARQHLDRFCQIVFRSDYVGQLPDYVFHKEETPYGGPILRATVTL 654
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EXK34908.1          PGGLPLNLRKFKSACGWKSERNAIRDAAFQAYVALYEAGLVTDNLAPLNAKSEKVEEKTD 714
                    *********.*************:************************************

XP_031068194.1      LMPEHLFDPWIQIAQRWKSGCDKQFYLFEFTDDEFPGPCLFDVMLPALIDLPRKITLYPG 774
MW344087            LMPEHLFDPWIQIAQRWKSGCDKQFYLFEFTDDEFPGPCLFDVMLPALIDLPRKITLYPG 774
EMT62501.1          LMPEHLFDPWIQIAQRWKSGCDKQFYLFEFTDDEFPGPCLFDVMLPALIDLPRKITLYPG 780
EXM25129.1          LMPEPLFDPWVQIAQRWKSGCDKQFYLFEFTDDEFPGPCSFDVMLPALIDLPRKITLYPG 774
RYC87782.1          LMPEPLFDPWVQIAQRWKSGCDKQFYLFEFTDDEFPGPCSFDVMLPALIDLPRKITLYPG 774
XP_018253198.1      LMPEPLFDPWVQIAQRWKSGCDKQFYLFEFTDDEFPGPCSFDVMLPALIDLPRKITLYPG 774
EXK34908.1          LMPEPLFDPWVQIAQRWKSGCDKQFYLFEFTDDEFPGPCSFDVMLPALIDLPRKITLYPG 774
                    **** ***** :************************** ********************

XP_031068194.1      NGSKWSIVCDSFVDIPDEVSSKEPDQTSALLAMNFQHRWPVEDREHVIKMSIASTTIFPA 834
MW344087            NGSKWSIVCDSFVDIPDEVSSKEPDQTSALLAMNFQHRWPVEDREHVIKMSIASTTIFPA 834
EMT62501.1          NGSKWSIVCDSFVDIPDEVSSKEPDQTSALLAMNFQHRWPVEDREHVIKMSIASTTIFPA 840
EXM25129.1          NGSKWSIVCDSFVDIPDEVSSKEPDQTSALLAMNFQHRWPVEDREHVIKMSIASTTIFPA 834
RYC87782.1          NGSKWSIVCDSFVDIPDEVSSKEPDQTSALLAMNFQHRWPVEDREHVIKMSIASTTIFPA 834
XP_018253198.1      NGSKWSIVCDSFVDIPDEVSSKEPDQTSALLAMNFQHRWPVEDREHVIKMSIASTTIFPA 834
EXK34908.1          NGSKWSIVCDSFVDIPDEVSSKEPDQTSALLAMNFQHRWPVEDREHVIKMSIASTTIFPA 834
                    ************************************************************

XP_031068194.1      HHVTRDDMGALPFRGDGVEEAKKHRVLVRDQYKTPFHYVGTIPSKPDFYDVQRPFYEYDK 894
MW344087            HHVTRDDMGALPFRGDGVEEAKKHRVLVRDQYKTPFHYVGTIPSKPDFYDVQRPFYEYDK 894
EMT62501.1          HHVTRDDMGALPFRGDGVEEAKKHRVLVRDQYKTPFHYVGTIPSKPDFYDVQRPFYEYDK 900
EXM25129.1          PHVTRDDMGALPFRGYGVEEATKHRVLVRDQYKTPFHYVGTIPSKPDFCDVQRPFYEYDK 894
RYC87782.1          PHVTRDDMGAFPFRGDGMEEAKKHRVLVRDQYKTPFHYVGTIPSKPDFYDVQRPFYEYDK 894
XP_018253198.1      PHVTRDDMGAFPFRGDGMEEAKKHRVLVRDQYKTPFHYVGTIPSKPDFYDVQRPFYEYDK 894
EXK34908.1          PHVTRDDMGAFPFRGDGMEEAKKHRVLVRDQYKTPFHYVGTIPSKPDFYDVQRPFYEYDK 894
                     *********:**** *:***.************************* ***********

XP_031068194.1      GPLNEEYLVLERWGLRVDLLHDLKDGQAKSSTKPYECVLPISYARVDRVKRRVVKCGLFI 954
MW344087            GPLNEEYLVLERWGLRVDLLHDLKDGQAKSSTKPYECVLPISYARVDRVKRRVVKCGLFI 954
EMT62501.1          GPLNEEYLVLERWGLRVDLLHDLKDGQAKSSTKPYECVLPISYARVDRVKRRVVKCGLFI 960
EXM25129.1          GPLNEEYLVLERWGLRVDLLHDLKDGQAKSSTKPYECVLPISYARVDRVKRRVVKCGLFI 954
RYC87782.1          GPLNEEYLVLERWGLRVDLLHDLKDGQAKSSTKPYECVLPISYARVDRVKRRVVKCGLFI 954
XP_018253198.1      GPLNEEYLVLERWGLRVDLLHDLKDGQAKSSTKPYECVLPISYARVDRVKRRVVKCGLFI 954
EXK34908.1          GPLNEEYLVLERWGLRVDLLHDLKDGQAKSSTKPYECVLPISYARVDRVKRRVVKCGLFI 954
                    ************************************************************

XP_031068194.1      PHIIHELEVQLIASELSSTLLKPVGIKNLQLVLEAVSSPSAREPVDYQRLEFLGDSILKF 1074
MW344087            PHIIHELEVQLIASELSSTLLKPVGIKNLQLVLEAVSSPSAREPVDYQRLEFLGDSILKF 1074
EMT62501.1          PHIIHELEVQLIASELSSTLLKPVGIKNLQLVLEAVSSPSAREPVDYQRLEFLGDSILKF 1080
EXM25129.1          PHIIHELEVQLIASELSSTLLKPVGIKNLQLVLEAVSSPSAREPVDYQRLEFLGDSILKF 1074
RYC87782.1          PHIIHELEVQLIASELSSTLLKPVGIKNLQLVLEAVSSPSAREPVDYQRLEFLGDSILKF 1074
XP_018253198.1      PHIIHELEVQLIASELSSTLLKPVGIKNLQLVLEAVSSPSAREPVDYQRLEFLGDSILKF 1074
EXK34908.1          PHIIHELEVQLIATELSSTLLKPVGIKNLQLVLEAVSSPSAREPVDYQRLEFLGDSILKF 1074
                    *************:**********************************************

                                                                  RIBOc

XP_031068194.1      CTVTQAYSEHPSWPEGLLNHFKDRLVSNERLQRVCLEKGLSKFILSKPFTALKWRPRYLD 1074
MW344087            CTVTQAYSEHPSWPEGLLNHFKDRLVSNERLQRVCLEKGLSKFILSKPFTALKWRPRYLD 1074
EMT62501.1          CTVTQAYSEHPSWPEGLLNHFKDRLVSNERLQRVCLEKGLSKFILSKPFTALKWRPRYLD 1020
EXM25129.1          CTVTQAYSEHPSWPEGLLNHFKDRLVSNERLQRVCLEKGLSKFILSKPFTALKWRPRYLD 1074
RYC87782.1          CTVTQAYSEHPSWPEGLLNHFKDRLVSNERLQRVCLEKGLSKFILSKPFTALKWRPRYLD 1074
XP_018253198.1      CTVTQAYSEHPSWPEGLLNHFKDRLVSNERLQRVCLEKGLSKFILSKPFTALKWRPRYLD 1074
EXK34908.1          CTVTQAYSEHPSWPEGLLNHFKDRLVSNERLQRVCLEKGLSKFILSKPFTALKWRPRYLD 1074
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Figure 3.14 : Amino acid sequence alignment of the predicted *Foc* TR4 DCL2 with homologs of other Fusarium formae specials. Conserved residues among species are indicated in stars, Gray colour shade residues are not conserved. XP_031068194.1: *Fusarium odoratissium*, MW344087: *Fusarium odoratissium* (UPM), EMT62501.1: *Fusarium odoratissium*, EXM25129.1: *Fusarium oxysporum* f.sp. *vasinfectum*, RYC87782.1: *Fusarium oxysporum* f.sp. *narcissi*, XP_018253198.1: *Fusarium oxysporum* f.sp. *lycopersici*, EXK34908.1: *Fusarium oxysporum* f.sp. *melonis*

3.3.8 Secondary and tertiary structures

A graphical representation of secondary structures with helices, sheets and turns/ random coils are given in Figure 3.15 and 3.16. DCL1 protein was composed of 63 α-helices, 33 β-sheets and 19 coils whereas 60 α-helices, 32 β-sheets and 19 random coils were predicted in DCL2. The secondary structure prediction result showed that the percentage of alpha helix was much greater than sheets and turns/ random coils. Psipred server predicted DCL1 has 27.18% α-helices, 15.81% β-sheets and 57.01% random coils, while in DCL2 has 27.68% α-helices, 16.56% β-sheets and 55.76% random coils.

Fold recognition with Phyre2 software identified the crystal structure of the human endoribonuclease dicer as the most hit with 100% confidence and 25% identity (c5zamA). Three-dimensional shape of a protein is important for its activity and biochemical functions. (Figure 3.17).

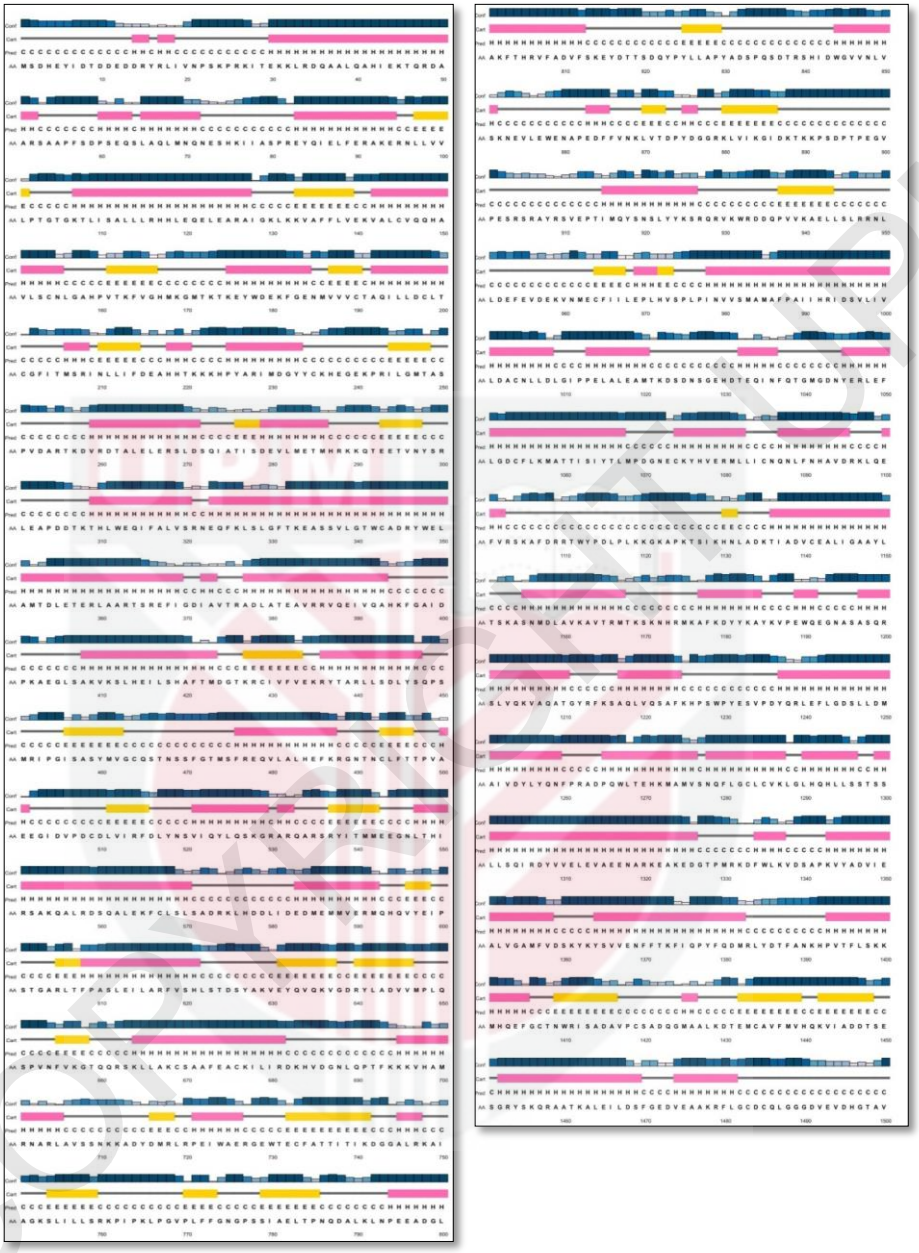


Figure 3.15 : Secondary structure of *Foc* TR4 DCL1 protein predicted with Psipred server. Pink colour- alpha helix, Yellow colour – Beta sheets and Line – Random coil

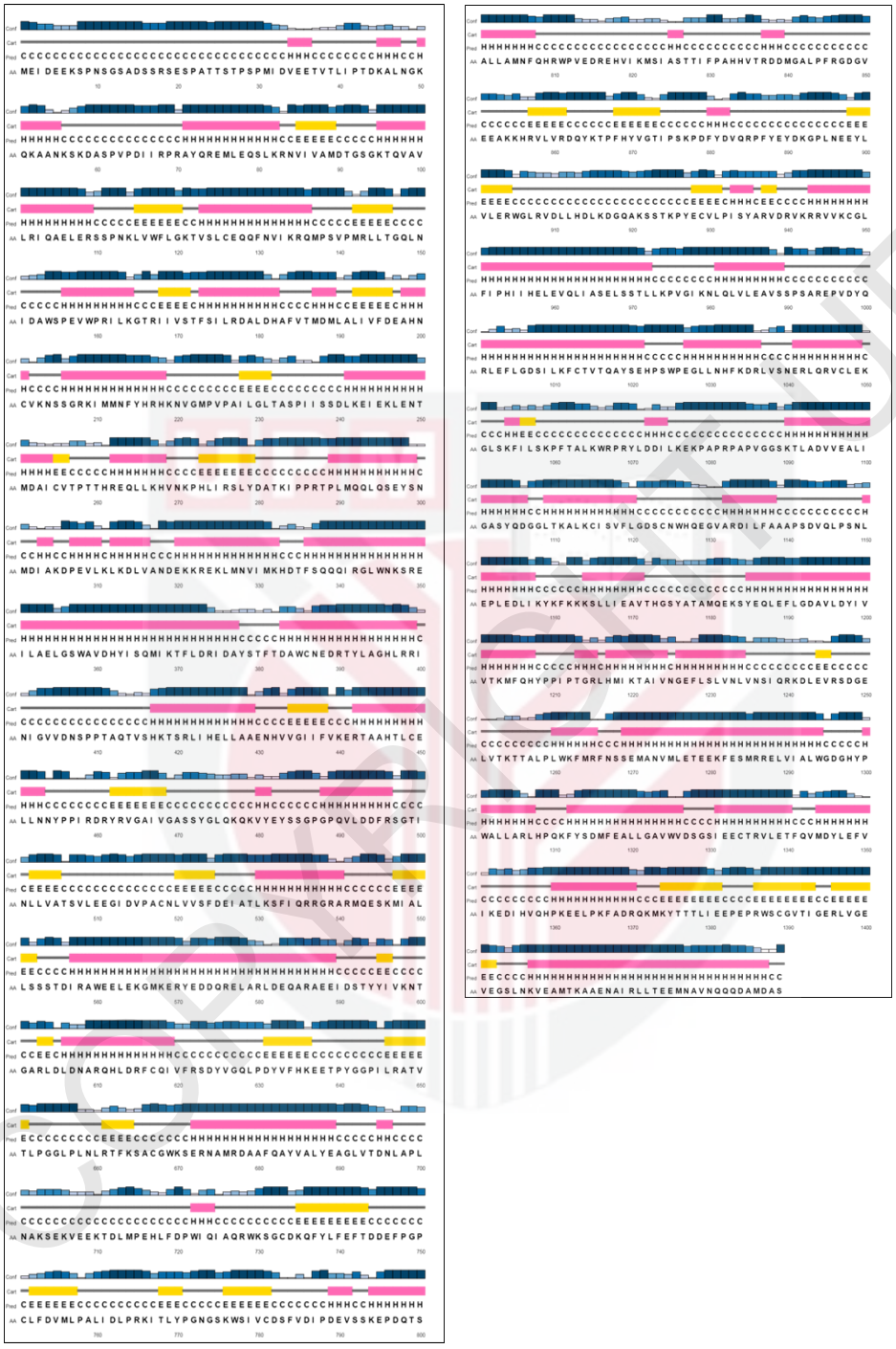


Figure 3.16 : Secondary structure of *Foc* TR4 DCL2 protein predicted with Psipred server. Pink colour- alpha helix, Yellow colour – Beta sheets and Line – Random coil

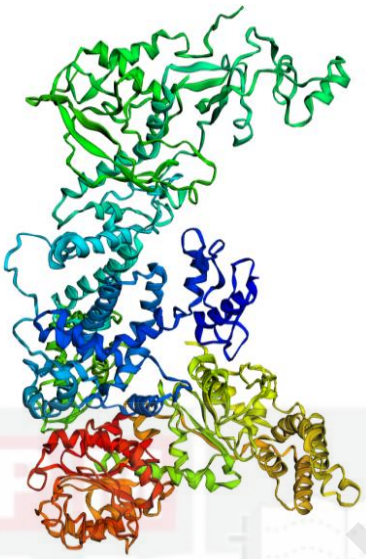


Figure 3.17 : 3D structure of *Foc* TR4 DCL proteins predicted by Phyre2

3.3.9 Phylogenetic analysis of DCL proteins

The phylogenetic trees constructed for DCL1 and DCL2 with DCL proteins of ascomycete fungi using MegaX software with 1000 bootstrap replicates are shown in Figure 3.18 and 3.19. The *Puccinia graminis f. sp. tritici* and *Rhodotorula* sp. were classified as distinct outgroups and separated from primary clades in DCL1 and DCL2 respectively. All *Fusarium oxysporum* species under Nectriaceae family were classified under separate clades in both genes. Identified *Foc* TR4 DCL1/2 also grouped under nectriaceae family clades. DCL1/2 primary clades divided into four subgroups of sodariomycetes, leotiomycetes, dothideomycetes and eurotiomycetes.

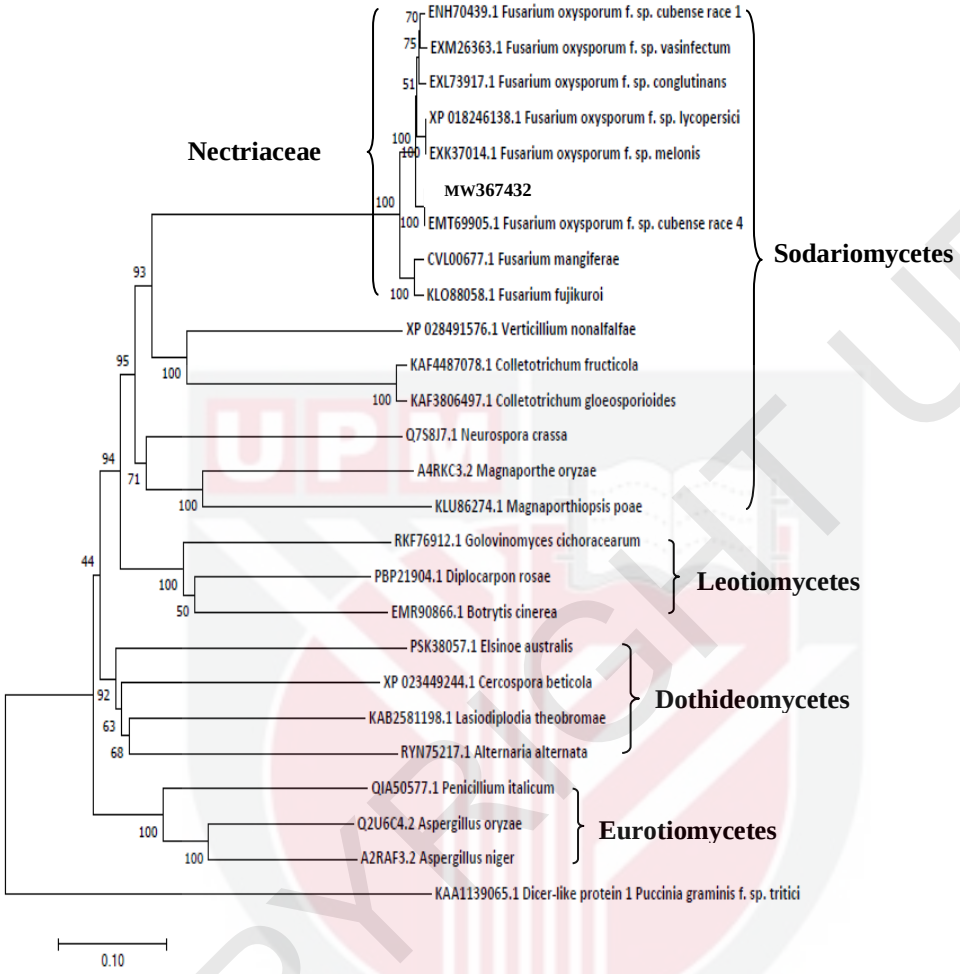


Figure 3.18 : Phylogenetic analysis of *Foc* TR4 DCL1 gene with the selected plant pathogenic ascomycete fungi, Neighbour joining trees with 1000 bootstrap replicates

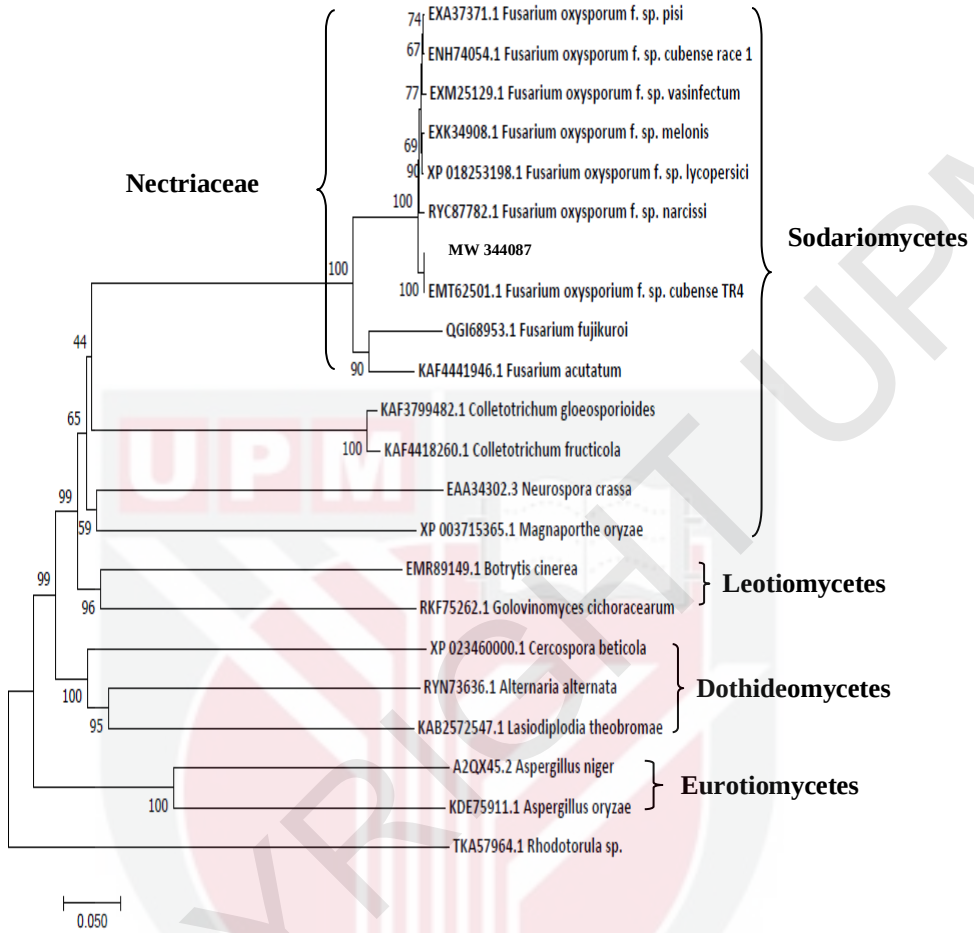


Figure 3.19 : Phylogenetic analysis of *Foc* TR4 DCL2 with the selected plant pathogenic ascomycete fungi, Neighbour joining trees with 1000 bootstrap replicates

3.4 Discussion

This study reports the identification, characterization, and phylogenetic status of two DCL transcripts that encodes DCL proteins in *Foc* TR4 which are considered as key proteins in sRNA production from dsRNAs. DCL proteins have been identified in many plant pathogenic fungi, such as *F. graminearum*, *C. gloeosporioides*, *M. oryzae*, *P. triticina*, *P. infestans*, *P. italicum* and *V. nonalfalfae* (Chen et al., 2015; Dubey et al., 2020; Jeseničnik et al., 2019; Kadotani et al., 2004; Vetukuri et al., 2011; Wang et al., 2018; Yin et al., 2020). The number of DCL proteins differs among distinct organisms whereas its functional diversity varies even in closely related organisms (Kadotani et al., 2004). *M. oryzae* and *N. crassa* encoded two DCLs while *Aspergillus oryzae* and *Aspergillus flavus* encoded three DCLs (Catalanotto et al., 2004; Hammond et al., 2008; Kadotani et al., 2004). *Fg*DCL1 is responsible for sexual ascosporogenesis while

*Fg*DCL2 for micro-like RNA production and asexual conidia formation and germination (Chen et al., 2015; Gaffar et al., 2019). DCL2 in *M. oryzae* is responsible for siRNA production through hairpin RNA (Kadotani et al. 2004). Both DCL1 and DCL2 in *C. gloeosporioides* are collectively govern the fungal growth and conidiation (Wang et al., 2018).

In the predicted protein sequences of *Foc* TR4-DCL1/2, there were highly conserved domain regions among different fusarium physiological races. Domain analysis of the identified *Foc* TR4-DCLs revealed the presence of similar domains as found in a typical DCL proteins except PAZ and dicer dimerization domains. These domains are required for precise cleavage of dsRNA into sRNAs. The available domains vary in different fungi. *P. triticina* has a RNA helicase domain, a dicer dimer domain and two RNase III domains. *Saccharomyces castellii* has one RNase III domain and dual dsRNA binding domains (Dubey et al., 2020). *P. infestans* has a DEXDc, a DUF283 and dual RNase III domains.

The IP is the pH value at which the net overall surface charge of protein is equivalent to zero. It is important for separation and purification of proteins using isoelectric focusing, free flow electrophoresis, capillary electrophoresis and 2-D gel electrophoresis and characterization of physiochemical properties like surface charge and solubility (Audain et al. 2016; Łapińska et al. 2017). According to the IP value results of 6.57 and 5.98 of *Foc* TR4-DCL1 and *Foc* TR4-DCL2 respectively revealed that they are acidic. Previous findings have estimated *P. triticina* DCLs' molecular weight ranged from 155.52 to 185 kDa and IP values ranged from 6.08 to 6.58 (Dubey et al., 2020; Itsathitphaisarn et al. 2017). Also molecular weights of 170.04 kDa and 163.26 kDa in *Foc* TR4 DCL1/2 respectively lied in *P. triticina* IP range (Dubey et al., 2020).

The primary polypeptide sequences of the DCL proteins were utilized to predict its secondary structures. It is the first level of protein folding. The predicted secondary structures are composed of many α-helices, *ß*-strands and random coils. A prediction of the secondary structures of DCL proteins were carried out using PSIPRED server. PSIPRED performed by incorporating output profile of position specific iterated BLAST with two feed forward neural networks (McGuffin et al., 2000). The secondary structures of the DCL proteins are regular repeating folding patterns within these proteins which are stabilized by hydrogen bonds between the amino and keto groups of the peptide bonds. Certain properties of random coils have significant functions in proteins folding and conformational variations.

Homology structure modelling on the Phyre2 server predicted the 3D structures of the DCL proteins. Phyre2 predicts the ligand binding sites and analyse the effect of amino acid variants by comparing homology structures (Kelley et al., 2016). Homology modelling was performed to predict the 3-D structures of DCL proteins, since there is no available experimental record in the protein data bases. Protein 3-D structures aid to the understanding of protein function and active regions.

A phylogenetic tree was constructed to identify evolutionary relationship among *Foc* TR4 DCL proteins and other ascomycetes' DCL proteins using MEGA X software. The consistency of the constructed phylogenetic tree was assessed with bootstrap analysis of 1000 replications. Bootstrap values are showed next to the branches. The scale bar displays the number of substitutions per base. The full-length protein sequences of DCL for selected plant pathogenic ascomycete fungi and one basidiomycete fungi were retrieved from the NCBI database as out group. Within the ascomycetes plant pathogens, four subclusters were observed as sodariomycetes, leotiomycetes, dothideomycetes and eurotiomycetes. Further, within sodariomycetes subgroup, species of family nectriaceae formed a separate group from the rest of the species. All fusarium species grouped together suggesting a common ancestral relationship among these pathogens. In evolutionary relationship of *Fg* DCL1/2 with other ascomycetes fungi showed similar results to this study as species of single class were classified into one clade.

3.5 Conclusion

In this study, two DCL genes were identified and characterized in *Foc* TR4. RACE PCR, cloning and subsequent sequence analysis of *Foc* TR4 DCL1/2 confirmed the presence of typical fungal DCL genes in *Foc* TR4 for the first time. ORF of *Foc* TR4-DCL1 has 1501 a.a. and *Foc* TR4-DCL2 has 1441 a.a. The multiple sequence alignment of DCL1/2 protein sequences from fusarium formae specials showed the conserved domain regions and those formae specials were classified under one clade known as nectriaceae family in phylogenetic analysis. SMART analysis predicted that *Foc* TR4-DCL1 has four domains while *Foc* TR4-DCL2 had five domains. Secondary structure prediction and 3D modelling further provided insight into the spatial arrangement of the amino acids in the proteins.

CHAPTER 4

RNAi-MEDIATED GENE SILENCING OF DICER LIKE 2 GENE AND ITS EFFECT ON FUNGAL GROWTH AND CONIDIATION OF *Foc* TR4 *In Vitro*

4.1 Introduction

RNAi is a conserved cellular defence mechanism mediated by sRNAs which regulate protein expression through targeted destruction or modulation of mRNA molecules (Andika et al., 2015; Fukudome and Fukuhara, 2017). It is a fundamental cellular defence mechanism against invading pathogens and it can be exploited by introducing *in vitro* synthesized dsRNAs or molecules produced in planta against pathogens.

Introduction of RNAi molecules to pest cells and assessing its effect on gene silencing and phenotype changes is the primary step in RNAi based crop protection. *In vitro* assessment of synthetic dsRNA molecules homologous to selected target genes on *Foc* and *Mycosphaerella fijiensis* was performed and adenylate cyclase, DNA polymerase alpha subunit and DNA polymerase delta subunit genes showed high levels of spore germination inhibition in both pathogens (Mumbanza et al., 2013). Similarly *F. graminearum in vitro* studies found *Fg*DCL1 and *Fg*AGO2 mediate sexual ascosporogenesis, *Fg*DCL2 and *Fg*AGO1 contribute to asexual conidia formation and germination while RdRps, QDE3, and QIP strongly affect ascosporogenesis (Gaffar et al., 2019). Preliminary *in vitro* studies help to identify pathogenicity related genes of amino acyl tRNA ligase, thioredoxin reductase and TIM44 of *S. sclerotiorum* could be RNAi silenced effectively and its relative transcript abundance varied with dsRNA dose and time from application (McLoughlin et al., 2018).

The critical step for RNAi-based crop protection strategies is the identification of effective target genes. RNAi silencing of DCLs in some ascomycete pathogen controls its mycelial growth, conidiation and ultimately disease severity. *F. graminearum*, *P. italicum*, *B. cinera*, *C. gloeosporioides* and *P. viticola* were successfully controlled through RNAi of DCLs (Haile et al., 2021; Wang et al., 2016; Wang et al., 2018; Werner et al., 2020; Yin et al., 2020). Also the size of long dsRNA determines the effectiveness of RNAi. Longer dsRNAs exhibited a higher gene silencing efficiency and a stronger disease resistance than shorter dsRNAs. (Werner et al., 2020). RNAi molecules derived from different regions of a gene had different effects on mycelial growth, conidiation and virulence (Gu et al., 2019). Due to these variations, *in vitro* assessment of dsRNAs is important to select effective one rather than expend high cost for production of stable dsRNA for field evaluation.

The objective of this study was to identify the ability of synthesized dsRNA molecules to suppress the gene expression of DCL2 gene and its effect on fungal growth and conidiation of *Foc* TR4 under *in vitro* conditions.

4.2 Materials and Methods

4.2.1 Fungal culture

Mycelial discs of 0.7 cm diameter from 7 days old actively growing mycelia of *Foc* TR4 on PDA were transferred to 250 mL conical flasks, which containing 100 mL of PDB. The cultures were incubated at 25-28°C and 12 h lighting.

4.2.2 Total RNA extraction and cDNA synthesis for preparation of dsRNA

All glasswares used for RNA extraction were dipped in diethyl pyrocarbonate treated water (0.1% V/V) overnight and autoclaved at 120˚C and 15 psi for 20 min. All the surfaces of RNA extraction working area were wiped with RNase inhibitor to inhibit ribonuclease activity. *Foc* TR4 liquid cultures were filtered through a muslin cloth and washed with sterile distilled water to remove PDB media and excess salts. Then fungal mass was kept on a filter paper until evaporating water. Total RNA was extracted using One step RNA reagent (Bio basic) by following manufacturer's protocol. Fungal mass was put into 1.5 mL eppendorf tube and ground to fine powder in liquid nitrogen using micro pestle. One step reagent (1000 µL) and chloroform (200 µL) were added to ground fungal sample just after grinding and shaken vigorously. The sample was then centrifuged at 12,000 g for 15 min at 4˚C and transferred the aqueous phase to new eppendorf tube. For RNA precipitation, 500 µL of isopropyl alcohol was added and kept for 10 min at 15-30˚C. The sample was centrifuged at 12,000 g for 10 min at 4˚C. Pelleted RNA was washed two times with 75% ethanol (1000 µL) by centrifuging at 7,500 g for 10 min at 4°C. The RNA pellet was air dried for 10 min at 15-30˚C and resuspended in 20 µL RNase-free water. RNA sample was stored at -80°C until use.

DNA contamination in extracted RNA sample was degraded using DNase 1 (Thermo scientific, Califonia, USA) according to manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop UV/Vis spectrophotometer whereas integrity was observed on 1% (w/v) agarose gel in TAE buffer using gel electrophoresis. RNA purity was determined by the absorbance ratios of 260/280 (1.8 - 2.0) and 260/230 (> 2.0). After that, total RNA samples were subjected to cDNA synthesis using GoScript Reverse Transcription System (Promega, Madison, WI, USA) by following the manufacturer's specifications. The synthesized cDNA was stored at -20°C until use.

4.2.3 *In vitro* synthesis of long dsRNA

The dsRNAs were synthesized using MEGAscript RNAi Kit (Invitrogen, Carlsbad, CA, USA) following manufacturer's protocols. Gene specific forward and reverse primers for preparation of cDNA amplicon to synthesis dsRNA were designed using SnapDragon online dsRNA design program (http://www.flyrnai.org/snapdragon). The sequence of selected region for dsRNA was analysed using NCBI BLAST tool to confirm divergence from other organisms to avoid potential off-target effects. The complete coding sequence of *Foc* TR4-DCL2 (NCBI accession number: MW344087) was used as the template

sequence for designing primers. The two opposing T7 promoters were attached to 5`end of both forward and reverse primers (Figure 4.1). dsRNA synthesis primers were F: TAATACGACTCACTATAGGGAGAGCCATGCAGGAAAAATCCTA and R: TAATACGACTCACTATAGGGAGACTGTCAAAAGTCGAATCGCA. Annealing temperature of entire PCR primer with the T7 promoter was calculated using New England Biolabs Tm calculator (https://tmcalculator.neb.com) for PCR amplification of dsRNA template. The PCR reaction contained 12.5 µL of high fidelity Q5 2× master mix (New England Biolabs), 2 µL of cDNA (1 µg of total RNA), 0.4 µM of each primer and NFW up to total volume of 25 µL. Thermal cycling conditions were: initial denaturation at 98˚C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 30 sec, extension at 72˚C for 30 sec, then final extension at 72°C for 2 min and soaking at 4°C for infinite. The PCR amplicon was examined on 1.5% agarose gel in TAE buffer (1st Base) stained with Fluoro safe (1st Base) to estimate concentration and verify the single band at expected size. DNA loading dye (1st Base) and 100 bp ladder (1st Base) were used as indicator of PCR product migration and molecular weight size maker respectively. Then the long dsRNA amplicon sequence was confirmed by Sangar sequencing.

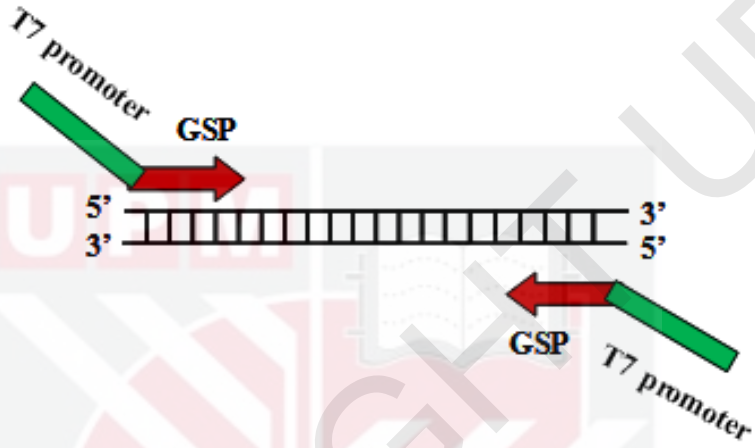
For *in vitro* transcription, *Foc* TR4-DCL2 PCR product (2 µg template RNA), 2 µL from each of 10 × T7 reaction Buffer, ATP solution, CTP solution, GTP solution, UTP solution and NFW up to total volume of 20 µL were added to eppendorf tube. The reaction mixture was mixed thoroughly by pipetting up and down and spun down briefly to collect the reaction mixture at the bottom of the tube. The sample was incubated at 37˚C for 6 h. The complementary RNAs were then annealed by incubating the reaction mixture at 75˚C for 5 min and followed by cooling to room temperature on the bench. Finally, the integrity and efficiency of duplex formation of dsRNAs were assessed by running 1/400th of the dsRNA on 1% agarose gel in TAE buffer using gel electrophoresis.

For digestion of DNA and ssRNAs, the digestion reaction was proceeded by adding 21 µL of NFW, 5 µL of 10× digestion Buffer, 2 µL from each DNase I and RNase to transcription solution. Then the reaction mixture was incubated at 37°C for one h.

For purification of dsRNAs, the dsRNA binding mix was assembled in a 1.5 mL eppendorf tube by adding 50 µL of transcribed dsRNA, 50 µL of 10 × binding buffer, 150 µL of NFW and 250 µL of absolute ethanol. Then the reaction mixture was mixed gently by pipetting up and down. The entire 500 µL dsRNA mixture was put on-to the filter membrane in the filter cartridge using a pipette and centrifuged at 13,500 rpm for 2 min. Then the flow-through was discarded and replaced the filter cartridge in the collection tube. The washing step was repeated two times with 500 µL of 2× wash solution. Wash solution was poured on to the filter membrane in the filter cartridge using a pepette and centrifuged at 13,500 rpm for 2 min. Then the flow-through was discarded and replaced the filter cartridge in the collection tube. After that, last trace of liquid was removed by centrifuging at 13,500 rpm for 30 sec. For recovering the dsRNAs, 50 µL of elution solution was added on to the filter membrane in the filter cartridge and incubated in a heat block set at 65°C for 2 min. Then the dsRNA was drawn out by centrifuging at 13,500 rpm for 2 min. The remaining dsRNA was eluted by repeating the elution step with 30 µL of elution solution. The quantity and purity of dsRNAs were assessed using

nanodrop UV/Vis spectrophotometer and gel electrophoresis by running 1/400th of the dsRNA on a 1% agarose gel in TAE buffer respectively. The eluted dsRNA was stored at -20°C until use.

Figure 4.1 : Annealing of gene specific primers containing T7 promoters to template cDNA



4.2.4 Determination the effective dose of dsRNAs for DCL2 gene silencing *in vitro*

The relative transcript abundance of DCL2 gene in *Foc* TR4 was evaluated after co-incubation with different doses of dsRNAs. A series of 100, 500, 1000, 1500 and 2000 ng/mL of dsRNAs were added to 5 mL PDB in 100 mL conical flasks. A half of 5 mm diameter size mycelial plug taken from the leading edge of the freshly cultured 72 h old fungal colony was put into PDB. Liquid cultures were maintained at 25 ± 2°C in a rotary shaker at 200 rpm in 12 h lighting. Then the tissues were collected 72 h after dsRNA application for relative transcript abundance analysis. Another set of liquid cultures were maintained adding NFW instead of dsRNA as untreated control. These tested doses were selected based on previous studies conducted for controlling different plant pathogens using SIGS (Koch et al., 2018; McLoughlin et al.,2018; Ruiz-Jimenez et al., 2021).

4.2.5 Determination of effective duration of dsRNAs for DCL2 gene silencing *in vitro*

A half of 5 mm diameter size mycelial plug taken from the leading edge of the freshly cultured 72 h old fungal colony was put into 5 mL PDB in 100 mL conical flasks and kept for 24 h. Then DCL2 dsRNA was added to 24 h old PDB cultures at the dose of 1000 ng/mL. Liquid cultures were maintained at 25 ± 2°C in a rotary shaker at 200 rpm in 12 h lighting. The tissues were then collected at 0, 24, 48, 72, 96 and 120 h after

dsRNA application for relative transcript abundance analysis. Another set of liquid cultures without adding dsRNA was maintained as untreated control.

4.2.6 Quantification of relative transcript abundance following dsRNA application

The relative transcript abundance of real time quantitative PCR (RT-qPCR) experiments were designed according to the guidelines of Minimum information for publication of quantitative real time PCR experiments (Bustin et al., 2009). The reference genes were selected based on the previous studies in *Foc* TR4. These include isocitrate dehydrogenase (IDH) and glucose-6-phosphate-1-dehydrogenase (G6DH) (Sutherland et al., 2013). The sequences of reference genes were retrieved from NCBI. The qPCR primers were designed using Primer3 software and analysed in OligoAnalyzer tool in integrated DNA technologies online programme (https://sg.idtdna.com).

The primer specificity for these reference genes and target DCL2 gene were determined by melting curve analysis and gel electrophoresis. Primer sets that amplified a single specific product were chosen for RT-qPCR amplification efficiency test. The standard curves were established for respective genes to assess the PCR amplification efficiency (%). The standard curves for DCL2, IDH and G6DH were generated using a five folds serial dilution of template cDNA in six different concentrations corresponding from 0.072 to 225 ng. The standard curves were generated by plotting the $\log_{10}$ value of template concentration in the dilution series (x-axis) against the quantification value (Cq) obtained (y-axis) using Bio rad CFX manager software. PCR efficiency % was calculated using linear regression equation (Y= mX +C) and coefficient of determination (R^2). The transcript levels of DCL2 gene and two reference genes were determined using Maxima SYBR green qPCR master mix (2×) according to the manufacturer's protocol (Thermo Fisher Scientic Inc., Waltham, MA, USA) in Bio-Rad CFX96 connect real time system. Melting curve analysis was executed straightaway after the PCR reaction from 60°C to 95°C with temperature increment of 0.5°C to assess nonspecific amplification, primer dimers and aberrant amplification. The primer efficiency analysis was carried out with three replicates for each concentration and non-template control (NTC) to detect cross contaminations. The amplicons were purified using GeneJET gel extraction kit (Thermo Scientific) following manufacturer's protocol and the sequences were verified by Sangar sequencing.

The experiment reactions contained 26 ng of first strand cDNA, 0.3 µM of forward primer, 0.3 µM of reverse primer, 5 µl of 2× Maxima SYBR green qPCR master mix and NFW in a total volume of 10 µL. The RT-qPCR conditions were 50°C for 2 min, 95°C for 10 min, followed 40 cycles with each cycle at 95°C for 15 sec, 59°C for 30 sec and 72°C for 30 sec. A NTC was also included in each run for each gene.

Transcript abundance of DCL2 gene was normalized using two reference genes, IDH and G6DH. Geometric mean of two reference genes was used as normalization factor.

The relative fold change of transcript abundance was calculated according to the Livak $2^{-\Delta\Delta Cq}$ method. The relative transcript abundance of DCL2 gene after dsRNA application was obtained by comparing with transcript level of NFW control samples. For statistical accuracy, three technical replicates from each biological sample were used and the average Ct values of technical replicates were calculated. Also, non-template control was used to check the formation of primer dimers with amplified PCR product. (Vandesompele et al., 2002).

4.2.7 Assessment of dsRNAs against mycelial growth

In vitro inhibition effect of DCL2 dsRNAs on mycelial growth of *Foc* TR4 was determined on PDA medium. *Foc* TR4 mycelial plugs of 5 mm diameter and 72 h old were dipped in dsRNA concentration series of 1,000, 5,000, 10,000, 20,000 and 30,000 ng/mL for 24 h at room temperature and blocks were placed on centre of the PDA petri plates. The cultures were incubated at 25-28°C and 12 h lightning. The diameter of mycelia was measured at 1, 3, 5 and 7 days. NFW was used as a negative control instead of dsRNAs.

4.2.8 Assessment of dsRNAs for conidiation

dsRNA was added to PDB at the concentration of 1,000 and 2,000 ng/mL. Spores (micro conidia) were harvested from *Foc* TR4 grown in PDB for 7 days and inoculated into 1.0 mL of PDB with dsRNA to final concentration of 3×10^4/mL spores. Cultures were maintained for three days at 25-28°C in 12 h lightning. Fungal culture was mixed with 3 mL of PDB and filtered through a muslin cloth. Then microconidia density was counted under an optical microscope using a hemocytometer. Similar set of treatment was maintained adding NFW instead of dsRNA as untreated control.

4.2.9 Experimental Design and statistical analysis

dsRNA dose and effective duration evaluation experiments were carried out with three biological replicates in complete randomized design (CRD). In dose level experiment, the tested treatments were 100 ng/mL, 500 ng/mL, 1000 ng/mL, 1500 ng/mL, 2000 ng/mL and untreated control (without dsRNA). In effective duration experiment, treatments were 0 hours post dsRNA application (hpda), 24 hpda, 48 hpda, 72 hpda, 96 hpda and 20 hpda. Also another set of treatments were maintained without dsRNA application and collected fungal mycelia in same time intervals. Conidiation assessment trial was carried out with seven replicates and two parallel experiments were executed for each treatment. Treatments were dsRNA at1000 ng/mL, 2000 ng/mL and untreated (without dsRNA). All the data were analysed using SAS 9.4. Mean separation was done using Tukey's studentized range test with significance levels set at $p < 0.05$.

4.3 Results

4.3.1 Designing and *in vitro* synthesis of long dsRNAs

The Snapdragon programme designed long dsRNA sequence length was 736 bp for *Foc* TR4-DCL2 gene. It was complementary to the ribonuclease III family domain II region and DSRM region located between 3537 bp and 4273 bp within coding sequence of DCL2. In the synthesis of long dsRNAs, cDNA amplicon size and single band availability was verified before *in vitro* transcription using gel electrophoresis while the sequence was confirmed by Sangar sequencing. Band size of cDNA template used for dsRNA synthesis was 782 bp including long dsRNA and T7 promoter on 1% agarose gel (Figure 4.2). Although, the amplified dsRNA templates of DCL2 gene fragment size was expected to be at 782 bp level, it was positioned near to 800 bp on 1% agarose gel after dsRNA preparation, because slow movement of dsRNAs on gel due to high molecular weight (Figure 4.3a). Later, purified dsRNA was again run on gel electrophoresis in 1% agarose gel in TAE buffer to confirm the absence of impurities like DNAs and ssRNAs (Figure 4.3b). The average concentration of extracted dsRNAs was 1360 ng/µL and average total dsRNA yield per dsRNA synthesis kit was 65-70 µg.

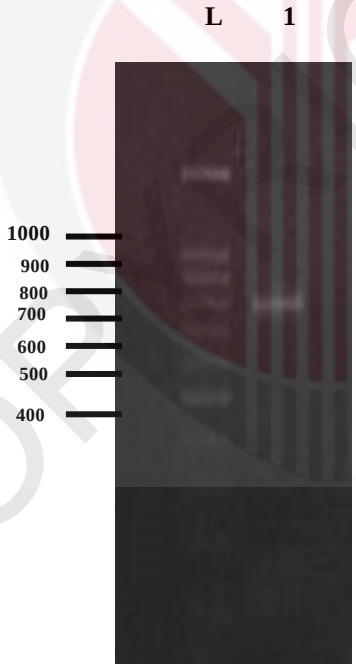


Figure 4.2 : PCR product of *Foc* TR4-DCL2 cDNA template used for dsRNA synthesis. Gel electrophoresis on 1.5% TAE agarose gel stained with Fluoro safe. L = 100 bp DNA ladder, 1 = PCR product of dsRNA synthesis template

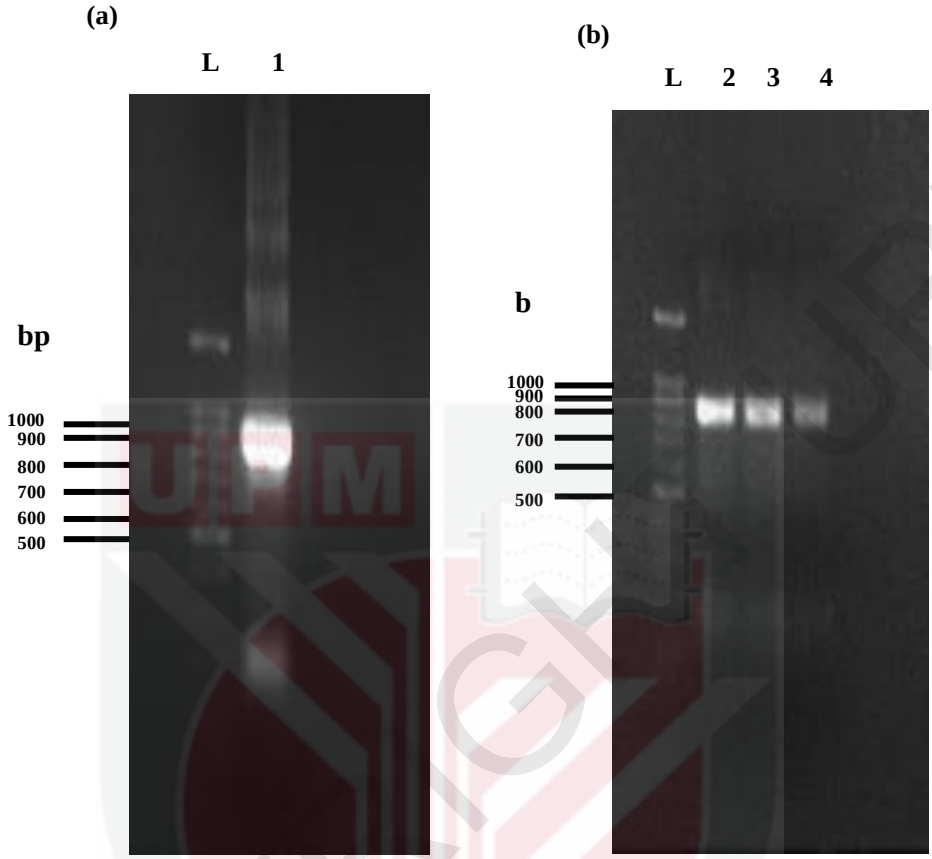


Figure 4.3 : Synthesized *Foc* TR4-DCL2 long dsRNAs. 1/400 diluted dsRNA product in 6× DNA loading dye was electrophoresed on 1% agarose gel stained with Fluoro safe. L= 100 bp DNA ladder, 1,2,3,4 = *Foc* TR4-DCL2 dsRNA products. (a) before purification, (b) after purification

4.3.2 Assessment of relative transcript abundance of *Foc* TR4-DCL2 gene following dsRNA application

4.3.2.1 Quality of extracted total RNAs

The absorbance of 260/280 was between 1.8-2.0 and 260/230 was > 1.7 in all extracted samples. RNA integrities were assessed on 1% agarose gel electrophoresis and it showed 28S:18S rRNA bands with 2:1 intensity ratio (Figure 4.4).

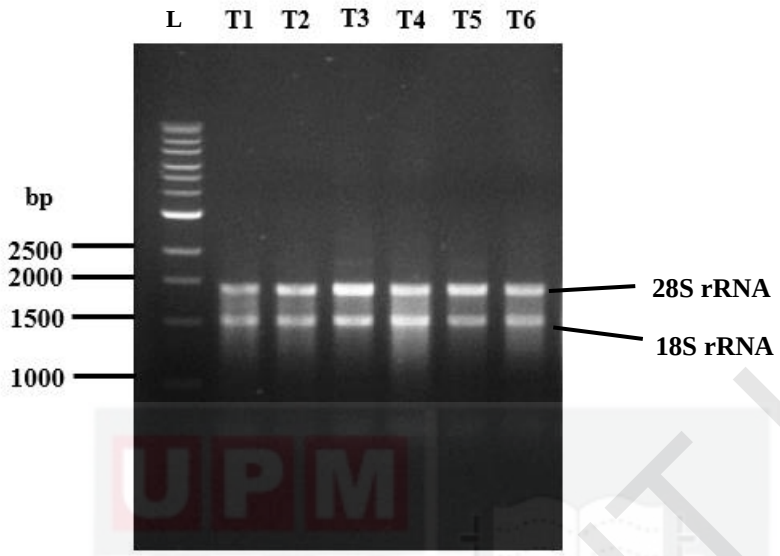


Figure 4.4 : Total RNA extracted from dsRNA treated samples. RNA samples (0.5 µg per well) were run on 1 % TAE agarose gel stained with Fluoro safe. L = 1 kb DNA ladder, T1= 0 ng/mL, T2= 100 ng/mL, T3=500 ng/mL, T4=1000 ng/mL, T5=1500 ng/mL and T6=2000 ng/mL

4.3.2.2 Evaluation of PCR amplification efficiency of target and reference genes

For DCL2 gene expression analysis following dsRNA application, two reference genes of IDH and G6DH was selected based on previous studies on *Foc* TR4 and designed primers using Primer3 (Table 4.1) (Sutherland et al., 2013). The primer annealing temperature was optimized at 59°C for all primer pairs. Standard curves of target and reference genes were performed by 5-fold dilution series of six concentrations. The amplification efficiency of all primer pairs in this experiment were in the range of 94.3 to 98.3% with R^2 value from 0.98 to 0.99 and slope value of -3.46 to -3.36 (Table 4.2) (Diagrammatic explanation is in Appendix D). Accepted parameters for PCR efficiency were ranged from 90 - 110% corresponding to a slope of -3.6 to -3.1 and R^2 >0.98 (Oh and Jang, 2020; Taylor et al., 2015). PCR efficiency of 90.0 to 110.0% and R^2 >0.98 of each primer pair is important to get similar amplification efficiency for accurate comparison each other (Bustin et al., 2009). Amplification efficiency 100.0% indicates that number of target gene molecules is doubled in each amplification cycle during exponential phase. R^2 value indicates the degree to which the experimental replicates fit to regression line. It determines the variation among replicates and whether the efficiency of different initial concentrations is similar. In primer specificity identification, the PCR amplicon of each primer pair exhibited a single peak in the melting curve and a single band with the anticipated size on agarose gel by electrophoresis (Figure 4.5). The amplified region by primer pairs were confirmed by Sangar sequencing (Appendix E).

Table 4.1 : List of primers used in RT-qPCR

Accession number	Gene	Function	Primer sequence	Amplicon size (bp)	Ta (°C)
XM_031196823.1	G6DH	Dissimilation of carbohydrates	F-CAAGCCTGACGAGGCAACAA R-CCCAGAAGCAAGTGCCCATC	110	59
XM_031213248.1	IDH	Oxidative decarboxylation	F-ACCAAGGCCATCCTCGACAA R-CGGCTCCCGCTTTACATACG	136	59
MW344087	DCL 2	Synthesis of siRNA from dsRNA	F-ACCTGGCCCCTCTCAATGCT R-CACAACCCGACTTCCAGCGT	114	59

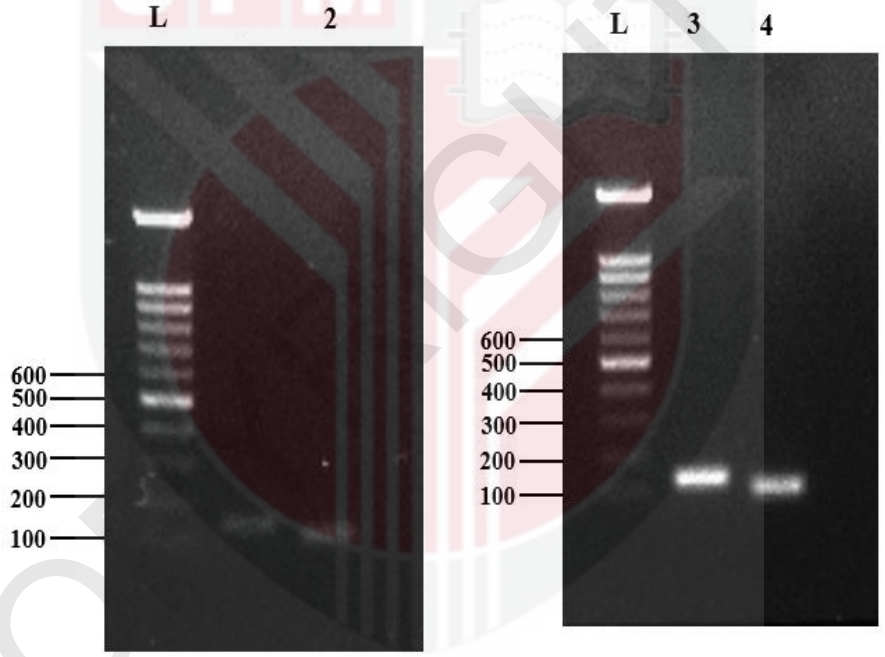


Figure 4.5 : PCR amplified target gene and reference genes of *Foc* TR4. PCR products was electrophoresed on 1.5% TAE agarose gel stained with Fluoro safe. 2= DCL2, 3= IDH, 4=G6DH

Table 4.2 : Primer amplification characteristics of target gene and reference genes in RT-qPCR

Gene	Primer efficiency (%)	R^2 value	Slope value
DCL2	98.30	0.98	- 3.36
G6DH	97.20	0.98	- 3.39
IDH	94.30	0.99	- 3.46

4.3.2.3 Effect of dsRNA dose on relative transcript abundance of *Foc* TR4-DCL2 gene

Foc TR4 liquid cultures were exposed to a range of dsRNA concentrations as 100, 500, 1000, 1500 and 2000 ng/mL. The DCL2 gene showed 22-55% reduction in relative transcript abundance in all tested dsRNA doses except 100 ng/mL compared to the water control after 72 h from dsRNA application. Although, DCL2 gene expression increased in 100 ng/mL, 1000-2000 ng/mL dose range could reduce the DCL2 transcript abundance significantly compared to water control. Also, the target transcript abundance was not decreased further from 1000 ng/mL and it was the minimum dsRNA dose required to elicit significant reduction in relative transcript abundance of *Foc* TR4-DCL2 gene (Figure 4.6).

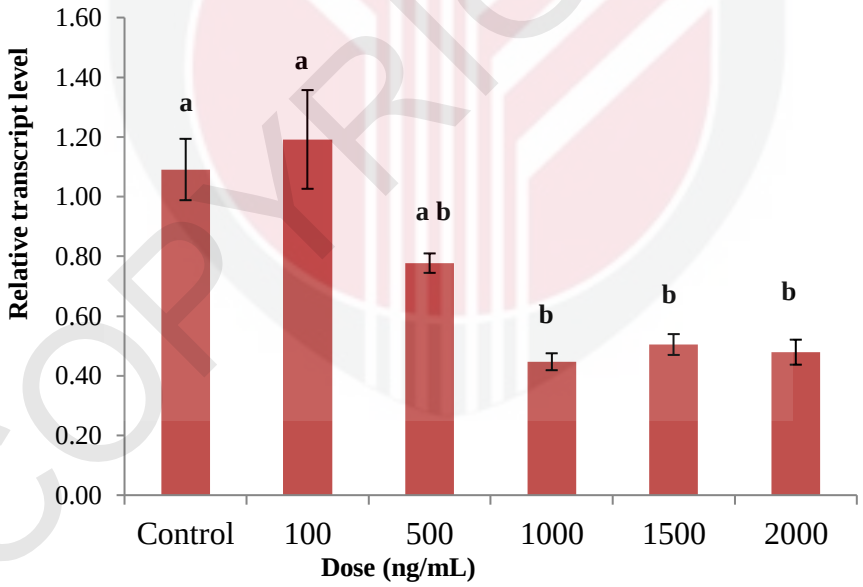


Figure 4.6 : The effect of dsRNA dose on relative transcript abundace of *Foc* TR4-DCL2 gene in vitro. The bars represent ± standard error. One-way ANOVA ($p < 0.05$) was performed and followed by Tukey's studentized range test to compare means, where significant differences are indicated with different letters

4.3.2.4 DCL2 gene knockdown duration following dsRNA application

Foc TR4 liquid cultures were co-incubated with dsRNA at 1000 ng/mL concentration, because this dose was identified as effective for significant transcript abundance reduction. The transcript abundance was reduced significantly within 72 h post dsRNA application compared to water control and thereafter no significant difference up to 120 h. Therefore, the results revealed that 72 h were required for significant *Foc* TR4-DCL2 gene knockdown in *Foc* TR4 *in vitro* (Figure 4.7).

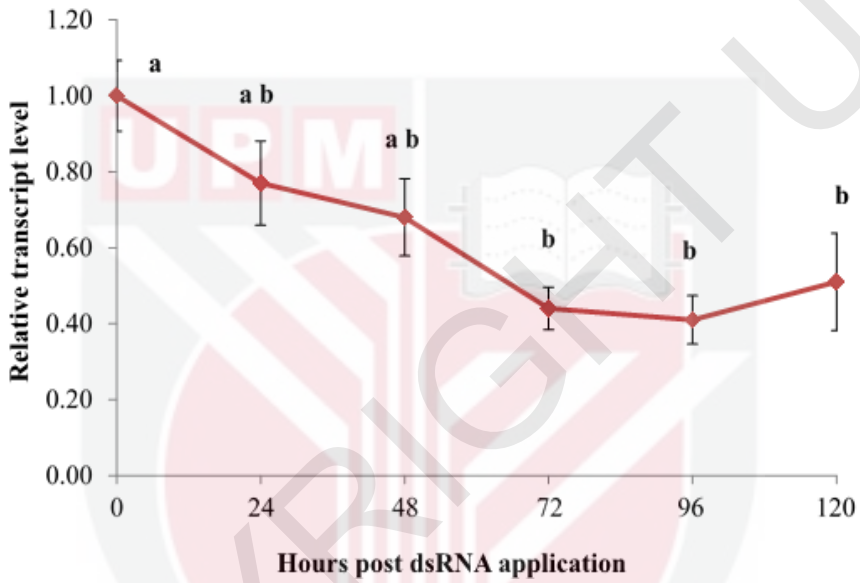


Figure 4.7 : The effective duration of RNAi silencing in *Foc* TR4-DCL2 gene *in vitro*. The bars represent ± standard error. One-way ANOVA ($p < 0.05$) was performed and followed by Tukey's studentized range test to compare means, where significant differences are indicated with different letters

4.3.3 Effect of dsRNAs on mycelial growth of *Foc* TR4

The mycelial plugs of *Foc* TR4 dipped in different doses of dsRNA (10,00, 5,000, 10,000, 20,000 and 30,000 ng/mL) were grown on PDA media. The result showed that the mycelial growth was not responded to selected doses of DCL2 dsRNAs (Figure 4.8).

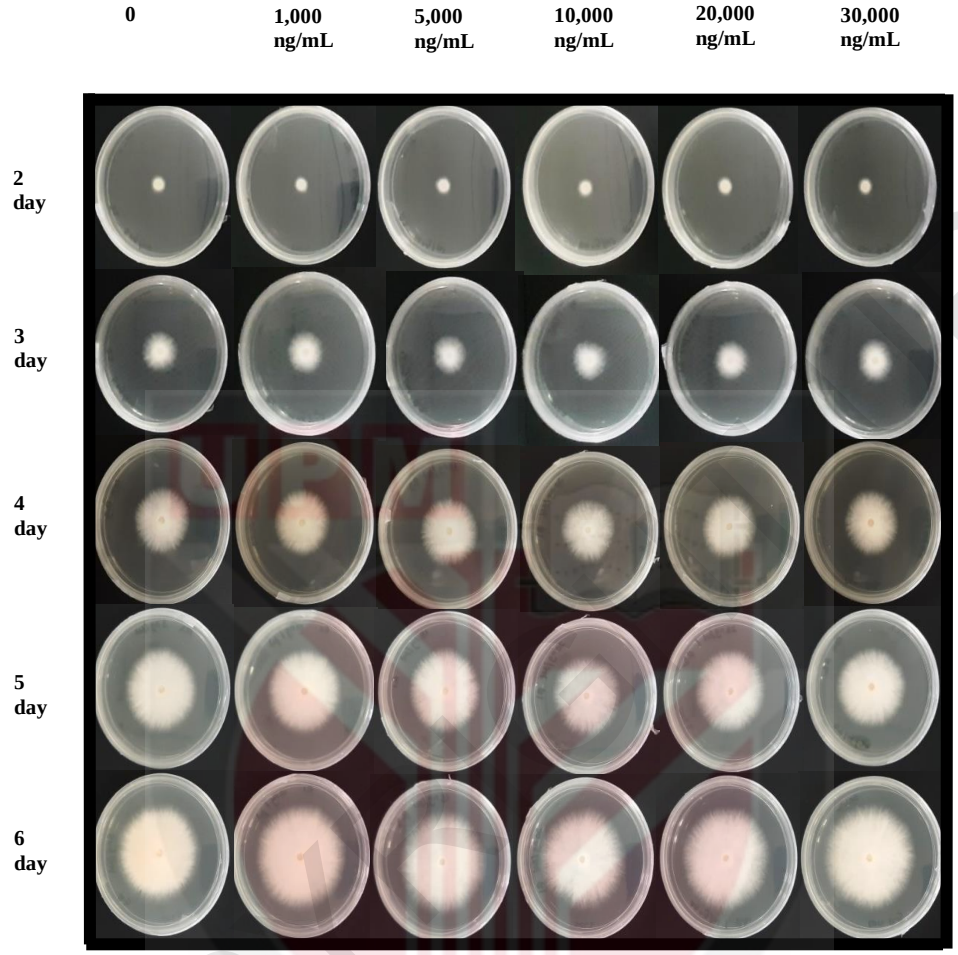


Figure 4.8 : Effect of dsRNA concentration on growth of *Foc* TR4 mycelia. Mycelial plugs were placed on PDA plates which dipped in different doses of *Foc* TR4-DCL2 dsRNAs. The plates were incubated at 25 -28°C

4.3.4 Effect of dsRNA on conidiation of *Foc* TR4

Results of conidiation with different selected doses of dsRNA revealed that micro conidia of *Foc* TR4 was significantly reduced at 1000 and 2000 ng/mL levels compared to without dsRNAs at 3 days after dsRNA application. Micro conidiation was suppressed equally in 1000 ng/mL and 2000 ng/mL dose levels at 3 days after dsRNA application (Figure 4.9).

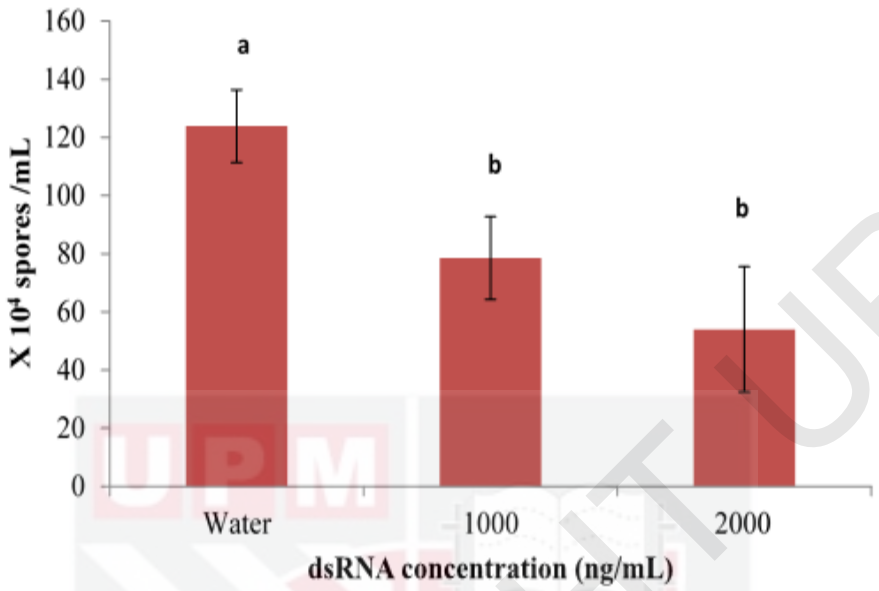


Figure 4.9 Effect of dsRNA concentration on conidiation. The bars represent ± standard error. One-way ANOVA ($p < 0.05$) was performed and followed by Tukey's studentized range test to compare means, where significant differences are indicated with different letters

4.4 Discussion

In this study, focusing on DCL2 gene for gene knockdown was judicious because reported findings proved the fungal pathogens can be controlled through RNAi mediated gene silencing of DCL2 gene (Haile et al., 2021; Koch et al., 2016; Yin et al., 2020). Silencing of DCL2 gene stop the long dsRNA break into siRNAs leading to cease the RNAi mechanism. However, reported studies confer the functional variation of DCLs in pathogenic ascomycete fungi (Haile et al., 2021; Koch et al., 2016; Yin et al., 2020). The role of DCL2 in growth and development of *Foc* TR4 is lack. This study reveals the exogenously applied long dsRNAs can be act on target gene in *Foc* TR4 and leads to gene silencing in first time.

Relative transcript abundance results showed the dsRNA mediated gene silencing in *Foc* TR4-DCL2 gene *in vitro* was success. The expression of DCL2 gene was unaffected at 100 and 500 ng/mL dsRNA doses compared to control. Doses of 1000-2000 ng/mL dsRNA co-incubated with *Foc* TR4 could reduce relative transcript abundance of *Foc* TR4-DCL2 gene confirms the uptake of long dsRNA in to fungal cells and RNAi mediated gene silencing. However, significant transcript abundance reduction was not observed with increasing dsRNA concentration from 1000 ng/mL. It may be due to RNAi silencing components could be saturated with dsRNA molecules and unable to silence all molecules at one time. Transcript level reduction varies 22-55% in this study. Previous studies demonstrated that could be achieved 79-85% transcript level reduction

from 100-1000 ng/mL dose range for amino acyl tRNA ligase gene in *S. sclerotiorum* by dsRNA application. The required minimum dose for significant gene silencing varies with gene. Amino acyl tRNA ligase gene requires 200 ng/mL while thioredoxin reductase, TIM44 and necrosis/ethylene-inducing peptide 2 genes require 100 ng/mL in *S. sclerotiorum*. However, in the same study, the increasing dsRNA gives more transcript level reduction in necrosis/ethylene-inducing peptide 2 gene (McLoughlin et al., 2018). The optimum dsRNA concentration for certain target gene and organism has to be determined for effective RNAi silencing because of oversaturation leads for poor gene silencing (Joga et al., 2016).

Although transcript abundance decreased within 72 h and no changed afterwards in this study, time taken to gene expression reduction varies in different organism. Gene expression of amino acyl tRNA ligase, thioredoxin reductase, and translocase of inner mitochondrial membrane 44 start to reduce within 24 h and do not reduce with increasing respective dsRNA concentrations thereafter in *S. sclerotiorum* under *in vitro* conditions (McLoughlin et al., 2018). It was recorded that MAP kinase Bmp3 gene expression was reduced 24 h after dsRNA incubation and the gene expression reduction increased until 96 h after dsRNA co-incubation in *B. cinerea* (Spada et al., 2021). MAP kinase Bmp3 gene target dsRNAs suppressed the fungal growth in *B. cinerea* within 24 h and achieved 67.5% reduction after 72 hr dsRNA application (Spada et al., 2021).

Silencing of DCL2 did not affect the mycelial growth of *Foc* TR4 while conidiation is reduced in dsRNA treated samples *in vitro*. In this study results match with other ascomycete DCL2 function of regulating sporulation. Conidiation intensifies the reproduction and pathogenicity. This result implies that DCL2 was important for vegetative reproduction of *Foc* TR4. These results suggest that the reduction of conidia production decreased pathogenicity of the *Foc* TR4. The result was accordant with earlier observations on plant pathogenic fungi *F. graminearum*, *P. italicum*, *Botrytis cinera*, *C. gloeosporioides* and *P. viticola* implying that this gene may be a possible target gene for RNAi based plant disease control strategy (Haile et al., 2021; Wang et al., 2016; Wang et al., 2018; Werner et al., 2020; Yin et al., 2020).

The efficiency of gene knockdown of dsRNA depends on sequence length which determines the number of stable siRNAs can be produced. Many siRNAs can be bound with many loci of target mRNA for effective degradation (McLoughlin et al., 2018). The required length of dsRNA to achieve effective silencing varies with insect species. In *Tribolium castaneum*, 60 and 30-bp dsRNAs achieved 70 and 30% of gene silencing respectively (Joga et al., 2016). However, most of the studies tested 140 to 500 nucleotides in length and achieved significant gene silencing (Joga et al., 2016). Although more siRNA duplexes can be bound to more locai in target gene which ensuring optimum gene silencing, multiple dsRNAs create competition for cellular uptake (Joga et al., 2016). Also, dsRNAs with multiple mismatches to target gene would be effective due to no need of 100% base pairing (Gu et al., 2019).

RNAi response relies on the uptake ability of dsRNA molecules by cells. The effectiveness of topical application depends on the RNA uptake efficacy of pathogens (Cai et al., 2019). The uptake mechanism of dsRNA by fungal cells is not elucidated up

to now. Mechanism of movement of small RNAs within fungal colonies is not explored and also studies on this aspect are basically absent. Fungi do not have well defined cellular transportation systems for the transferring nutrients and metabolites in cell to cell. However, movement of long dsRNA or siRNA to fungal cells leads to target gene silencing and sometimes it was continued for several months, suggesting presence of gene silencing, signal amplification and transportation in fungal colonies (Haile et al., 2021; Wang and Dean, 2020; Wang et al., 2016; Wang et al., 2018; Werner et al., 2020; Yin et al., 2020).

Further, location of target gene used for dsRNA synthesis may varies the level of silencing (Joga et al., 2016). Different segments of dsRNA along the β2-tubulin gene of *Fusarium asiaticum* have developed different effects on *F. asiaticum* growth and development. Phenamacril resistance in *Fusarium* spp. is associated with mutations in the myosin-5 gene. This gene based eight different dsRNA segment included eight RNAi transformants showed different response to phenamacril resistance in *F. asiaticum* (Song et al., 2018). However, in the pea aphid, *Acyrthosiphon pisum* study reported that no variation in mortality of insect fed with dsRNA complementary to the 5′ or 3′ end of the hunchback gene. To identify best dsRNA length, need for screening several locations of a gene to identify highly specific target gene or have a broad spectrum activity on closely related species (Joga et al., 2016).

4.5 Conclusion

Foc TR4 DCL2 gene expression reduction confirms the movement of dsRNA in to fungal cells and mRNA degradation of DCL2 gene in this pathogen first time. Co-incubation of dsRNA targeting *Foc* TR4 DCL2 gene effectively reduces the conidiation of the pathogen, supporting the idea that SIGS technology could be used to control fusarium wilt in banana in a sustainable and environmentally friendly manner.

CHAPTER 5

EFFECT OF INJECTED NAKED *Foc* TR4-DCL2 dsRNA ON PATHOGENICITY OF *Foc* TR4

5.1 Introduction

Banana fusarium wilt disease is one of the most widespread and destructive banana disease and a major production constraint to banana worldwide. There was no control measure identified for this disease and only prevention practices were followed to avoid spread the disease to new grounds through quarantine and exclusion, use of pathogen free tissue cultured plants, and preventing replanting of susceptible bananas in infected soils (Ploetz, 2015b; Thangavelu et al., 2019).

The pioneers of RNAi pathway called sRNAs, act on sequence-specific manner in transcript reduction causing translational control. DCL proteins are responsible for production of miRNA and siRNA from hairpin structures or long dsRNAs respectively. Also reported studies proved the DCL proteins are accountable for growth and virulence of plant pathogenic fungi. DCL proteins are responsible for growth, conidiation and pathogenicity of *C. gloeosporioides* in *Hevea brasiliensis* (Wang et al., 2018). Silencing DCL proteins in *B. cinerea* reduce pathogen virulence on fruits, vegetables and flower petals (Wang et al., 2016). *P. viticola* DCL proteins highly affect virulence of pathogen on grape leaves increasing disease progression (Haile et al., 2021). Gene knockdown of DCL impaired pathogenicity of *P. italicum* on citrus fruits (Yin et al., 2020). The 'cross-kingdom RNAi' can act as a natural plant defence mechanism, which allows movement of sRNAs from host to pathogen and vice versa to silence pathogenicity related genes in pathogen and host defence genes (Nowara et al., 2010; Wang et al., 2016). This mechanism based SIGS technology is a prospective crop protection tool among conventional plant protection ways. In this method, dsRNAs and siRNAs are sprayed onto plant surfaces as a topical application by targeting essential genes of pathogens (Wang and Jin, 2017).

Since there is no control measure for *Foc* TR4, studying RNAi based fungicide against this pathogen is an alternative to investigate. Prior to development of RNAi based fungicide for field application, the preliminary glasshouse study should be carried out to confirm the efficacy of the product. Thus, the aim of this study was to identify the effect of naked DCL2 dsRNAs on pathogenicity of *Foc* TR4 in banana plants. In addition, to determine the effective duration of DCL2 dsRNAs in plants and stability of naked dsRNA under glasshouse conditions.

5.2 Materials and Methods

5.2.1 *Foc* TR4 conidial solution

Foc TR4 cultures recovered on PDA medium was used to prepare liquid cultures. Three days old and 5 mm diameter fresh mycelial plugs were transferred to PDB and maintained at 200 rpm, at 25-28°C and 12 hours lighting for 5 days. Sterilized distilled water was added to cultures and dislodged the conidia using a glass rod. Liquid cultures were then filtered through two layers of muslin cloth. Conidia density of the suspension was adjusted to 1×10^5 conidia/mL using hemocytometer (Al-Hetar et al., 2011).

5.2.1.1 Determination of dsRNA application timing

Tissue culture 'Berangan' banana plants which are susceptible to banana fusarium wilt were purchased by Horus green SDN BHD, Klang, Malaysia. Two months old banana plants were re-potted into 35 * 35 cm size black colour poly bags containing sterilized coco peat as potting media. Plants were maintained under 12 h light and dark cycle for six months at glasshouse under 28-35°C temperature and 70-80% relative humidity. At the plant establishment, 10 g of commercially available fertilizer mixture (Nitrogen: phosphorus: potassium fertilizer 15-15-15 with trace elements) was added and continued in one month interval as 20 g per pot. Plants were watered every other day. Banana plants were maintained for six months to allow for ample root development before they were used in the in vivo evaluations of dsRNAs.

Plants with roots of more or less uniform diameter (0.7 mm ±0.2 mm) were selected for glasshouse evaluation. All roots selected for dsRNA injection were surface sterilized with 70% ethanol. A small wound was made using a needle for injection of dsRNAs and spores to roots using a 10 µL volume pipette. Three dsRNA application times were tested, namely 24 h before, 24 h after and simultaneous injection of *Foc* TR4 spore solution and dsRNA. dsRNA was diluted in NFW at the concentration of 25 ng/µL and injected 2 µg dsRNA per application. This dose level was selected based on previous research findings on SIGS of ascomycetes plant pathogens (McLoughlin et al., 2018; Hu et al., 2019; Ruiz-Jiménez et al., 2021). Also, one set of plants were maintained injecting NFW instead of dsRNA as untreated control. For pathogen infection, 20 µL of 1×10^5 spores/mL was injected to same wound of dsRNA injected. Lesion length was measured 7 days post infection (DPI). Plants were laid out CRD with five replicates in glasshouse. Average lesion lengths were recorded and percentage (%) lesion length reduction compared to water control was calculated.

$$\% \text{ Lesion length reduction} = \frac{\text{Lesion length of water control} - \text{Lesion length after dsRNA injection}}{\text{Lesion length of water control}} * 100$$

5.2.1.2 Determination the effective duration of dsRNAs

Berangan banana plants were maintained for six months until root development as described in previous section in this study. Selected roots were surface sterilized with 70% ethanol and made a small wound using a needle for injection of dsRNAs and spores to roots. dsRNA was injected into roots 24 h before *Foc* TR4 spore solution injection at the rate of 2 µg dsRNA per application. For infection, 20 µL of 1×10^5 spores mL^{-1} was injected to same wound of dsRNA injected. Also another set of plants were maintained injecting NFW instead of dsRNA as untreated control. Lesion length and relative transcript abundance were measured at 3, 5, and 7 days after fungal spore inoculation. Plants were laid out in CRD with five replicates in glasshouse. AUDPC values were calculated for disease severity assessment. For RT-qPCR assessment, three randomly selected plants were used for each time interval.

5.2.1.3 Determination the dsRNA efficiency to suppress fusarium wilt in banana plants

The de-flasked tissue culture banana plants planted in cell trays were purchased. Plants were then potted into 12.5 cm height and 14.5 cm width plastic pots containing sterilized coco peat as potting media. Plants were maintained under 12 h light and dark cycle for 8 weeks at glasshouse under 28-35°C. At the plant establishment, 3 g of commercially available fertilizer mixture (Nitrogen: phospourus: potassium fertilizer 15-15-15 with trace elements) was added to each pot and plants were watered every other day.

Potting media was removed from one side of pot to appear rhizome area and surface sterilized with 70% ethanol. dsRNA diluted in NFW at the concentration of 25 ng/µL was injected to rhizome area at the rate of 2 µg dsRNA per application. In the following day 24 h after, the plants were dipped in *Foc* TR4 spore solution at the concentration of 1×10^5 spores/mL for 15 min. Another set of inoculated plants were treated with sterile distilled water instead of dsRNA as untreated control. Plants were uprooted at 07 and 14 days intervals from pathogen inoculation. Disease severity as a percentage of total corm area were recorded using ImageJ software and relative transcript abundances were recorded at 07 and 14 days intervals from pathogen inoculation. Experiment was carried out CRD with five replicates in glasshouse. Randomly selected three plants were used for RT-qPCR analysis in each time interval.

5.2.1.4 Total RNA extraction and cDNA synthesis of plant samples

Root and rhizome samples were destructively collected in an aseptic manner by sterilizing knives and gloves with a solution of 15% (v/v) sodium hypochloride (NaOCl) between samples to prevent cross contamination. Samples from the field were separately washed in running water and then surface sterilized using 15% v/v NaOCl mixed few drops of Tween 20 to eliminate external contamination. Samples were thoroughly rinsed with sterile distilled water to remove excess NaOCl and blotted dry using paper towels. Root and rhizome samples including pathogen infected area were cut using sterilized scalpel blade. Each sample was cut to small species and weighted 1.5 g from each. Root

and rhizome samples were ground to fine powder in liquid nitrogen using prechilled sterile mortars and pestles.

Total RNA was extracted from root and rhizome samples using One Step RNA reagent (Bio basic) by following manufacturer's procedure. NFW diluted RNA samples were treated with DNase 1 (Thermo scientific, Califonia, USA) by following manufacturer's instructions to degrade DNA contamination. RNA concentration and purity was recorded using a NanoDrop UV/Vis spectrophotometer while integrity was assessed using gel electrophoresis on 1% (w/v) agarose gel in TAE buffer. The extracted total RNA samples were used for cDNA synthesis using GoScript reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's specifications and synthesized cDNA was stored at -20°C until use.

5.2.1.5 Quantification of relative transcript abundance in plant samples

Adhering to minimal MIQE guidelines, RT-qPCR was carried out using IDH and G6DH as reference genes, as previously reported. Any contamination was verified using NTC. RT-qPCR system with the Maxima SYBR green qPCR master mix (2×) (Thermo Fisher Scientic Inc., Waltham, MA, USA) were performed in a Bio-Rad CFX96 Connect Real-Time system. The 10 µL reaction mix contained 2.0 µL cDNA (26 ng of total RNA) templates, 5.0 µL 2× Maxima SYBR green qPCR master mix, 1.0 µL each primer (10 µM) and NFW 1.0 µL. Three independent biological replicates and three technical replicates for each biological replicate were arranged in RT-qPCR assessing. The RT-qPCR conditions were 50°C for 2 min, 95°C for 10 min, followed 40 cycles with each cycle at 95°C for 15 sec, 59°C for 30 sec and 72°C for 30 sec. Relative transcript abundance of DCL2 gene was normalized using geomean value of IDH and G6DH Ct values. The relative transcript abundance of target gene was calculated according to the Livak 2-ΔΔCq equation. The relative transcript abundance of DCL2 gene in dsRNA treated samples were obtained by comparing with its transcript level in NFW control samples (Vandesompele et al., 2002).

5.2.2 Assessment the stability of dsRNA in controlled environmental conditions

In order to evaluate the dsRNA stability in open environment condition, 10 µL of known dilution of dsRNAs (35 ng/µL) was put into each 200 µL volume PCR tubes and kept inside the glasshouse. Daily average temperature and relative humidity were 28-35°C and 75-80% respectively in the glasshouse. The dsRNA samples were collected at 0, 3, 5, 7 and 14 days after and stored in -20°C freezer. For visualization the dsRNA stability, 5 µL of dsRNAs were mixed with 1 µl of 6×loading dye and loaded on 1% agarose gel. The gel picture was photographed using UV gel documentation imaging system (Gel Doc XR+ system - Bio Rad). Experimental design was CRD with four replicates. Experiment was repeated two times.

5.2.3 Assessment the stability of dsRNA for UV radiation

To investigate dsRNA stability under UV rays, 10 µL of dsRNAs (35 ng/µL) was put into each 200 µL volume PCR tubes and kept inside laminar flow cabinet under direct exposure of UV rays at 254 nm intensity. The dsRNA samples were collected at 0, 2, 4, 6 and 8 h after sample establishment and stored in -20°C freezer. Naked dsRNA band intensity was visualized on 1% agarose gel by loading 5 µL of dsRNAs were mixed with 1 µl of 6×loading dye. The gel picture was photographed using UV gel documentation imaging system (Gel Doc XR+ system - Bio Rad). Assessment was carried out CRD with four replicates. Experiment was repeated two times.

5.2.4 Statistical analysis

Lesion length and infected area results were reported as mean ± SE of five independent biological replicates and differences of gene expression between dsRNA treated and water control were compared by student's t-test. Lesion length, infected area and AUDPC values were analyzed using one-way ANOVA ($p < 0.05$) and followed by Tukey's studentized range test to compare means. All statistical analyses were performed using SAS 9.4 software with $p < 0.05$ considered significant.

5.3 Results

5.3.1 Quality of RNA samples

In assessing the RNA purity, the absorbance of 260/280 was recorded between 1.8-2.1 and 260/230 was > 1.7 in all extracted dsRNA treated and untreated samples. Samples run on 1.0% agarose gel electrophoresis in TAE buffer appeared the presence of clear visible 28S and 18S rRNA bands in 2:1 ratio indicating good quality RNA in all extracted samples (Figure 5.1).

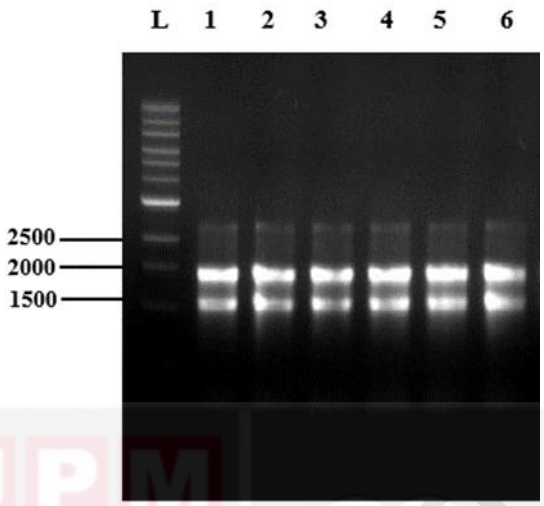


Figure 5.1 : Representative total RNA samples run on 1% TAE agarose gel stained with Fluoro safe. RNA samples in one well contained 0.5 µg of total RNAs. L = 1 kb DNA ladder, 1-6: Extracted total RNA samples

5.3.2 Time of dsRNA application

The pathogenicity of the *Foc* TR4 with time of DCL2 dsRNA injection was tested in roots. The lesion lengths at different dsRNA application times with respect to time of pathogen infection were not significantly differ at 7 DPI, but they were significantly low compared to water control. There was a reddish brown small lesion on sterilized distilled water applied treatment without *Foc* TR4 in images due to root tissues damaged by needle (Figure 5.2 and 5.3). By 7 DPI, disease development was low 56.40% in 24 h pre-treatment, 51.38% in 24 h post treatment and 58.22% in dsRNA and *Foc* TR4 simultaneous treatment compared to water control in terms of % lesion length reduction. Thus. The ability of *Foc* TR4 to infect roots was significantly limited in DCL2 dsRNA injected roots.

Pre treatment | **Post treatment** | **Simultaneous application** | **Water control** | **Sterilized distilled water (No *Foc* TR4)**

Figure 5.2 : Representative images of infection lesion lengths of roots at 7 DPI following dsRNA application

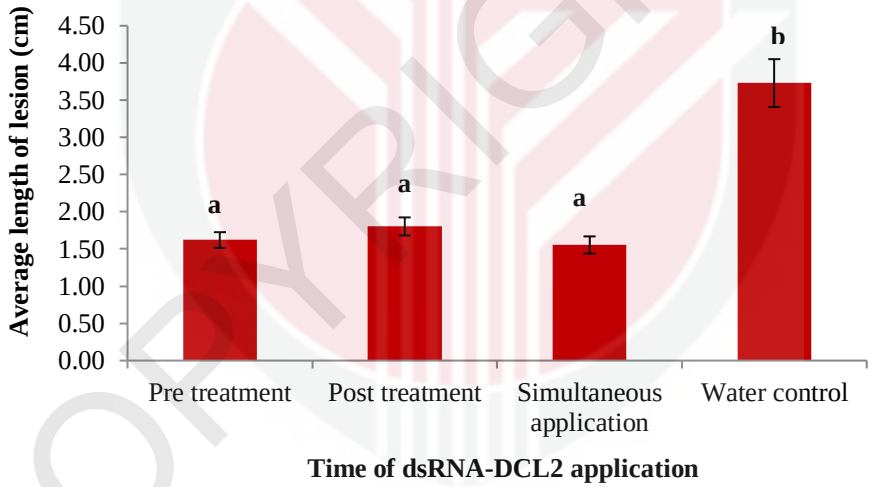


Figure 5.3 : Variation of average lesion length at 7 DPI following dsRNA application at different time. The bars represent ± standard error. One-way ANOVA ($p < 0.05$) was performed and followed by Tukey's studentized range test to compare means, significant differences are indicated with different letters

5.3.3 Effective duration of dsRNA following root injection

A preliminary inoculation assay was conducted to determine the effective duration of DCL2 dsRNAs that could silence *Foc* TR4 DCL2 gene and consequently suppress disease development. Inoculated roots were monitored on 3, 5, and 7 DPI. The lesion

lengths were significantly decreased on 5 and 7 DPI in dsRNA treated samples compared water control (Figure 5.4). The disease development was low 7.63%, 20.63% and 24.84% in 3, 5 and 7 DPI respectively than water control in terms of % lesion length reduction. According to AUDPC values, DCL2 dsRNA can suppress fusarium wilt disease progression in roots (Figure 5.5). Also relative transcript abundance of DCL2 gene in infected root tissues revealed that DCL2 was significantly decreased on 5 and 7 DPI compared to water control (Figure 5.6). These results suggested that DCL2 gene participates in pathogenicity of *Foc* TR4 and dsRNA-DCL2 was effective for 7 DPI under *in planta* conditions.

Figure 5.4 : Representative images of infection lesion lengths of roots at 3, 5 and 7 DPI following dsRNA application

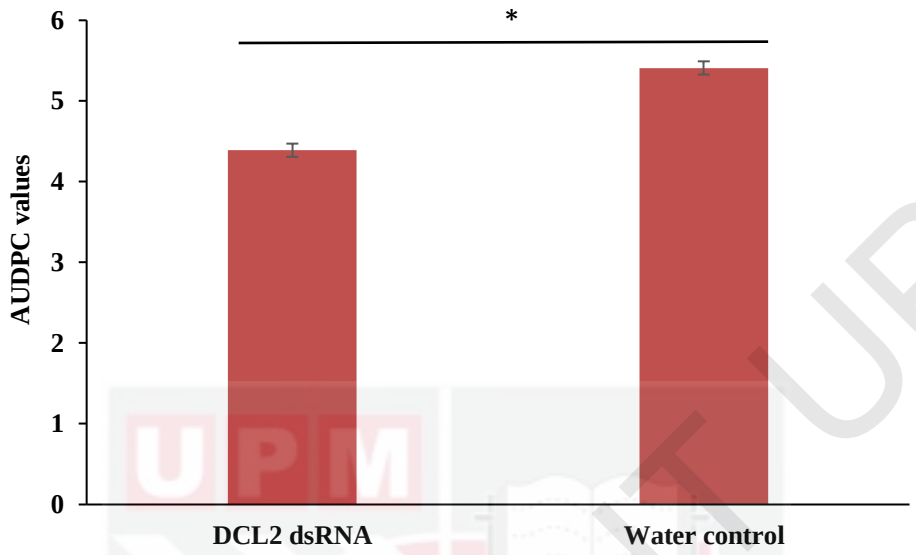


Figure 5.5 : AUDPC values on 7 DPI in roots following dsRNA application. The bars represent ± standard error. Differences were assessed using Student's t-test. where '*' mark represents significant difference

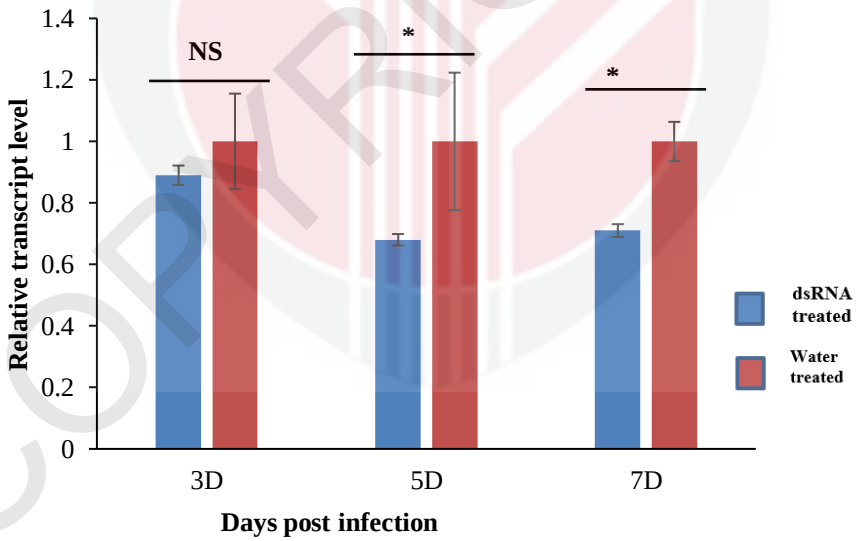


Figure 5.6 : Relative transcript level measured at 3,5 and 7 DPI in roots following dsRNA application. The bars represent ± standard error. Differences at each time interval were assessed using Student's t-tests. where '*' marks represent significant difference

5.3.4 Disease development in rhizome following DCL2 dsRNA injection

Banana rhizomes injected with dsRNA targeting DCL2 gene being inoculated with *Foc* TR4 were found to have lower infection compared to water control on 7 DPI and thereafter no disease suppression observed on 14 DPI (Figure 5.7 and 5.8). The disease severity reduction was 36.5% on 7 DPI compared to water control. However, the control banana rhizome injected with water and dsRNA injected rhizome equally infected on 14 DPI. The expression of DCL2 gene was also reduced to 26.0% of that in banana rhizomes treated with dsRNA on 7 DPI (Figure 5.9). This disease severity and transcript abundance variation with time, suggesting that DCL2 dsRNA was effective for 7 DPI and no regeneration of siRNAs to silence DCL2 gene therafter.

Figure 5.7 : Representative images of infection of rhizomes at 7 and 14 DPI following dsRNA application

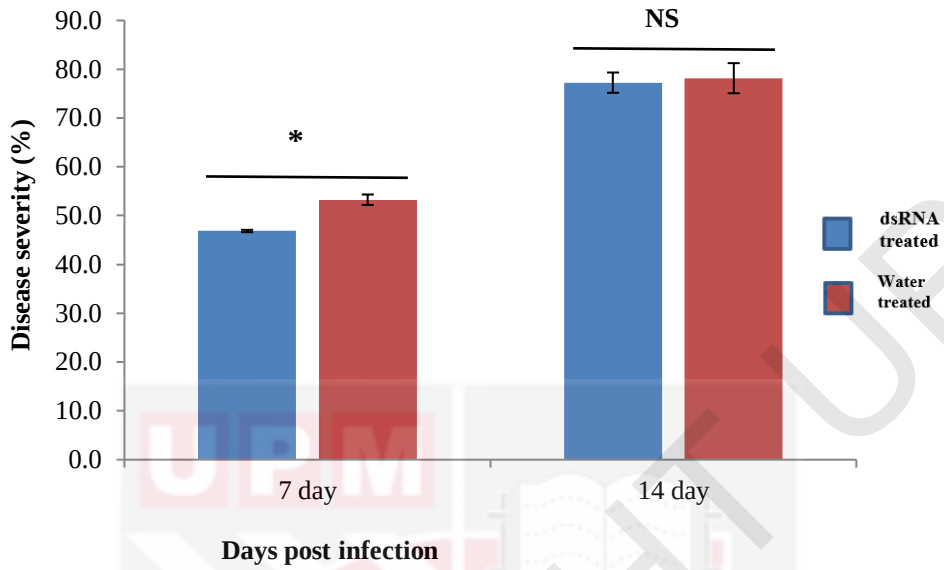


Figure 5.8 : Disease severity variation in rhizomes on 7 and 14 DPI following dsRNA application. The bars represent ± standard errors. Differences were assessed using Student's t-test in each time interval, where '*' marks represent significant difference

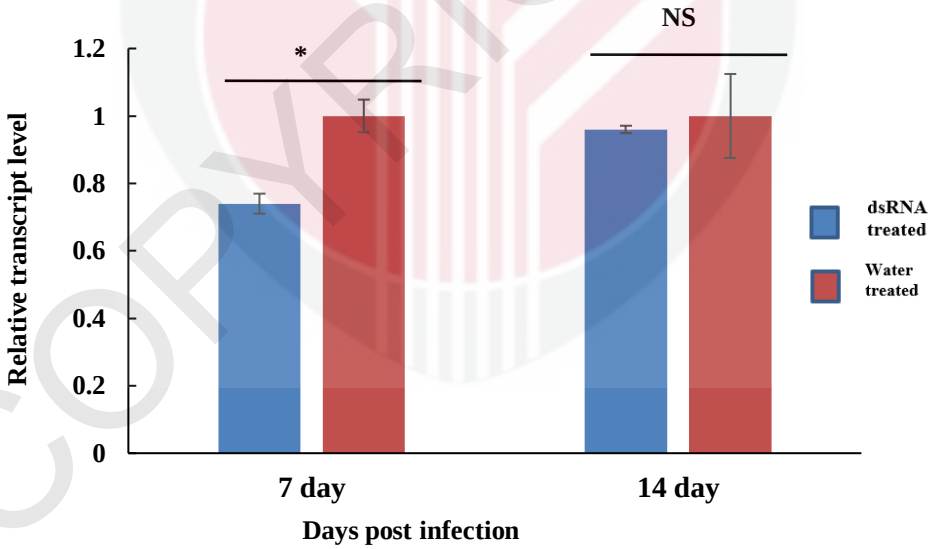


Figure 5.9 : Relative transcript levels measured at 7 and 14 DPI in rhizomes following dsRNA application. The bars represent ± standard error. Differences at each time interval were assessed using Student's t-tests in each time interval. where '*' marks represent significant difference

5.3.5 Stability under controlled environmental condition

The environmental stability of dsRNA is a key factor that affects the application of RNAi fungicides under field level. To examine field stability of naked dsRNA from degradation, an experiment was set up to test the integrity of dsRNA, using gel electrophoresis after incubation in glasshouse. DCL2 dsRNA solution incubated under glasshouse condition was stable after 14 days and showed no signs of degradation on 1% agarose gel. Band intensities were observed in equal intensities on 0, 3, 5, 7, 14 days after exposure to glasshouse conditions (Figure 5.10).

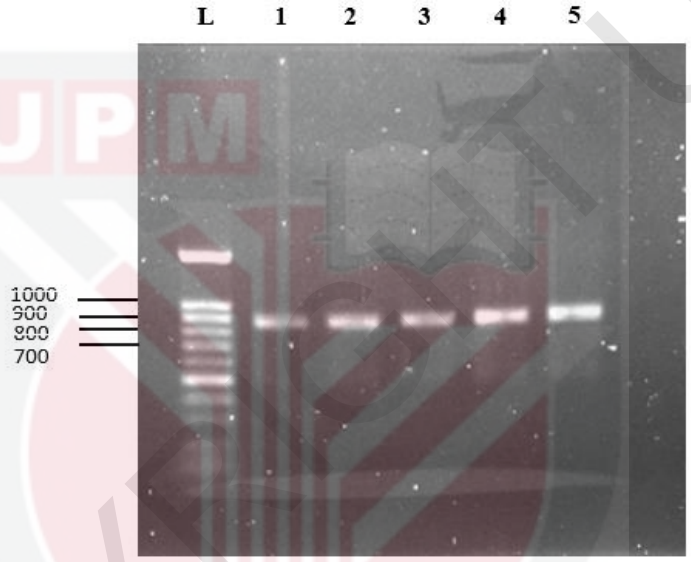


Figure 5.10 : dsRNA samples kept under controlled environmental conditions run on 1% agarose gel in TAE buffer. L- 100 bp ladder, 1 - 0 day, 2 – 3 day, 3 – 5 day, 4 – 7 day and 5 – 14 day

5.3.6 Stability under UV exposure

To examine naked dsRNA stability under UV light, a similar experiment to above was conducted inside laminar floor cabinet and observed dsRNA band intensities using gel electrophoresis. After 6 h exposure to UV rays, naked DCL2 dsRNAs degraded rapidly in all replicates and was no longer detected on 1% agarose gel after 8 h. However, dsRNA band intensities were similar until 6 h after exposure to UV light (Figure 5.11).

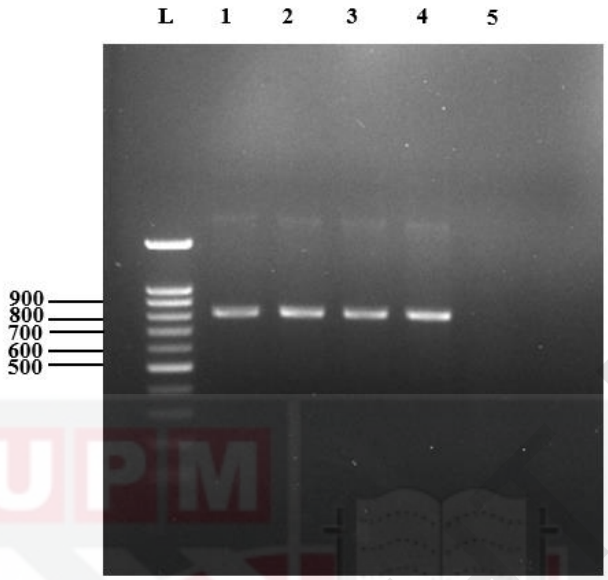


Figure 5.11 : dsRNA samples kept under UV exposure run on 1% agarose gel in TAE buffer. L- 100 bp ladder, 1 - 0 hr, 2 – 2 h, 3 – 4 h, 4 – 6 h and 5 – 8 h

5.4 Discussion

This study demonstrated dsRNA mediated in vivo knockdown of *Foc* TR4-DCL2 transcripts and root and rhizome injection of dsRNAs conferred significant protection to banana plants from fusarium wilt. When dsRNAs were injected to *Foc* TR4 infection area, reduction of transcript abundance of pathogen DCL2 gene and suppression of disease symptom development were observed in all experiments, providing clear evidence of uptake and an RNAi response of pathogen.

Previous results suggested *Foc* TR4 can be controlled 1000, 1500 and 2000 ng/mL DCL2 dsRNAs *in vitro*. However, plant topical application dsRNA doses are higher compared to doses used in *in vitro* study in reported plant fungal disease control. It may be due to degradation under open environment condition. Amino acyl tRNA ligase gene in *S. sclerotiorum* was suppressed at 0.1 ng/µL dsRNA dose significantly *in vitro* while 8 ng/µL dose was used for its leaf application for significant disease reduction (McLoughlin et al., 2018). For *B. cinerea*, 20 µL of Bc-DCL1/2–dsRNAs containing 400 ng total dsRNAs at the rate of 20 ng/µL were applied on fruits, vegetables and rose flower petals (Wang et al., 2016). Further, target genes AGO and DCL in *F. graminearum* were knocked down by spraying 500 µL of dsRNA at the dose level of 20 ng/µL on barley Leaves (Werner et al., 2020). Most of the dsRNA spray application studies used 20 ng/µL to achieve different plant disease suppression. Also, topical application of dsRNA tagetting common transcript of *S. sclerotiorum* and *B. cinerea* reduces disease severity in *A. thaliana* and *B. napus* leaf surfaces, but the required dsRNA amounts were 200 ng and 500 ng respectively (McLoughlin et al., 2018).

Therefore, the required dsRNA dose depends on pathogen gene expression level. Considering these findings, the 2000 ng at the rate of 25 ng/µL for root and corm injection was selected in this study based on *in vitro* results.

According to previous chapter results, DCL2 dsRNA did not impact on mycelial growth while it changes the conidiation which is directly influence on pathogenicity. All in planta sub experimental results showed significant difference between dsRNA applied and water treated control plants in terms of disease severity and relative transcript abundance. It confirms that DCL2 gene contribute to *Foc* TR4 pathogenicity. This result was match with reported studies on controlling plant pathogenic fungi, *F. graminearum*, *B. cinerea*, *P. italicum* and *C. gloeosporioides* by spray induced gene silencing of DCL genes (Gaffar et al., 2019; Wang et al., 2016; Wang et al., 2018; Yin et al., 2020).

Application of dsRNAs 24 h before was followed to check the protective activity of dsRNA. Also application of dsRNA and pathogen inoculation together and pathogen infection 24 h after dsRNA application were done to determine the curative activity of dsRNA. However, the time of dsRNA application with respect to pathogen infection did not influence on disease severity in this study. Therefore DCL2 dsRNA has both protective and curative activity. *P. viticola* DCL1/2 dsRNA displayed a curative action by reducing the disease progression of downy mildew in grapevine (Haile et al., 2021). Both AUDPC values and relative transcript abundance were reduced on 7 DPI in roots and rhizomes compared to control suggested that exogenous application of *Foc* TR4-DCL2 can be used as a banana fusarium control biofungicide. Also, there was no significant difference between dsRNA treated and control in 14 DPI. It may be due to no secondary siRNA amplification in *Foc* TR4 or degradation of siRNA by nucleases.

Stability of naked dsRNA under open field conditions and UV light are important factors to consider in field application. dsRNA was stable for 14 days under glasshouse condition in this study. One disadvantage of RNAi mediated topical applications is short lasting of dsRNAs (Gu et al., 2019). The field environment is more complex and constantly changing, and the dsRNA is degraded or taken up by multiple pathways before being absorbed by fungi (Rank and Koch, 2021). Environmental factors like nucleases, rainwater, UV rays, and microorganisms directly affect the stability of dsRNA (Yang et al., 2022).

Although sRNA transfer mechanism into fungi bodies are not established, exosomes are considered important for communication between cells via sRNAs. It is reported wound derived citrus exosomes aid to spray induced gene silencing against *P. italicum* (Cai et al., 2019; Yin et al., 2020). Previous studies demonstrated that wounded plant surfaces enhanced the efficiency of dsRNA uptake than non-wounded (Yin et al., 2020). Likewise, wounded banana roots and rhizome may produce exosome and support to transfer dsRNA to pathogen. To achieve potent RNAi response in host plants, the absorbed dsRNA required to transport within plant. sRNAs transport from cell to cell and long distances systemically inside plants by plasmodesmata and phloem tissues respectively (Mingels et al., 2020; Nowara et al., 2010).

The dsRNA molecules are considered environmentally friendly as it is easily degraded in nature as a natural biological molecule (Mezzetti et al., 2020). It minimizes environmental hazards. Although transgenic plants release more dsRNA and increase the potency for resistance development, topical application limits the pathogen exposure to dsRNA due to transient character resulting avoiding development of resistance in pathogen. RNAi resistance may be developed due to reduction in cellular uptake, mutations in transcript, RNAi suppressors, up regulation of the target gene, down regulation of RNAi machinery genes, nuclease activity and behavioral changes (Cagliari et al., 2019).

RNAi mediated gene silencing may be prevented by the innate regulatory system responsible for pathogenicity like lengthy protein half-life, negative pathogenicity regulation and transcript accumulation level. Low transcript level may not activate the RISC mechanism. Also, high transcript level fail to silence due to higher dose requirement of dsRNAs. Therefore, moderately expressed genes are effectively controlled in planta (McLoughlin et al., 2018). The efficiency of dsRNA application could be enhanced through higher dsRNA amount. However, high cost of production and stability of naked dsRNA under field level are main barriers in development of commercial grade RNAi based fungicide (Gu et al., 2019). Therefore, more research on nano particle coupled dsRNA and low cost production techniques have been performed (Pugsley et al., 2021).

5.5 Conclusion

Foc TR4-DCL2 mediated siRNAs perform an important role in *Foc* TR4 pathogenicity. DCL2 dsRNA could potentially suppress the pathogen as protective and curative biofungicide. Although, direct UV exposure degrade the dsRNA after 6 h, naked dsRNA molecules can survive up to 14 days under glasshouse condition. However, naked dsRNA can be formulated by adding stabilizing agents. Direct exogeneous application of DCL2 dsRNA has a great potential for RNAi mediated control of *Foc* TR4. Thus, for the first time, it is reported that the exogenous application of DCL2 dsRNA demonstrated its potential to be used in controlling banana fusarium wilt disease.

CHAPTER 6

SUMMARY, GENERAL CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

Full length sequences of DCL1 and DCL2 transcripts were isolated and characterized from *Foc* TR4 and performed RNAi mediated gene silencing of DCL2 gene to assess its effect on pathogenicity of *Foc* TR4. Full length DCL1 gene consisted of 4503 bp ORF encoding 1501 amino acids while DCL2 gene has 4323 bp ORF encoding 1441 amino acids. The DCL1 protein sequence contained four domains: DExDc domain, HELICc domain and dual RIBOc domains whereas DCL2 included five domains: DExDc domain, HELICc domain, dual RIBOc domains and double stranded RNA binding motif. DCL1 proten consisted 27.18% alpha helix and 15.81% beta sheets while DCL2 comprised 27.68% alpha helix and 16.56% beta sheets according to secondary structure prediction by Psipred server system. Further, Phyre2 software recognized the crystal structure of human endoribonuclease dicer was more similar to *Foc* TR4 dicers with 100% confidence and 25% identity. In phylogenetic analysis, both DCLl and DCL2 genes were classified into four subgroups of sodariomycetes, leotiomycetes, dothideomycetes and eurotiomycetes. All *Fusarium oxysporum* species were grouped under nectriaceae family clade in both genes.

Long dsRNA sequence for RNAi-mediated silencing of *Foc* TR4-DCL2 gene was predicted using online available Snapdragon software. The designed region length was 736 bp which was complementary to part of ribonuclease III family domain II region and DSRM region. The minimum dsRNA dose required for RNAi silencing of DCL2 gene was 1000 ng/mL and 1000-2000 ng/mL dose levels significantly reduced the relative transcript abundance of DCL2 compared to water treated control *in vitro*. Relative transcript abundance of DCL2 was significantly reduced 72 h after dsRNA treatment at 1000 ng/mL and it continued to 120 h compared to water treated control *in vitro*. dsRNA did not affect on *Foc* TR4 mycelial growth. However, micro conidiation was significantly reduced at 1000 ng/mL at 3 DAT. The reduction of *Foc* TR4 DCL2 gene expression confirms the movement of dsRNA molecules into fungal cells and RNAi-mediated gene silencing of *Foc* TR4 DCL2 gene for the first time.

Equal disease severities were observed in application of dsRNA as pre, post or simultaneously with respect to pathogen inoculation. Its lesion length parameter was reduced between 51.38 - 58.22% compared to control. AUDPC values and relative transcript abundance were reduced in dsRNA treated root samples until 7 DPI compared to control suggesting the effective duration of naked dsRNA molecules was 7 DPI in planta. Also, disease severity and relative transcript abundance was low on 7 DPI in rhizome compared to water control. In naked dsRNA stability assessment, uniform band intensities were observed until 14 days and 6 h under controlled environmental condition and UV exposure respectively.

Full length DCL1/DCL2 genes were isolated and characterized for the first time in this study. The designed long dsRNA sequence was effective to silence the *Foc* TR4-DCL2 gene and it affected to micro conidia production of *Foc* TR4 *in vitro*. *Foc* TR4-DCL2

gene is important for pathogenicity of *Foc* TR4. DCL2 dsRNA molecules could act as protective and curative biofungicide against *Foc* TR4. Exogenous application of DCL2 long dsRNA molecules could be used to suppress banana fusarium wilt and has a potential to control fusarium wilt in banana in a sustainable and environmental- friendly manner via RNAi-mediated gene silencing.

6.1 Recommended future research

1. Optimization of soil drenching of DCL2-dsRNA to control banana fusarium wilt via mass production of dsRNA
2. Investigation on dsRNA embedded more stable delivery compounds to pathogens under environmental conditions.
3. Development of low cost dsRNA mass production systems for field application
4. Identification of the movement mechanism of dsRNA or siRNA between host and pathogen.

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APPENDICES

Appendix A

NCBI deposited 28S-18S ribosomal RNA intergenic spacer sequence of *Fusarium odoratissimum*

***Fusarium odoratissimum* voucher JPT-2020-1 28S-18S ribosomal RNA intergenic spacer, partial sequence**

GenBank: MW122510.1
LOCUS MW122510 472 bp DNA linear PLN 24-NOV-2020
DEFINITION Fusarium odoratissimum voucher JPT-2020-1 28S-18S ribosomal RNA intergenic spacer, partial sequence.
ACCESSION MW122510
VERSION MW122510.1
KEYWORDS .
SOURCE Fusarium odoratissimum (Fusarium oxysporum f. sp. cubense tropical race 4)
ORGANISM Fusarium odoratissimum
Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Fusarium; Fusarium oxysporum species complex.
REFERENCE 1 (bases 1 to 472)
AUTHORS Jayasekara, S.S. and Wong, M.Y.
TITLE Identification of Fusarium odoratissimum (previously Fusarium oxysporum f. sp. cubense Tropical Race 4)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 472)
AUTHORS Jayasekara, S.S. and Wong, M.Y.
TITLE Direct Submission
JOURNAL Submitted (10-OCT-2020) Department of Plant Protection, Universiti Putra Malaysia, Jalan Universiti 1 Serdang, Seri Kembangan, Selangor 43000, Malaysia
COMMENT ##Assembly-Data-START##
Sequencing Technology: Sanger dideoxy sequencing
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//

NCBI deposited Fusarium odoratissimum dicer-like protein 1 mRNA, complete cds

GenBank: MW367432.1
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DEFINITION Fusarium odoratissimum dicer-like protein 1 mRNA, complete cds.
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VERSION MW367432.1
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SOURCE Fusarium odoratissimum (Fusarium oxysporum f. sp. cubense tropical race 4)
ORGANISM Fusarium odoratissimum
Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Fusarium; Fusarium oxysporum species complex.
REFERENCE 1 (bases 1 to 4521)
AUTHORS Jayasekara,S.S. and Wong,M.Y.
TITLE Molecular characterization of Dicer-like proteins in Fusarium ordoratissium
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 4521)
AUTHORS Jayasekara,S.S. and Wong,M.Y.
TITLE Direct Submission
JOURNAL Submitted (13-DEC-2020) Department of Plant Protection, Universiti Putra Malaysia, Jalan Universiti 1 Serdang, Seri Kembangan, Selangor 43000, Malaysia
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NCBI deposited Fusarium odoratissimum dicer-like protein 2 mRNA, complete cds

LOCUS MW344087 5053 bp mRNA linear PLN 19-APR-2022
DEFINITION Fusarium odoratissimum dicer-like protein 2 mRNA, complete cds.
ACCESSION MW344087
VERSION MW344087.1
KEYWORDS
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ORGANISM Fusarium odoratissimum
Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae; Fusarium; Fusarium oxysporum species complex.
REFERENCE 1 (bases 1 to 5053)
AUTHORS Jayasekara,S.S. and Wong,M.Y.
TITLE Molecular characterization of dicer-like proteins in Fusarium odoratissimum
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 5053)
AUTHORS Jayasekara,S.S. and Wong,M.Y.
TITLE Direct Submission
JOURNAL Submitted (05-DEC-2020) Department of Plant Protection, Universiti Putra Malaysia, Jalan Universiti 1 Serdang, Seri Kembangan, Selangor 43000, Malaysia
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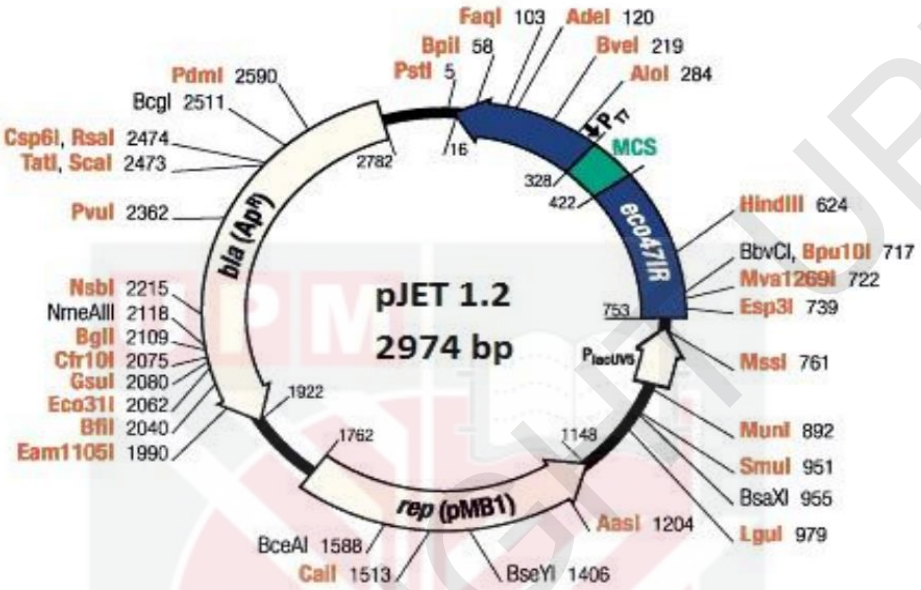
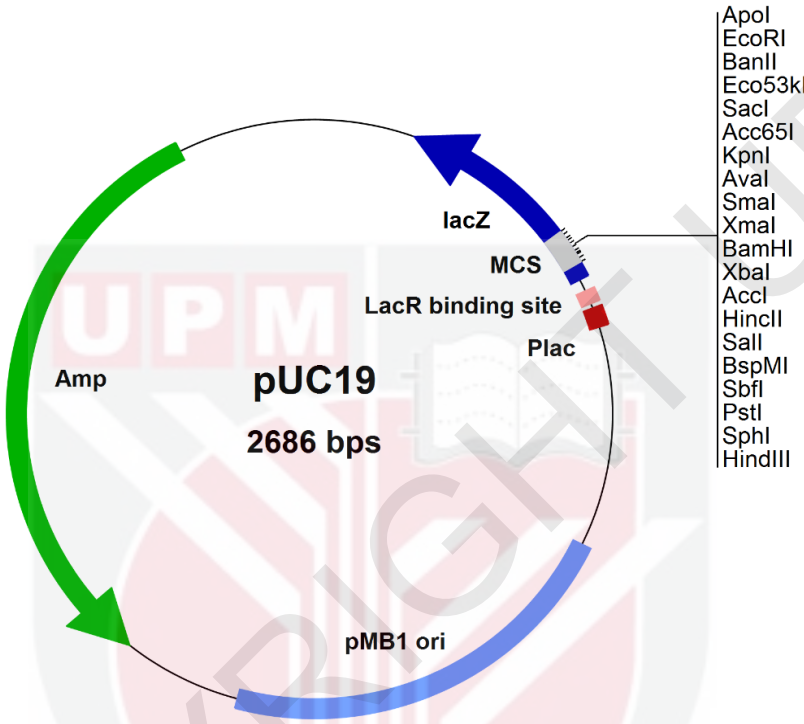
Appendix B

Map of pJET 1.2 cloning vector



Appendix C

Map of pUC19 cloning vector



Appendix D

Real time PCR standard curves and melt curves

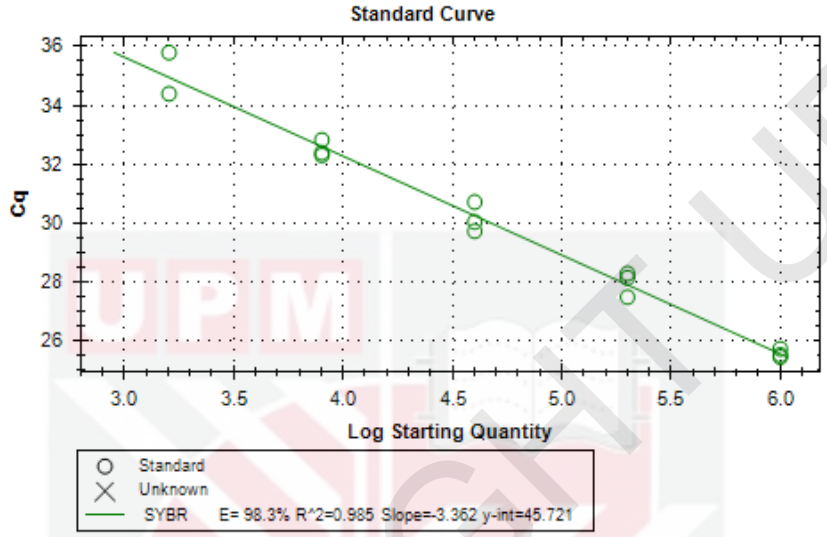


Figure 1 : Standard curve of DCL2 gene Amplification efficiency–98.30% and R^2 0.98

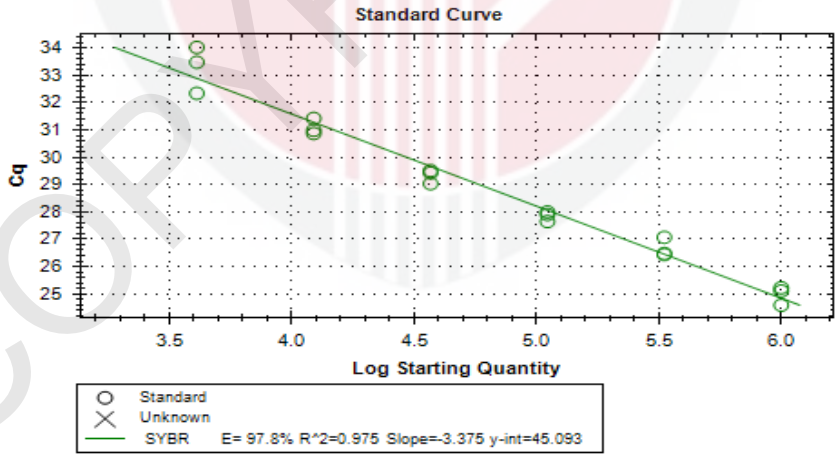


Figure 2 : Standard curve of DCL1 gene. Amplification efficiency–97.80% and R^2 0.97

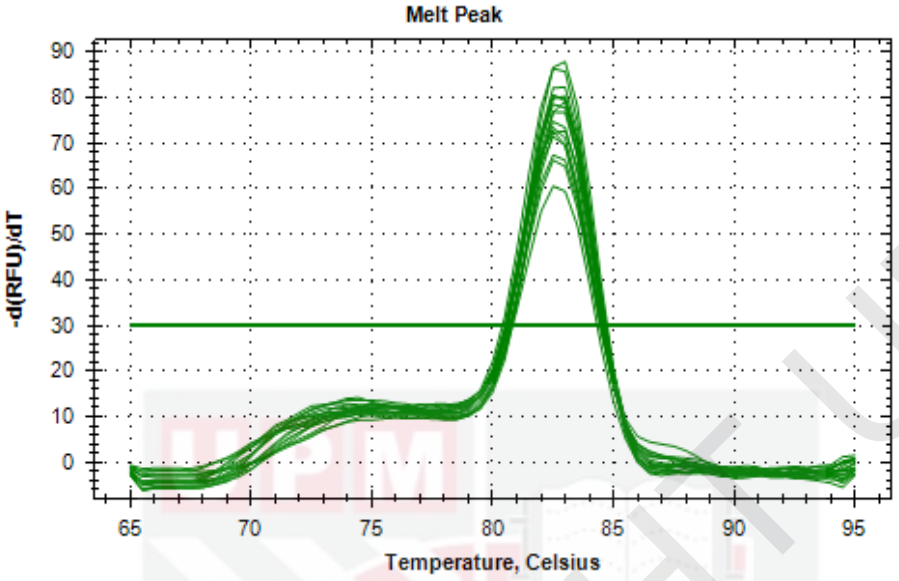


Figure 4 : Melting curve analysis of DCL1 gene

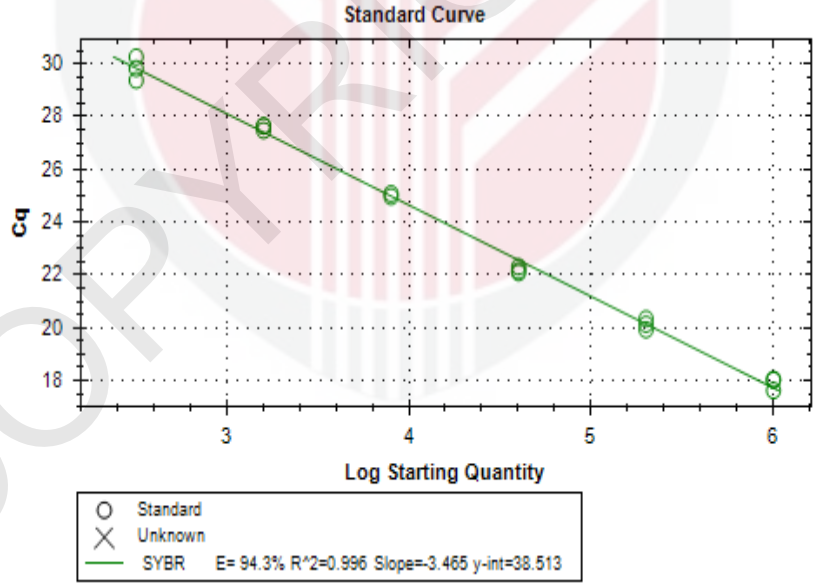


Figure 5 : Standard curve of IDH gene. Amplification efficiency–94.30% and R^2 0.99

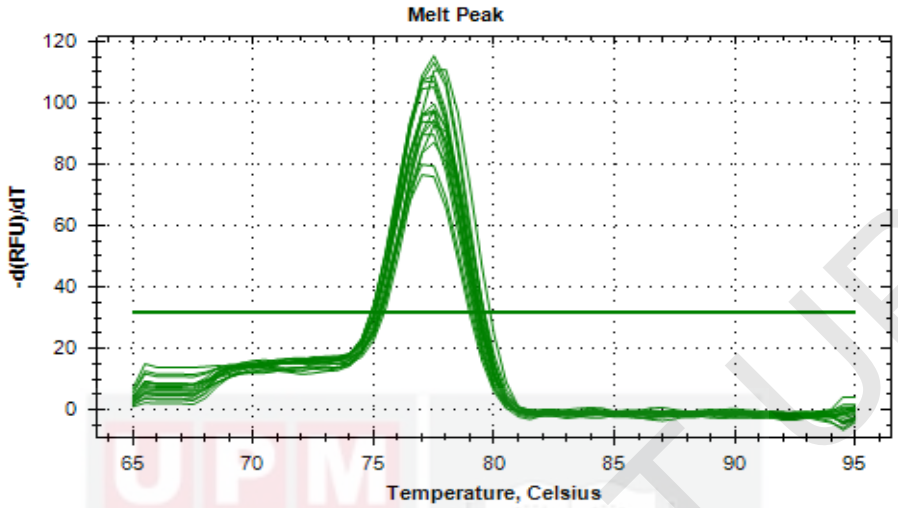


Figure 6 : Melting curve analysis of IDH gene

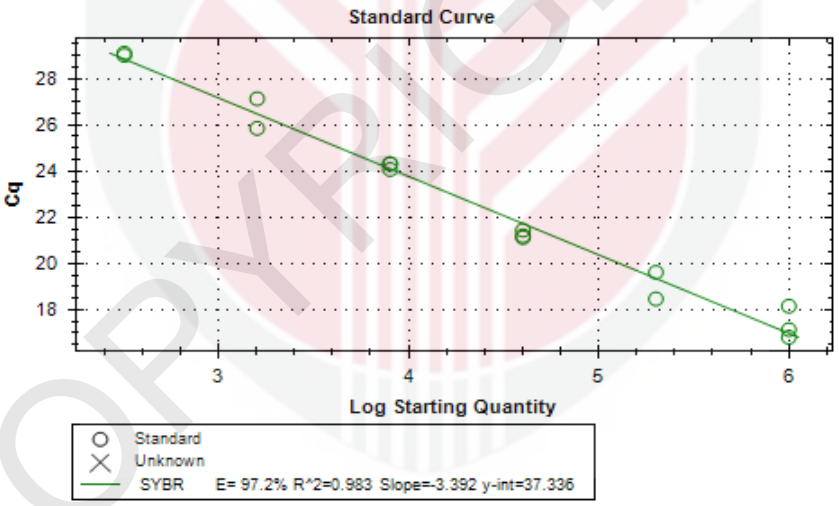


Figure 7 : Standard curve of G6DH gene. Amplification efficiency–97.20% and R^2 0.98

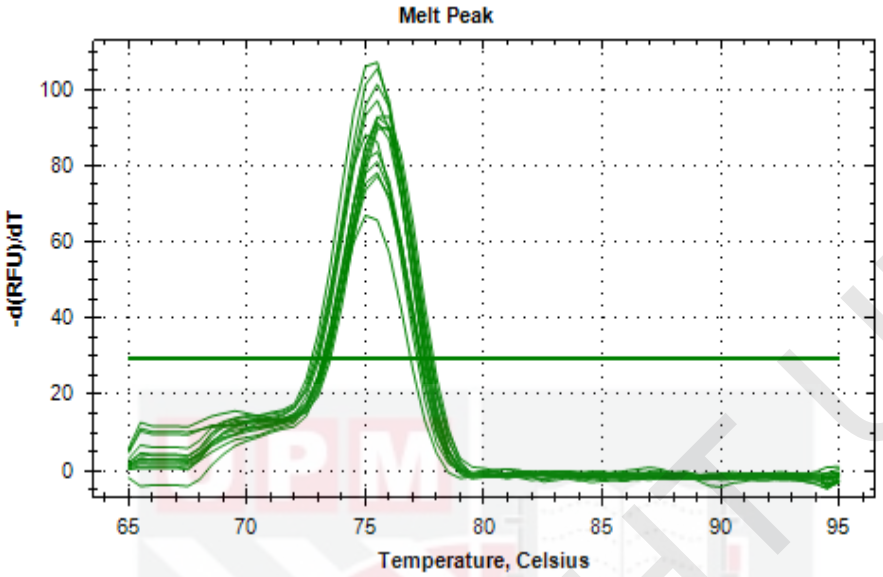


Figure 8 : Melting curve analysis of G6DH gene

Appendix E

Sequence results of target and reference genes used in qPCR by Sangar sequencing

1. DCL2

ACCTGGCCCCTCTCAATGCTAAGTCAGAGAAGGTGGAAGAAAAGACCG
ACCTAATGCCGGAGCACCTATTCGACCCCTGGATCCAGATCGCCCAAC
GCTGGAAGTCGGGTTGTG

2. IDH

TACCAAGGCCATCCTCGACAAGCTCGAGACTGTTTAAATGAAGCATCT
TTTGTGATATCCCCCTCCCAAAACAAACCATGCGAAACCACCATTTTTT
TATTTTTCGTCTATCTTTCCCGTATGTAAAGCGGGAGCCG

3. G6DH

TTCAAGCCTGACGAGGCAACAAAACACCAATCAATGATTTACTCTATA
TAAAAAAACGATGGAAACTGTTGTCGTAACTTTTGCGACATAATGATG
GGCACTTGCTTCTGGG

Appendix F

Table 1 : Relative transcript abundance of DCL2 gene in *Foc* TR4 calculated using Livak $2^{-\Delta\Delta Cq}$ method after application of different dose levels of dsRNA in vitro

dsRNA Concentrations (ng/mL)	Δ Cq control	Δ Cq Treated	ΔΔ Cq	Fold change	Mean
100	6.24	6.45	0.21	0.86	1.19
100	6.24	5.76	-0.48	1.39	±
100	6.24	5.84	-0.40	1.32	0.165
500	6.24	6.50	0.26	0.84	0.78
500	6.24	6.70	0.46	0.72	±
500	6.24	6.62	0.38	0.77	0.033
1000	6.24	7.39	1.15	0.45	0.45
1000	6.24	7.58	1.34	0.40	±
1000	6.24	7.26	1.02	0.49	0.028
1500	6.24	7.11	0.87	0.55	0.50
1500	6.24	7.44	1.20	0.44	±
1500	6.24	7.16	0.92	0.53	0.035
2000	6.24	7.11	0.87	0.55	0.48
2000	6.24	7.28	1.04	0.49	±
2000	6.24	7.55	1.31	0.40	0.042

Table 2 : Relative transcript abundance of DCL2 gene in *Foc* TR4 calculated using Livak $2^{-\Delta\Delta Cq}$ method at different time interval after application dsRNA in vitro

Days after dsRNA application	Δ Cq control	Δ Cq Treated	ΔΔ Cq	Fold change	Mean
0	6.26	6.44	0.18	0.88	
0	6.26	6.12	-0.14	1.1	1.00 ± 0.09
0	6.27	6.23	-0.04	1.03	
24	7.05	7.56	0.51	0.7	
24	7.45	8.14	0.7	0.62	0.77 ± 0.11
24	6.7	6.72	0.02	0.98	
48	6.71	7.01	0.3	0.81	
48	6.88	7.93	1.05	0.48	0.68 ± 0.10
48	7.36	7.76	0.41	0.75	
72	6.72	7.63	0.91	0.53	
72	7.39	8.95	1.56	0.34	0.44 ± 0.06
72	7.2	8.37	1.16	0.45	
96	6.45	7.69	1.24	0.42	
96	6.66	7.63	0.98	0.51	0.41 ± 0.06
96	7.25	9.03	1.78	0.29	
120	6.95	8.43	1.48	0.36	
120	7.77	8.73	0.97	0.51	0.55 ± 0.13
120	7.2	7.53	0.33	0.8	

Table 3 : Relative transcript abundance of DCL2 gene in *Foc* TR4 calculated using Livak $2^{-\Delta\Delta Cq}$ method at different time interval after application dsRNA on infected roots

Days after pathogen infection	Δ Cq control	Δ Cq Treated	ΔΔ Cq	Fold change	Mean
3	2.07	2.32	0.25	0.84	0.89 ± 0.03
3	1.58	1.66	0.08	0.95	
3	2.04	2.20	0.16	0.90	
5	0.61	1.21	0.60	0.66	0.68 ± 0.02
5	0.21	0.79	0.59	0.67	
5	1.33	1.79	0.46	0.73	
7	0.97	1.38	0.41	0.75	0.71 ± 0.02
7	2.21	2.74	0.53	0.69	
7	2.10	2.64	0.54	0.69	

Table 4 : Relative transcript abundance of DCL2 gene in *Foc* TR4 calculated using Livak $2^{-\Delta\Delta Cq}$ method at different time interval after application dsRNA on infected corms

Days from pathgen infection	Δ Cq control	Δ Cq Treated	ΔΔ Cq	Fold change	Mean
7	2.37	2.83	0.46	0.73	0.74 ± 0.03
7	2.78	3.30	0.52	0.70	
7	2.24	2.57	0.33	0.80	
14	2.94	3.03	0.09	0.94	0.96 ± 0.01
14	3.35	3.42	0.07	0.95	
14	2.79	2.82	0.03	0.98	

Appendix G

Effect of dsRNA dose on relative transcript abundance of *Foc* TR4-DCL2 gene

The SAS System

The GLM Procedure

Dependent Variable: GEDOSE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.62791956	0.23255994	9.65	0.0009
Error	10	0.24092689	0.02409269		
Corrected Total	17	1.86884644			

R-Square	Coeff Var	Root MSE	GEDOSE Mean
0.871083	20.73877	0.155218	0.748444

Source	DF	Type I SS	Mean Square	F Value	Pr > F
METHOD	5	1.61256444	0.32251289	13.39	0.0004
REP	2	0.01535511	0.00767756	0.32	0.7342

Source	DF	Type III SS	Mean Square	F Value	Pr > F
METHOD	5	1.61256444	0.32251289	13.39	0.0004
REP	2	0.01535511	0.00767756	0.32	0.7342

Effective DCL2 gene knockdown duration following dsRNA application

The SAS System

The GLM Procedure

Dependent Variable: GETIME

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.90016122	0.12859446	4.48	0.0166
Error	10	0.28690922	0.02869092		
Corrected Total	17	1.18707044			

R-Square	Coeff Var	Root MSE	GETIME Mean
0.758305	26.94813	0.169384	0.628556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
METHOD	5	0.81535578	0.16307116	5.68	0.0097
REP	2	0.08480544	0.04240272	1.48	0.2740

Source	DF	Type III SS	Mean Square	F Value	Pr > F
METHOD	5	0.81535578	0.16307116	5.68	0.0097
REP	2	0.08480544	0.04240272	1.48	0.2740

Effect of dsRNA on conidiation of *Foc* TR4

The SAS System

The GLM Procedure

Dependent Variable: CONIDIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	44108.37333	5513.54667	6.24	0.0026
Error	12	10596.05810	883.00484		
Corrected Total	20	54704.43143			

R-Square	Coeff Var	Root MSE	CONIDIA Mean
0.806303	34.77228	29.71540	85.45714

Source	DF	Type I SS	Mean Square	F Value	Pr > F
METHOD	2	17605.08857	8802.54429	9.97	0.0028
REP	6	26503.28476	4417.21413	5.00	0.0087

Source	DF	Type III SS	Mean Square	F Value	Pr > F
METHOD	2	17605.08857	8802.54429	9.97	0.0028
REP	6	26503.28476	4417.21413	5.00	0.0087

Effect of time of dsRNA application under *in planta*

The SAS System

The GLM Procedure

Dependent Variable: Time

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	10.35380650	2.07076130	49.79	<.0001
Error	6	0.24955117	0.04159186		
Corrected Total	11	10.60335767			

R-Square	Coeff Var	Root MSE	YIELD Mean
0.976465	9.368693	0.203941	2.176833

Source	DF	Type I SS	Mean Square	F Value	Pr > F
METHOD	3	9.74920833	3.24973611	78.13	<.0001
REP	2	0.60459817	0.30229908	7.27	0.0249

Source	DF	Type III SS	Mean Square	F Value	Pr > F
METHOD	3	9.74920833	3.24973611	78.13	<.0001
REP	2	0.60459817	0.30229908	7.27	0.0249

BIODATA OF STUDENT

E.A.E.S.S. Jayasekara was born on February 21 1980 in Ratnapura Sri Lanaka. She obtained Bachelor degree from Rajarata University of Sri Lanka in 2007 and master degree from University of Peradeniya, Sri Lanaka in 2012. She joined to the Department of Agriculture in Sri Lanka in 2011 as a research officer. She enrolled her study for Doctor of philosophy in September 2018 at Faculty of Agriculture, Universiti Putra Malaysia.

LIST OF PUBLICATIONS

Jayasekara, E. A. E. S. S., Vadamalai, G., Saad, N. B., Hailing, J., & Mui-Yun, W. (2025). RNA interference-based gene silencing of dicer-like 2 in *Fusarium oxysporum* f. sp. *cubense* tropical race 4 mitigates Fusarium wilt disease in banana. *Biocatalysis and Agricultural Biotechnology*, *65*, 103541.

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