EFFICACY OF dsRNA IN LATE BLIGHT *(Phytophthora infestans)* OF TOMATO

Dissertation submitted in partial fulfillment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

BY

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UNDER THE GUIDANCE OF

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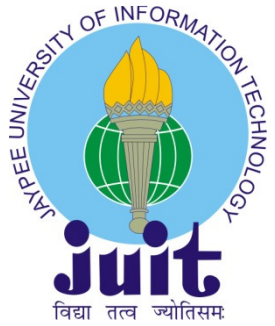

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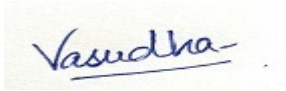
DECLARATION BY THE SCHOLAR

We hereby declare that the work reported in B.Tech project report entitled "**Efficacy of dsRNA in late blight *(Phytophthora infestans)* of tomato"** submitted at **Jaypee University of Information Technology, Waknaghat, Solan (H.P.)** is an authentic record of our work carried out under the supervision of **Dr. Anil Kant.** We have not submitted this work elsewhere for any other degree or diploma.



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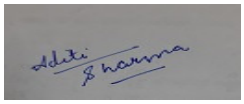

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SUPERVISOR'S CERTIFICATE

This is to certify that the work, which is present in the project report, entitled "**Efficacy of dsRNA in late blight *(Phytophthora infestans)* of tomato".** It is submitted in partial fulfillment of the requirements for the degree of Bachelor of Technology in Biotechnology at Jaypee University of Information Technology; Waknaghat is an authentic record of work carried out by Vasudha Porwal (161805) and Aditi Sharma (161821) under the supervision of Dr Anil Kant Professor, Department of Biotechnology, Jaypee University of Information Technology, Waknaghat. The above statement made is correct to the best of our knowledge.

Signature of Supervisor:

Name of Supervisor Dr. Anil Kant

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Date:

ACKNOWLEDGEMENT

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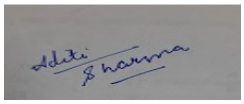
We would like to thank our parents for their continuous support and motivation. Finally, we would like to thank those who directly or indirectly helped us in completing this project.

Signature of Students:  

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ABSTRACT

Late blight disease is caused by Phytophthora infestans, which is one of the most devastating pathogens for tomato cultivation in India. To reduce the loss caused by this plant pathogen, plant researchers have adopted various methods to produce resistance in plants such as chemical fungicides. Among many methods used, RNAi based technique has been an upcoming technique and is an asset i.e., utilized to engineer resistant plants. This technology is designed to accomplish gene silencing. However, loss of function never accomplished the expression of the targeted gene that could be reduced to practically 70% of the first levels. This study focuses on in-vitro production of dsRNA. It inhibits the expression of genes by binding with complementary nucleotide sequences. Hence, reducing the production of proteins that enables the pathogen to make the host disease susceptible.

In this project, we conducted experiments using dsRNA on tomato leaves and petioles. Different concentrations of constructed dsRNA were used to check the growth of this pathogen via a detach leaf assay. It was observed that in leaves the highest concentration of dsRNA produced more infection due to off-target effects whereas the spread of infection was limited when the concentrations were reduced. Whereas in petioles the dsRNA concentration that showed limited growth of infection in leaves was taken and was diluted further which showed that at the highest concentration of dsRNA produced the minimum infection on the petioles.This method can be proved as a very effective way to help the farmers to protect tomatoes and potatoes from the late blight disease.

CHAPTER- 1

Introduction

Phytophthora infestans is a devastating plant pathogen known for causing the infection, late blight. This pathogen caused the Potato Irish famine during late 1980s. M.J. Berkeley first described this pathogen during the outbreak. After that, Anton de Bary named it as Phytophthora infestans in 1870s. The occurrence of this pathogen is seen in many plants other than potato such as tomato, and many related Solanaceae species. This pathogen is spread worldwide causing yield losses of many major crops *(Fry, 1993)*. In recent times, late blight of tomato is evidently increasing in India. High humidity and cool temperature can cause up to 100% loss of tomato crops in areas such as Himachal Pradesh (mainly Shimla, Simour and Kullu) (*Kaushal and Sonia, 2019)*.

This infection starts to spread by producing water soaked lesions having chlorotic borders on leaves of the tomatoes. These lesions though small expand very rapidly when present in humid conditions. The entire plant can destroyed by this pathogen within few days *(Bhimanagoud Kumbar, 2017).*The late blight pathogen is grown in moist humid conditions. It prefers from cool to moderate temperatures. Night temperature to 10°C to 20°C and day temperature of 15-20°C are general for disease advancement. Free water from rain, dew and irrigation systems give the necessary water for the pathogen advancement *(Andy and Neil, 2017).* Spores start to form in 3-5 days and require 12 hours of free dampness for contamination to occur *(Allen, 2012).* Poor agriculture practices lead to expanded infection occurrences in the tomato fields. It leads to cause decrease of product quality and increasing yield losses. It is observable that upon ideal natural conditions late blight can cause high yield losses even up to 100% (*Guenthner et al., 2001*).

There are numerous alternatives in farming for the control of this infection. The most widely recognized foliar-applied fungicides are used. The disadvantage of the use of hazardous chemicals is that the pathogens become resistant to chemical pesticides, environmental pollution and ecological imbalances may occur *(Rashidul.et.al, 2013)*. Other method consists of organic method the microorganism inoculums and compost extracts excised leaves with copper oxychloride showed different results in the management of late blight (*Wilcockson and Leifert, 2005)*. Although the result produced were very inconsistent. Efficacy of compost tea and poultry litter extract is tested in controlling late blight of tomato under natural infection condition. Significant effects of different organic amendments are observed on the incidence and severity of

late blight of tomato as compared to control *(Rashidul.et.al., 2013).* However, some of these approaches were efficient but they did not really served the purpose.

After not having much of the satisfactory results, the scientist moved towards the RNAi approach for the management of late blight. This technology is based to accomplish gene silencing. However, loss of function was never accomplished; the expression of the targeted gene could be reduced to practically 70% of the first levels *(Ratna.et.al, 2007).* A double stranded RNA is introduced in an organism. The regulation of gene expression is controlled by using dsRNA. This dsRNA inhibits the expression of genes with complementary nucleotide sequences (*Sanjeev, 2014).*

The plant uses a mechanism to control the viral infection by making hairpin RNAi or antisense constructs *(Nowara.et.al, 2010).* The way RNA interference works involves many enzymes and proteins such as Dicers and RISCs (RNA induced silencing complexes). Dicers activity whereas is similar to ribonucleases and cleaves the dsRNA into smaller duplexes called small interfering RNA. Antisense strand of each the duplexes is subjected to RISC where it recognizes the target molecule. Thus, degrading the mRNA or suppressing the translational activity will take place (*Waterhouse and Fusaro, 2006).*

One of the ways to introduce gene silencing is through virus. It shows an effective way to reduce the expression or knock out the expression of gene without making any genetic changes. The first step is to introduce the recombinant DNA (containing host partial gene sequences, which are to be silenced) into virus vector and cause the infection in host plant *(Liu.et.al, 2002).* After that RNA, dependent RNA polymerase enzyme will form dsRNA molecules (SiRNA). These siRNAs play the major role in Post Transcriptional gene silencing (PTGS). PTGS leads to endogenous mRNA degradation. When, there is relative high similarity in nucleotide sequences between RNA transcripts and target endogenous gene sequences there would be sequence specific endogenous mRNA degradation *(Bekele.et.al, 2019).*

Another way for gene silencing is host introduced. It was developed to form a multiple crop system to control the diseases caused by oomycetes *(Song.et.al, 2018).* Here, the plants transformed with dsRNA constructs, are introduced into host. It either targets the pathogen genes which are vital for its functioning or host susceptibility *(Ghag, 2017).* Recently the development in HIGS has led to spray induced gene silencing. In SIGS, there is no need to transform the plant. When dsRNA is sprayed on the plant, it can act two ways. The one where dsRNA is taken up by

the plant cells and then it is introduced in the fungal cell indirectly or the fungus can take up the dsRNA directly*(Song.et.al, 2018).*We had used the Spray induced technology for the one reason that it is chemical free and other it provides high sequence dependent specificity *(Wang and Jin 2016)*

Objectives:

1. Production of multi si-RNA construct from invitro transcription of 640 base pairs developed at CPRI.
2. In vitro testing of efficacy of siRNA, on growth of *Phytophthora infestans* on frozen pea agar.
3. Testing efficacy of si-RNA on *Phytophthora infestans* infection in tomato via detach leaf assay and on infected seedling.

CHAPTER 2

Review of literature

2.1. Classification of *Phytophthora infestans*

Late blight of potatoes is the disease that was responsible for the Irish potato famine in the mid-nineteenth century. It is caused by the fungus-like pathogen *Phytophthora infestans (Haas.et.al, 2009).* With the advancements in the technology, *Phytophthora infestans* came out as not a true fungus but is regarded as a fungus like organism. It belongs to Domain eukaryota and Kingdom Stramenopila (chromista). It comes under phylum Oomycota and class Oomycetes, also known as 'lower fungi' (*Rossman and Palm, 2006).* This group of organism is kept under a different kingdom because all true fungus has a cell wall of chitin but in Oomycetes, the cell wall is majorly made up of cellulose and beta glucans. Oomycota has a diploid nuclear state of mycelium and where as true fungus has haploid or dikaryotic stage. Oomycetes are closely related to heteroknot algae where sexual reproduction is through heterogametangia whereas in true fungus it is through zygospore, ascospores and basidiospore. The zoospores present in lower fungi are biflagellated and higher fungus may or may not be flagellated. The lysine synthesis is done by Diaminopimelic acid pathway in oomycetes and where as in other higer fungus it is Alpha-aminoadipate pathway. Then, there is difference in miochondria cristae, the lower fungus has tubular and whereas higher fungus has it flattened. The reason that these many differences occur between higher and lower (oomycetes), the lower class of fungus closely relates to brown algae or diatoms. Therefore, these are kept in different kingdom which is Kingdom Straminopila *(Dahlin, 2017)*

2.2. Symptoms of late blight in Potato and Tomato

The first symptoms of late blight are seen on the tomato leaves as small inconclusive, water-soaked lesions that broaden quickly into light green to brownish-black spots and can cover enormous regions of the leaf *(Allen,2012).* During the wet climate, lesions on the abaxial surface of the leaf are covered with white cottony growth where sporangia develop. On the undersides of larger lesions, a ring of moldy development of the pathogen is observed during a humid climate. As the infection advances, the lesions enlarge causing leaves to brown, shrivel, and die. On petioles and stems lesions start with inconclusive, water-soaked spots that extend quickly into brown to dark lesions that spread at enormous areas of the stems and petioles*(Nelson,2008).* During the wet climate, lesions are visible as powdery, whitish rings around the margins of the blighted areas. The whitish material consists of mycelia and sporangia of the pathogen. Affected petioles and stems collapse at the point where the infection is caused, leading to the death of all the distal plant parts. Tomato fruits infected turnout to be greasy, olivaceous-brown, decay, can shrivel up and fall off the plant and never ripen. Such infected fruits are not fit for human consumption and should be removed and destroyed. The fruit damage occurs in a few days or

less under the right weather conditions *(Robinson.et.al, 2017).*

2.3. Reproduction of *Phytophthora infestans* for potato and tomato

As we speak of reproduction in *Phytophthora infestans*, both sexual and asexual reproduction is reported. With respect to sexual reproductions, oopsores formation is in notice. The presence of oospores makes this pathogen soil borne. Sexual reproduction causes increased genetic variations as the level of sexual recombination increases. However, the quantity of oospores required to cause infection cannot be know. But, the some properties related to epidemics have proved it as an evidence that oospores serves as a 1° inoculums for the infection to occur *(Anderson,2007).* When it comes to mating both A1 and A2 types were found in this fungus like diploid organism. These both types represent the compatibility types but not the dimorphic stages of the pathogen. Some of the chemical compounds called pheromones are released. These chemicals help to differentiate the diploid vegetative mycelium into male and female gametangia (antheridium and oogonium) through meiosis and oospore is formed *(Judelson.1995).* The sex hormones when released by A1 isolates can induce only A2 isolates (not A1 isolates) for sexual reproduction. Likewise, the sexual reproduction happening in A1 isolates is regulated by A2 isolates sex hormones (*W.H.Ko, 1978).* As, it comes to the sexual reproduction, the genetic recombination occurring in this diploid pathogen during meiosis is the cause for genetic diversity. There are other ways; this pathogen has adapted to cause the variability instead of genetic recombination. When we speak of the old populations of *Phytophthora infestans* there is no presence of A2 type mating. Then, it was the asexual reproduction, which was playing the role. The biflagellate motile zoospore is the main entity to produce the genetic variability during that time. Variability in the characteristics such as morphology, growth rate and even in virulence were seen. This type of variability is called as intraspecific variability, which is mainly caused by mutations, parasexual recombination and recombinations, caused by mitosis *(Samen, Secor, Gudmestad, 2002).*

2.4. Disease cycle of *Phytophthora infestans* for potato and tomato

The spread of this disease is mostly seen in wet and humid seasons. The white colour spores are mostly present in the lower surface of the infected leaves, stems and lesions present in the fruits*(Allen,2012).* The zoospores carried in the sporangia present on sporangiophore are released during cool and wet conditions and thus this is the indirect germination. The sporangia can germinate directly also in warmer conditions. The sporangia or the spores are dispersed through the wind or water (*Dahlin, 2017).* When the sexual reproduction is absent, the pathogen survives through tubers or tomato fruit which are already infected. Then there is sexual way of causing the disease cycle. When, both the mating types A1 and A2 are in close connection with each other. Meiosis will be done, fusion of antherdium and oogonium happens. After Karyogamy

(fusion of nucleus) in the oogonium, a thick-walled oospores are formed. These oospores will cause the infection through the soil and cause infection (*Schumann and Arcy, 2000).*

2.5. Geographical distribution of late blight for potato and tomato

Late blight is the infection caused by *Phytophthora Infestans.* The major host plants of this disease belongs to the Solanaceae family, those are, potato and tomato. It was primarily noticed in potato when the great Irish potato famine occurred in 1840's (*Schumann and Arcy,2000)*. For more than a century, this pathogen caused a huge threat to the potato production. This infection started to emerge from northern European countries those have wet and humid conditions. Until 1960, the major focus was to produce the resistance varieties, when they found about the mutations, which helps the pathogen to overcome this resistance. The situation became more complex in 1970's *(Cooke.et.al, 2011).* The old population, which was found only in European, parts contained A1 type (which only reproduced asexually) migrated towards central Mexico where A2 type was present. Both the types were in close proximity of each other and the sexual reproduction started in this pathogen hence the new population was formed. This population caused epidemics in America, Canada and Mexico. The first noticeable genetical diversity other than Mexico was seen in Switzerland, which showed A2 type. Hence, this strain and mode of reproduction were distributed globally *(Fry and Goodwin, 1997).* Before 1970's, only A1 type was seen in the Asian countries. From year 1992 to 1997 many strains were collected which showed the allozyme genotype which was specific to of A2 type. They were mainly present in Thailand, Nepal Korea, Thailand, Nepal, India, Taiwan, Indonesia, and China. Since 2008 in some parts of India, This disease has known to cause major destruction for potato and tomato crop plants. Alone in southern parts of India, in years from 2010 to 2012 there were 63 isolates form potato and 97 from tomatoes. Since 2008, few epidemics were seen which led to full loss of crop production. These epidemics where seen both in tomato as well potato plants in the states of Karnataka, Tamil Nadu and Andhra Pradesh. The first occurrence of type A1 was seen in 1993 and in 1994 the new population, which was A2 type on potato, was seen for the first time in northern India *(Chowdappa.et.al, 2014).* In Himachal Pradesh, the most prone areas of Late Blight of tomato are Shimla (60.4-23%), Sirmour (58.9-21.4%) and kullu(44.6-26.3%) districts (*Sonia and Sandeep, 2019).*

2.6. Economical Impact of Late blight in both potatos and tomatoes

During 1800, countries like Ireland were majorly dependent on the food crop like potato. The infection caused by *Phytophthora* infestans led to the fast destruction of this food crop all over country. Thus, leading to the death of 1 million people and another 1 million had to migrate. As the infection caused by this pathogen is rapid, it is responsible for much yield loss of potatoes and tomatoes. As of 2009, late blight is responsible for total yield loss in tomatoes estimating the

loss ranging from $46-66 billion. In 2012, the annual loss for potatoes was estimated in $ 6.7 billion *(Nowicki.et.al, 2012)*. From year 2012, the India is the second largest producer of Potato behind China producing 45 million ton from 1.9 million hectare area. Indian production for potato is going to be reduced by 9.6% and 161.1% for years 2020 and 2050 respectively as there is rise in global warming *(Rana.et.al, 2014).*

2.7. Different approaches of managing the disease of late blight

The effectiveness of the control strategies depends on the species *of Phytophthora* to survive, either as a dormant spore or as a saprophyte. At present, there is no resistant or tolerant potato and tomato variety available against late blight pathogen worldwide. New fungicides are introducing in the country every year against late blight pathogen but none of them found effective due to the dynamic changes of population structures of *P.infestans (Rashidul.et.al.,2013)* . There are a number of practices available for the management of *Phytophthora.*

Cultural practices are done in nurseries where potting mixtures are steamed to kill *Phytophthora* inoculum and only *Phytophthora* free plants should be grown and used. It is impossible to remove *Phytophthora* from the soil so if the *Phytophthora* is present, metalaxyl is added to minimize the disease growth but most strains of P. infestans became resistant to metalaxyl. To reduce the occurrence of resistance, a single-target fungicide such as metalaxyl along with carbamate compounds. Organic amendments and mulching can be done to prevent the growth of the disease. Mulching stimulates plants root growth, increase nutrient uptake, regulate soil temperatureand provide high level of nutrients for the *soil (Aryantha et al. 2000)*. Organic amendments can inhibit the growth of *Phytophthora* by adding alfalfa meal, cotton waste, wheat straw, soya bean meal, and urea. Ammonia and volatile organic acids released by decomposing organic matter kill *Phytophthora*, and the residual organic matter stimulates competitive and antagonistic microorganism in the soil.

Fungicide treatment can be done to protect the plants. Bordeaux mixture is the best example that has successfully control diseases caused by *Phytophthora* species. Bordeaux mixture is a combination of calcium hydroxide and copper sulphate so it is labour intensive to prepare and apply. This mixture cannot be used in a long run because it can easily be washed off by the rain.

Phenylamides (acylanilides) can also be used. This group contains chemicals, which include metalaxyl (Ridomil), benalaxyl (Galben) and furalaxyl (Fongarid) (*Drenth and Guest, 2004).* Metalaxyl has shown effectiveness at controlling infection caused by species of *Phytophthora*. Due to its systemic nature metalaxyl, it can control infection beyond the roots. The mode of action of matalaxyl involves inhibition of RNA synthesis. It is highly inhibitory to sporangium formation, and reduces oospore formation.

2.8. Why RNAi approach?

RNAi has come up as a breakthrough in the field of molecular biology, providing another face to the unexplored nature of the molecule "RNA". RNAi a wide term that can refer to the impacts of short or long noncoding RNAs on the expression of people or groups of protein-encoding genes. RNAi is a process by which cells utilize short, double stranded RNAs (dsRNAs) to recognize messenger RNAs (mRNAs) with its specificity, prompting their enzymatic destruction and translation into the protein. These inhibit gene function *(Karan.et.al, 2007).* For the exogenous control of this pathway, dsRNA is introduced into cells in different forms. The two methodologies mostly used to silence a given gene in vitro through RNAi are (1) the introduction into cells of in vitro small interfering RNA (siRNA) or (2) the utilization of DNA template known as short hairpin RNA (shRNA) that have cells used to create the siRNAs (*neema.et. al.,2003).* The latest methodology for the protection of the field crops is the gene silencing. The antisense RNA technology was also being used to achieve gene silencing. However, the loss of function was not achieved; partial loss of function was achieved in 70% of the first level. Double-stranded RNA is being used for the regulation of gene expression. The dsRNA inhibits the gene expression with a complementary nucleotide sequence. This technique was discovered to be successful in the case of potatoes. So, in this study, we centered on the use of RNA silencing in tomato plants to deliver the pathogen free plant (*Safe, 2013).*

2.9. Mechanism of RNAi

As RNAi machinery is being discovered, the mechanism of RNAi is developing and emerging more. Over the most recent years, significant insight has gained in explaining the mechanism of RNAi. Results acquired from a few in vivo and in vitro experiments have gelled into a two-step model for RNAi/PTGS. RNAi components involved in the processing of are Dicer, inducer, RNA-induced silencing complex (RISC), and RNA-dependent RNA polymerase (RdRp). All these components in coordination with other effector molecules work together in an organized fashion, resulting in the silencing of the target gene.(*Karan.et.al., 2007*). The initial step, known as the RNAi initiating step, in which RNA nucleases shows binding to a large dsRNA and its cleavage into discrete 21-to 25-nucleotide RNA fragments (siRNA). In the second step, these siRNAs join a multinucleate complex, RISC, in which degradation of homologous single-stranded mRNA occurs. A little is known about the RNAi intermediates, complexes of RNA-protein, and mechanism for the formation of complexes RNAi *(Farrell, 2010).*

2.10. Methods to introduce dsRNA in the host

There are multiple method used to introduce dsRNA into the host cell. Two methods that are popular for the introduction are explained below.

2.10.1. Host induced dsRNA

Host induced gene silencing (HIGS) is the RNAi based process. In this process, we consider small RNAs that are made in the plant to silence genes of pathogens or pests that infect the plant. These RNAs are made by double-stranded RNA that is introduced in plant cells with the help of viruses or agrobacterium which can easily replicate this dsRNA(*Qi.et.al.,2019*). HIGS has developed as a promising methodology for increasing plant resistance to pathogens by targeting the genes essential for control of the infection. Though it is a popular method it has numerous challenges, the selection of the target sequences so that they do not affect either the host or the non-target organisms and the difficulty of producing the stable transgenic plant that can be safe and is accepted by the consumer *(Yin.et.al., 2015).*

2.10.2. Spray induced dsRNA

The spray induce dsRNA is the non transformative technique to provide protection to the plants. The dsRNA is sprayed on the plant surface that is targeting the pathogen. The dsRNA is either taken up by the fungal pathogen or by the host and then induce either the fungal RNAi machinery or the plant RNAi machinery *(Koch et al. 2016).* Using the approach silences the pathogen gene without introducing any heritable changes in the genome. This technique is more advantageous as it is a disease control strategy and works on both monocot and dicots. This technique is fast, powerful and environment friendly which overcomes the problem of genetically modifies crops *(Sang and Kim, 2020).*

CHAPTER 3

Material and methods

3.1 Plant material

Young tomato leaves were taken from the plant, which was at least 6 weeks old. The leaves must not be treated with any fungicide. It should also not contain any infection caused by different pathogen. The plant should be free from any susceptible diseases present in those environmental conditions.

3.2 Culture requirements:

Phytophthora culture was long term preserved in Rye A media and was provided by CPRI-ICAR, Shimla. Further, our experiment was carried out in Rye agar B or frozen pea agar. *Phytophthora infestans* requires zoospores for the infection to occur. The minimum requirement for zoopsores is around 40,000-50,000 spores present in 300µl seen under inverted microscope in 10X.

3.3 Methodology:

3.3.1 Multi si-RNA

Six different genes were targeted for the construction of multi si-RNA. These genes code for the proteins of *Phytophthora infestans,* which makes the host organisms (tomato) susceptible. This genes are SOR (Green), TEF (Blue), HSP 90 (grey), GPI (Yellow), PL-D (Pink), and AvrBLB2 (red). These genes were linked together to form the multi-siRNA of 640 base pairs. All these six genes protein products target the host immune system and cause infection in that plant.

AACTGCACGCACGGCTGTATTGGCAAGTACGTGGTGGACAAACCCATGGTTCTAGG
ACACGCGTACGACGGAACATTGGCCAAGTTCTACCGCATCCCCGAGGATTTCTGCTA
CAAACTGAGAGTCCTGGCAACAAAGTTGTGGACCGGTCACTTGATCTACAAGTGGTC
ACCGTGACTTCATCAAGAACATGATTACGGGTACCTCGCTCCCTCTACTCGAACAAG
GACATTTTCCTGCGTGAGCTCATCTCGAACGCCTCGGATGCCCTCGACGAACGAGAA
GTACAACAAGTTCTACGAGTCGTTCAGCAAGAACCTCAAGCTGGGCATCCACGACG
AAGGCTGACCTGATTAACAAAACAGGCATGGACAGCAGCATGAACATGACGTCCAG
CGGCACGAAACCGAAGCGGATTTATCCGATGGAACGGACATCAAAGTCCGGCAGCC
TCTCCTTAAAGCAACAGACTGGTTCCTCACGCTACAGCCCTGATCCCATTAAAACCG
GATATCGACCCTATACCACTGGCAAATTCGGCAAACAGAGTGTCCAGATCACGAGA
ACATTCAGCTGCACCTACCCCTTGTCGAAGACATCTCCTGACACTGTGGCCCAAGAG
GCCGGATATCAAGATAGGC

Central Potato Research Institute- ICAR, Shimla, patents this technology.

3.3.2 Preparation of culture:

The 50 µl of previously cloned culture was prepared and kept for overnight incubation. The cloned culture consists of our gene of interest with T7 polymerase promoters present upstream of our gene. T7 polymerase is a RNA polymerase, which directs the formation of RNA from DNA in 5' to 3'direction. This promoter is extracted from bacteriophage. The incubation was done in 3 ml of Luria- Bertani broth containing 3µl of ampicillin.

3.4 Experiment 1- Isolation of Plasmid:

Plasmid DNA was isolated from the overnight grown culture. The cells were pelleted down at 10,000 rpm for 3 minutes. After the supernatant was discarded, the pellet was dissolved in 250µl re-suspension buffer with RNase A. The cells lysis was done by adding 250µl of lysis buffer. It was mixed by inverting the tube and was incubated for 5 minutes. Further 350µl of precipitation buffer was added, it was mixed by inverting the tube. The mixture was homogenized and was centrifuged at 10,000rpm for 10 minutes. The supernatant was loaded in a spin column and centrifuged at 10,000 rpm for 1 minute. Further, 600µl of wash buffer with ethanol was added and centrifuged at 10,000rpm for 1 minute. The spin column was then placed in 1.5ml recovery tube and 30µl of TE buffer was added. The incubation was done for 1 minute. The column was centrifuged at 10,000rpm for 1 minute. The flow through was collected in the micro centrifuge tubes. The flow through was checked and quantified using 1.2% agarose gel and nanodrop respectively.

3.5. Experiment 2- Gel extraction using NucleoSpin® kit:

After the bright bands are seen in the agrose gel of the plasmid isolated in experiment 1. The bands around 600 base pairs were excised. The excised DNA fragments along with the gel were weighed. Then, 200 µl Solubilization Buffer was added. The samples were incubated for 5-10 min in 50°C so that gel was completely dissolved in the buffer. The solution was transferred into Column (only upto 700µl) and was spun to 10,000 rpm for 1 min. Wash Buffer of 700µl was added and again spun down to 10,000 rpm for 1 min. Dry spin was done for 1 min for removing the extra residual ethanol. Finally, the DNA was eluted using clean 1.5 ml micro-centrifuge tube by adding 15-30 µl elution buffer and incubating it for 1 min. Centrifuge was done for 1 min at 10,000 rpm. The purity of the DNA extracted was checked on nanodrop.

3.6 Experiment 3- PCR amplification:

The PCR amplification was done of the positive clones, which contained the siRNA with T7 polymerase promoter sequences. PCR was performed using a Biorad PCR machine containing total volume of 20 µl. The different components used and their volumes is shown in table 1.

Plasmid DNA	1µl
Forward primer	1µl
Reverse Primer	1µl
Water	7 µl
Emrald PCR mix	10µl

Table 1: PCR mix of total volume of 20 µl

The thermal cycle programmed during was done as shown in table 2. This cycle further continued for 30 more repeats.

Initial denaturation	94°C	4 minutes
Final denaturation	94°C	1 minute
Annealing	51°C	30 seconds
Initial Extension	72°C	45 seconds
Final Extension	72°C	5 minutes

Table 2: Conditions for amplification of gene of interest

After checking the PCR product in 1.2% agarose gel. The bands were further solublized, done same as in 3.5.

3.7 Experiment 4- MegaScript RNAi preparation kit by Invitrogen:

For preparation of dsRNA 2ml tube was taken. Total volume was made upto 40 µl. The different components used for the synthesis of dsRNA used as shown in table 3. This mixture was kept for overnight incubation in 37°C. The major difference was that instead of dTTP, dUTP was used for the production of RNAi.

PCR product	20µl
dATP	2µl
dCTP	2µl
dGTP	2µl
dUTP	2µl
T7 polymerase enzyme	2µl
T7 reaction buffer	4µl
H2O	6µl

Table 3: Components for the synthesis of dsRNA

3.8 Experiment 5- Testing efficacy of multi si-RNA on the growth of *Phytophthora infestans:*

The dsRNA prepared in experiment 4 was diluted to get different concentration. The dsRNA prepared was spread on a rye agar B plates. The bit of *Phytophthora infestans* of previously grown culture was kept on them. It was incubated for 5 to 7 days to get proper growth. Different concentrations of dsRNA used are shown in the table 4.

Sample	**dsRNA (µl)**	**Nuclease free water (µl)**	**Concentration ng/µl**
A	5	5	15554.4
B	5	10	13576.5
C	5	15	1581.7

Table 4: Concentrations of dsRNA used for Rye B Agar plates for detached leaf assay

3.9Experiment 6- Detach leaf assay:

3.9.1 On leaves:

For detached leaf assay, fully grown tomato leaves were taken. *Phytophthora infestans* cultures were grown in different concentrations of dsRNA. The leaves were inoculated with cultures that were grown in Experiment 5. The leaves were kept in a water saturated florist foam. A drop of water was added on the leaves. The *Phtophthora infestans* bit from those cultures were kept on the young leaves. The leaves were incubated for over a period of week.

3.9.2 On petioles:

The leaf that showed least infection in the detached leaf assay on tomato leaves were taken. The concentration of that dsRNA was further diluted. It was mixed with nanoclay. Nanoclay helps to provide minerals and nutrients to the tomato leaves. Young leaves containing long petioles were dipped into the mixture and were kept until the leaves absorbs the solution completely. The leaves were kept in water-saturated foam tray. The leaves were inoculated with 300 µl of zoospore suspension. It was incubated for the time period of 1 week for the infection to spread. The different concentrations used in this method as shown in table 5.

Sample	dsRNA (µl)	Nuclease free H_2O (µl)	Concentration ng/µl
A	2.5	47.5	405
B	5	45	787.7
C	6	44	808

Table 5: Different concentrations of dsRNA used for Detach leaf assay used for petioles

CHAPTER-4

Results and Discussion:

4.1 Confirmation of multi siRNA construct:

The multi siRNA construct of 7 genes were confirmed on 1.2% agrose gel. Lane 1 shows the ladder of 1Kb and the lane 2 shows our siRNA construct of 640bp.

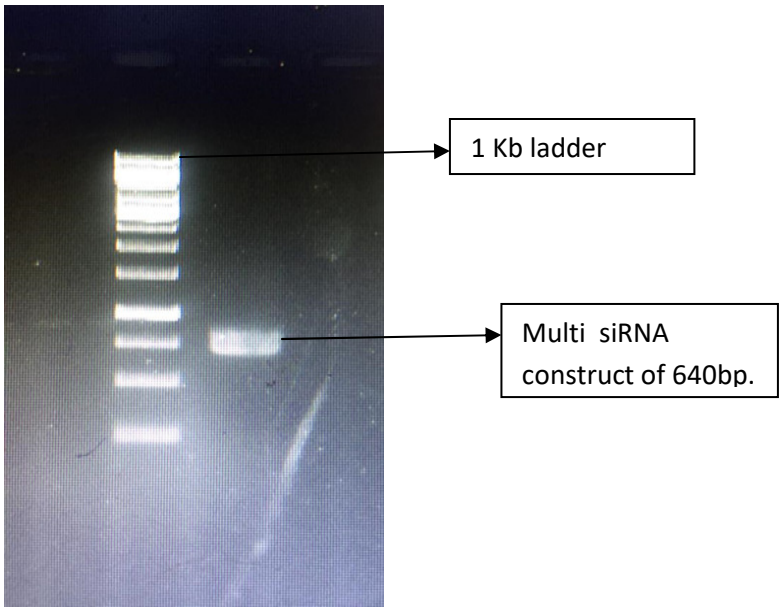


Figure1. The lane 1 shows the 1Kb ladder
and lane 2 shows the multi siRNA construct of 640bp
on 1.2% agrose gel.

4.2 Invitro transcription of dsRNA

dsRNA synthesis was done using the megascript RNAi preparation kit in which different components were taken as seen in experiment 4 (table 3). The lane 1 and 3 shows the 1Kb ladder and lane 2 shows the 640 bp multi siRNA construct and lane 4 shows the smear of dsRNA.

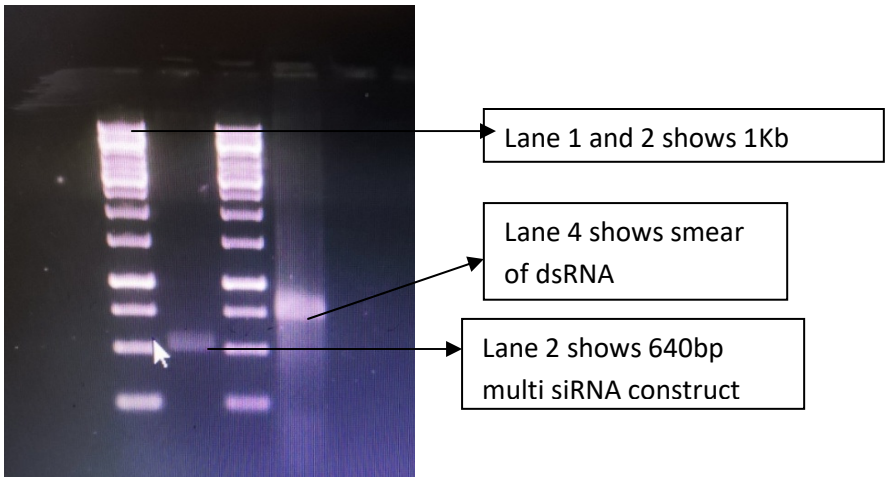


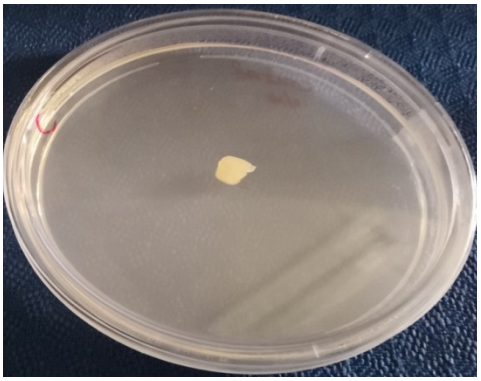
Figure 2. Lane 1 and 2 shows the 1Kb ladder,
lane 2 shows the multi siRNA of 640 bp. and
lane 4 shows the smear of dsRNA on 1.2% agrose gel.

4.3 Efficacy of multi si-RNA on the growth of *Phytophthora infestans:*

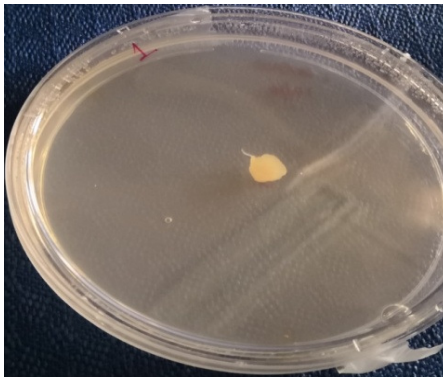
The Rye B agar plates were spread with dsRNA concentrations obtained in Table 4 (Experiment 5). The bit of *Phytophthora infestans* was kept and incubated for a period of one week. Three different concentrations and one negative control were used. Figure 1 shows the bits of *Phytophthora infestans* on the plates on 0^{th} day and figure 2 shows the growth of the *Phytophthora infestans* on 7^{th} day. The incubation was done in 25°C.

After seven days on visual analysis, The maximum growth was seen in Sample C(1581.7 ng/µl dsRNA) as the the circumference of the culture was the largest. The minimum circumference was seen in sample B(13576.5 ng/µl dsRNA)

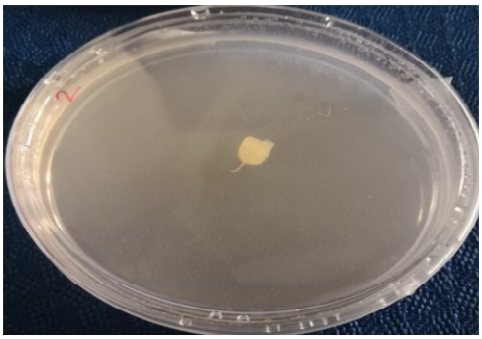
As the least growth was shown in sample B so the ideal concentration taken for further experiments was 13576.5 ng/µl dsRNA.



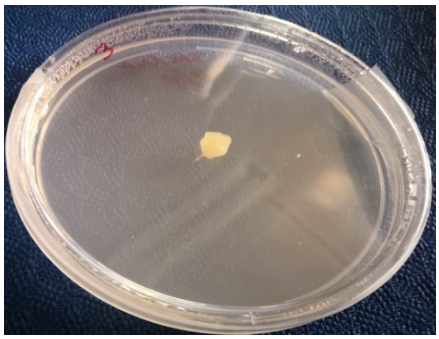
Control



Sample A: 15554.4 ng/µl of dsRNA

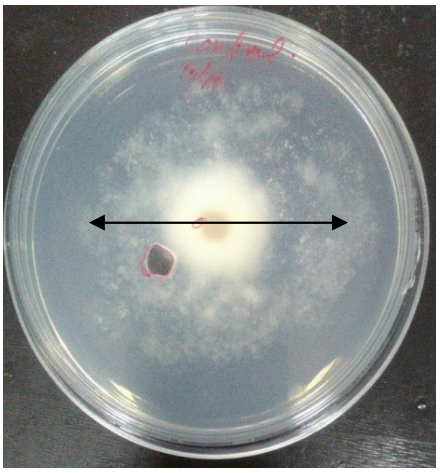


Sample B: 13576.5 ng/µl of dsRNA



Sample C: 1581.7 ng/µl of dsRNA

Figure 1. Rye B Agar plates containing bits of *Phytophthora infestans* on 0 day. There are 3 different concentrations were used of dsRNA. Sample A 15554.4 ng/µl, Sample B 13576.5 ng/µl and Sample C 1581.7 ng/µl. These plates were incubated for seven days in 25°C.



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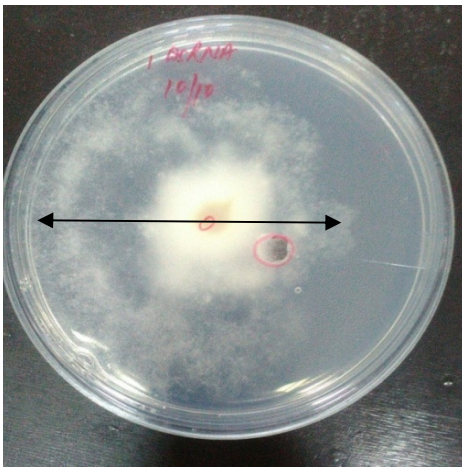
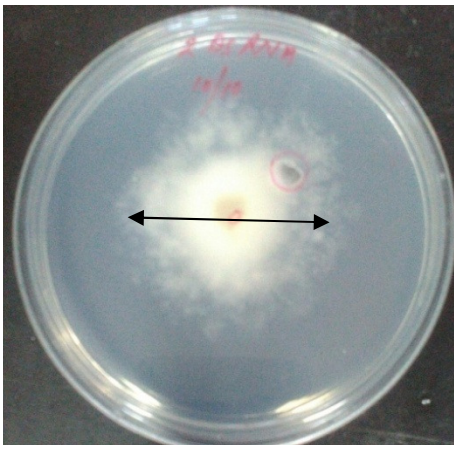
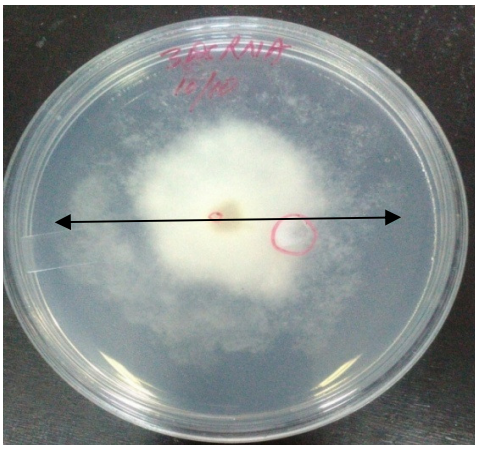


Sample A: 15554.4 ng/µl of dsRNA



Sample B: 13576.5 ng/µl of dsRNA



Sample C: 1581.7 ng/µl of dsRNA

Figure 2. Rye B Agar plates containing bits of *Phytophthora infestans* on 7th day. The maximum growth was seen in Sample C(1581.7 ng/µl dsRNA) as the culture was the more spread than in A and B. The minimum circumference was seen in sample B(13576.5 ng/µl dsRNA) Sample A shows less growth than sample C but more than Sample B. These results are based on visual analysis.

4.4 Detach leaf assay:

4.4.1 On leaves:

In experiment 6 (3.9.1) the detached leaf assay on leaves were carried out. Bits from culture as seen in figure 2 were kept on young tomato leaves and incubated for 7 days. Figure 3 Shows leaves on 0^{th} day and figure 4. shows the tomato leaves on 7^{th} day.

After seven days on visual analysis, the best result was seen in sample B as the spread of infection is limited to some region. Although, the concentration of dsRNA in Sample A was the highest but because of the off- targets effects it shows the maxium infection.

Figure 3: Young Tomato (2 weeks old) leaves having bits of *Phytophthora infestans,* from the culture shown in, figure 2 on 0^{th} day of incubation.

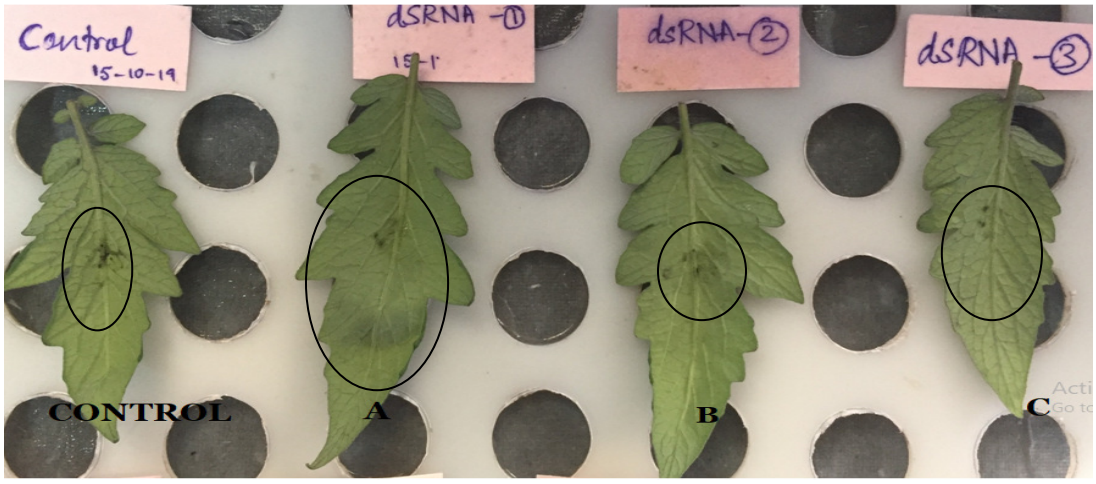
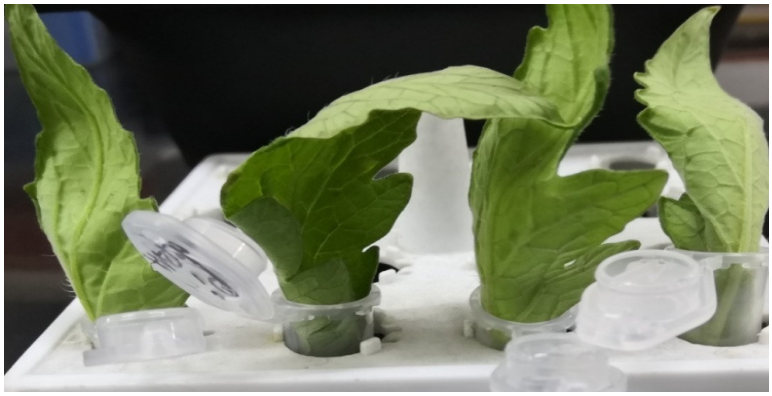


Figure 4: Detach leaf assay on leaves.Young Tomato leaves showing different level of infections during 7^{th} day of incubation. Spread of infection is maximum in sample A and in Sample B the spread of infection is minimum.

4.4.2 On petioles:

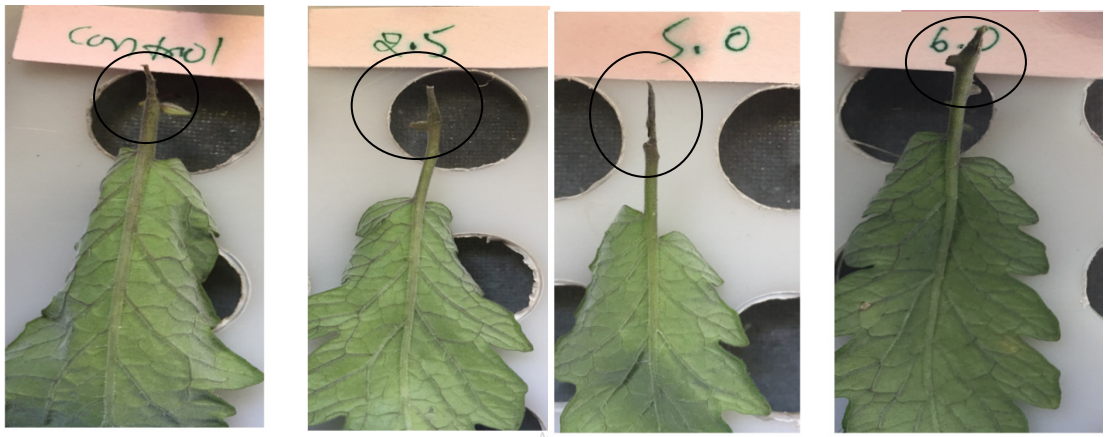
In experiment 6 (3.9.2), the detach leaf assay on petioles. The sample B in figure 4 contains the best result in controlling the spread of infection. The concentration (13576.5ng/µl of dsRNA) with respect to this sample was diluted further (as shown in table 5). These concentrations were mixed with nanoclay to optimize the infection in tomato petioles. Figure 5 is the 0^{th} day of the incubation and figure 6 shows the spread of infection on 7^{th} day.

After seven days, as the petioles of the young tomato leaves were observed for infection. The spread of infection was minimum on sample C that is 808 ng/µl. And the highest was seen in sample B (787.7 ng/µl).



A B C D

Figure 5: On 0^{th} day, Petioles of young tomato leaves were dipped in different concentrations of dsRNA with nanoclay (for minerals). Sample A 405 ng/µl Sample B 787.7 ng/µl Sample C 808 ng/µl



CONTROL A B C

Figure 6: Deatch leaf assay on petioles. On 7^{th} day, the spread of infection was minimum on sample C that is 808 ng/µl. And the highest was seen in sample B (787.7 ng/µl). Sample A (405 ng/µl)has infection relatively more than B and less than sample C

Conclusion

As the advancement and growth is occurring in the agricultural sector. Many different ways which are chemical free and more environmental friendly are been considered. Among many approaches, the one we chose was of RNA interference (RNAi) by spray induced method. This method ultimately leads mRNA degradation of the genes, those make the tomato plant susceptible of late blight. The Spray Induced method used is non invasive method as there is no induction of transgene into the plant. This method is already been tested on potato plant and showed positive results.

In this project, we conducted experiments using dsRNA on tomato leaves and petioles. Different concentrations of constructed dsRNA were used to check the growth of this pathogen via a detach leaf assay. It was observed that in leaves the highest concentration of dsRNA produced more infection due to off-target effects whereas the spread of infection was limited when the concentration was reduced. Whereas in petioles the dsRNA concentration that showed limited growth of infection in leaves was taken and was diluted further which showed that at the highest concentration of dsRNA produced the minimum infection on the petioles.

This method can be further optimized an can be used to check the result in large scale. This method can be proved as a very effective way to help the farmers to protect tomatoes and potatoes from late blight disease.

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