Current Research in Microbiology

Chapter 4

Resistance Strategies against Microbial Plant Pathogens

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Abstract

Major phytopathogens include viruses, fungi and bacteria are causing significant losses to the varieties of agroeconomical crops. Main causes of infection in plants are viruses, fungi and bacteria. These Plant pathogens are among the most significant challenges and causing severe damages especially to crop plants. Recent developments in the genetic engineering techniques and advancements in the understanding of plant defense mechanism have enabled the scientist to devise better strategies at the molecular level against plant pathogens additional to classical breeding tools or chemical control. Understanding the molecular mechanisms involved in the host plant defense has great potential for moderating the impact of plant disease outbreaks. Our discussion in this chapter will mainly deal with molecular strategies to generate transgenic plants against plant pathogens for sustainable resistance in crop plants. Beside this, bacterial and nematode resistance strategies are also discussed and possible solution to meet the recent challenges is proposed.

1. Plant Pathogens

Plants are infected by various pathogens including viruses, bacteria and fungi. Various studies have been published recently on the basis of their importance in crop plants [1,2]. The severity of attack and damages of plant pathogens varies due to different host and environmental factors. Among the plant pathogens viruses and fungi are of great importance but recently the survey on the bacterial pathogens was highly cited and international community allowed the construction of list of top damaging bacterial pathogens list too.

1.1 Plant Viruses

Viruses are submicroscopic agents that infect living organisms. They consist of nucleic acid (either DNA or RNA) and a protein coat. More complex viruses may additionally have a membranous envelope which is derived from the host but also contains virus-encoded proteins. Although the issue is debatable, viruses are generally not considered to be "living" and are probably best described as molecular pathogens. they do not have the ability to replicate outside the host cells are not functionally active outside host cells and require the biochemical machinery of a host cell to reproduce (multiply).

Viruses infect virtually every form of cellular life, including the simplest bacteria, animals and plants. The 9th report of the International Committee on Taxonomy of Viruses (ICTV) has classified viruses into 6 orders, 87 families, 19 subfamilies, 349 genera and more than 2200 species [3]**.**

The relationship of plant viruses with plant diseases was identified little more than a century ago. The first virus, *Tobacco mosaic virus* (TMV), was identified in 1898 by Martinus Beijerinck, who determined that plant sap from "mosaic disease" affected tobacco can cause infection even after passing through a porcelain filter which retained bacteria. Beijerinck referred this infectious fluid as a "contagium vivum fluidum", from which the term "virus" is derived. TMV was crystallized first by Wendell Stanley in 1935 [4]. He received the Nobel Prize in Chemistry in 1946. Major advances were made in the field of plant virology during the 1930s resulting in the publication of the first text book of plant virology in 1937 by Kenneth Smith [5].

The majority of plant viruses have RNA genomes. However, some plant viruses have DNA genomes, and these may be further sub-grouped into single-stranded DNA (ssDNA) and double-stranded DNA (ssDNA) viruses. Geminiviruses and nanoviruses are examples of ssDNA viruses [6] whereas badnaviruses and caulimoviruses are examples of plant viruses having dsDNA genomes [7]. Geminiviruses are one of the largest group of plant infecting viruses and causing economic losses to the various monocot and dicot crops all over the world. Large number of satellite molecules have also been identified that increase the pathogencity of plant

DNA viruses. These satellite molecules are approximately half the genome of monopartite or bipartite geminiviruses but can increase the movement and pathogencity of the viral infection in plants [8].

1.1.1 Transgenic Strategies for Countering plant viruses

The consideration of viruses in living organisms remained under debate for a long time due to absence of cellular structure and organelles. The only living character of these viruses is their genome which consists of either RNA or DNA. Plant viruses use the host cellular machinery for replication and control the defense mechanism. There is no chemical or physical way to control them. At this time the most efficient way to counter plant viruses is by killing the insect vectors using insecticides, preventing access of the vector to plants, for example by using screens or plastic film, or by breeding virus resistant plant varieties. The use of insecticides is expensive, environmentally unfriendly and also not durable since the insect vectors develop resistance. The use of screens is only practical on a small scale. In many cases the breeding of resistant varieties have not proven possible, due to the absence of suitable resistance sources, or have not been durable, such as the loss of resistance against the viruses causing CLCuD in Pakistan in the early 2000s [8-10]. It is for these reasons that researchers have increasingly looking at genetic engineering as a means of obtaining resistance against viruses, including geminiviruses which is one of the most important monocot and dicot infecting DNA viruses having more than 400 species. These plant viruses infection have been attributed with the reduction in leaf size, enation, leaf curling, vein yellowing and enation (Figure 2, Panel A-C). In essence the strategies used to obtain transgenic resistance to plant viruses can be divided in those that are pathogen-derived and those that are not.

1.1.1.1 Pathogen Derived Resistance (PDR)

Pathogen Derived Resistance (PDR) can be further divided into two subclasses - 1) Pathogen derived protein mediated resistance (PDPMR), in which a functional protein is used to mediate resistance and 2) pathogen derived non-protein mediated resistance (PDNPMR) for which viral sequences which donot encode a functional viral protein is used [11]. PDPMR failed to show prominent resistance showed resistance against distantly related virus species and ultimately plants become more susceptible to disease. Unfortunately the expression of these proteins in plants in some cases induced virus-like symptoms, one of the potential drawbacks of protein expression for obtaining resistance.

Extensive efforts have been made in the realm of PDNPMR to use viral genome to obtain resistance in plants. The most widely used and successful PDNPMR strategies which have been employed were based on RNA interference. This strategy involves the expression in plants of virus derived sequences which are processed by the plant to produce so-called small interfering (si)RNAs which mediate the effect of RNAi. The transgenes used could be virus

sequences expressed in sense, antisense or, more recently, hairpin constructs (expression of both sense and antisense sequences separated by an intron, which has been shown to deliver more efficient resistance [12-13]. A variety of sequences from a range of viruses have been used to generate resistance although in most cases, it was a truncated version of the Rep gene was chosen. Examples of this include *African cassava mosaic virus* [ACMV; [14]], *Mungbean yellow mosaic virus* (MYMV; [15]), *Tomato yellow leaf curl virus* (TYLCV; [16]). RNAi-mediated resistance has some advantage over protein mediated resistance since viral coding or non-coding sequences can be targeted and there is less likelihood of deleterious effects on plant growth.

1.1.1.2 Non-Pathogen Derived Resistance

NPDR is achieved by expressing proteins which can halt the virus at different level of infection has so far always involved the expression of proteins. Expression of a homologue of GroEL (a chaparonin produce by insect endosymbiotic bacteria) has been shown to impart resistance. GroEL has been shown to bind geminiviruses in insects where it is believed to stabilize (protect) the virus as it circulates through the insect's haemolymph. Plants transformed with GroEL were surprisingly protected from harmful effects of virus without reduction of virus titer [17-19].

Another novel approach for resistance against plant infecting DNA viruses utilises peptide aptamers. Peptide aptamers are short ~20aa peptides which bind their target protein and interferes with its function. Peptide aptamers can interfere with protein-DNA and protein-protein interactions [20-21]. Recently, various peptide aptamers have been identified which strongly interact with Rep protein of quite distinct plant viruses. Although not providing immunity, the aptamers had striking effect on symptoms in transgenic plants and significantly reduced virus DNA levels [22].

Artificial zinc finger (AZF) proteins, which are modified nucleic acid binding proteins, are another resistance strategy which have been investigated in last decade for resistance particularly against plant viruses [23-24]. Various techniques have been adopted previously to increase its affinity of AZF protein to bind with ssDNA viruses which resulted in reduced or no replication of the virus [25-26].

Although not entirely non-pathogen derived, [27,28] used the "inducible" properties of the begomovirus virion-sense transcription unit to express dianthin in transgenic plants. Thus, upon infection, the TrAP induced the expression of dianthin, a potent ribosome-inactivating protein, leading to cell death. This strategy was shown to lead to efficient resistance (just cell death at the site of inoculation). However, the approach has not found further use due to the fear of expressing such a toxic protein in plants for human or animal consumption. In most of the cases, against these strategies couldn't succeed much against DNA viruses but the resistance

against RNA genomes are quite significant.

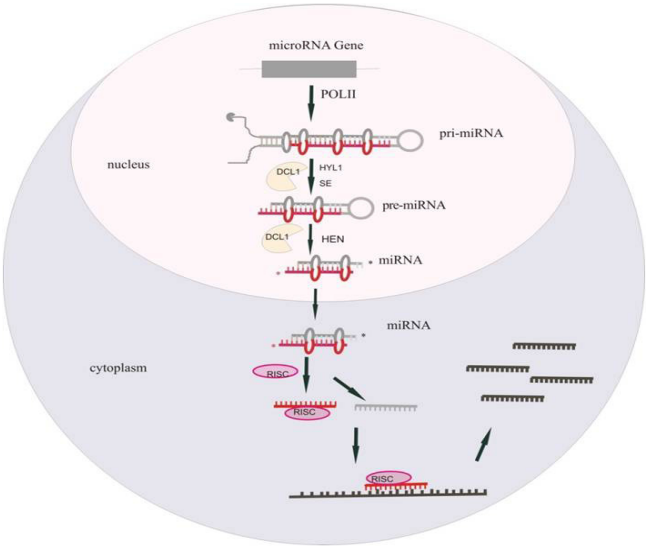

Figure 1: A generalized MicroRNA Pathway in Plants. miRNA genes are transcribed by DNA polymerase II (POL II) into primary (pri)-miRNA structures with poly-A tails which through the action of Dicer like protein1 (DCL1), HYPONASTIC LEAVES 1 (HYL1) and SERRATE (SER) are processed into precursor (pre)-miRNA. Further action by Hua Enhancer 1 (HEN1) and DCL1 process pre-miRNAs into mature miRNAs .miRNAs are then exported into the nucleus and one strand (the guide strand) is incorporated into the RNA induced silencing complex (RISC). The incorporated miRNA then guides the sequence-specific recognition of the target mRNA which is cut by the action of an Argonaute protein (AGO), part of the RISC.

The possible involvement of RNAi in plant host defense against viruses was first shown when transgenic tobacco plants carrying *Tobacco etch virus* sequences were found to recover from infection by the virus [29]. Subsequently RNAi was shown to be a natural component of innate antiviral immunity of plants when viruses were found to naturally induce a similar response in non-transgenic plants [30,31]. Since these initial investigations, RNAi-based strategies have become the "weapon" of choice in trying to develop resistance against phytopathogenic viruses [32,33].

These studies have met with a good degree of success with respect to viruses with RNA genomes, and transgenic, virus-resistant plant varieties with PTGS(siRNA)-mediated resistance are available commercially [34]. The first and most prominent of these is the use of transgenic papaya to overcome losses due to *Papaya ringspot virus* in Hawaii [35].

PTGS (siRNA)-mediated transgenic resistance has also been investigated as a means of providing plants with protection against plant-infecting viruses with DNA genomes. For geminiviruses a hand-full of studies have been published with varying levels of success [33]. Despite these efforts so far only a single success story has so far been described. Transgenic beans have been produced in Brazil with a hairpin RNAi construct targeting the Rep gene of *Bean golden mosaic virus*– a bipartite begomovirus [36]. Recently the bean variety has been approved for commercial cultivation [37]. This apparent lack of success in obtaining resistance against geminiviruses using the siRNA approach can be attributed to a number of factors including the high mutation rate of these viruses, the diversity of these viruses (with

usually multiple viruses in an area causing disease in each crop) and the fact that geminiviruses encode efficient suppressor of RNA silencing [38] and their genomes, being located in the nucleus [39] are immune from PTGS.

RNAi has become the technology of choice in efforts to develop transgenic resistance against phyto-pathogenic viruses but recent developments in the use of artificial miRNAs is giving new hope for developing resistance against DNA viruses. MicroRNAs are non-coding genes, transcribed in the nucleus and after sequential modifications, these sequences are modified into Primary miRNA (Pri-miRNA) and then in to Pre-miRNA which generate 20-24 nucelotide single stranded mature microRNA to cytoplasm with the help of nuclear proteins. These miRNAs are involved in the sequence specific regulation of genes. First use of artificial microRNA was applied against the RNA viruses in 2006 which showed much better resistance even at low temperature against multiple viruses. This strategy has been reported also successful against DNA viruses in the model plants. More recently engineered miRNAs have been investigated as a means of obtaining resistance following the demonstration that the targeting sequences of pre-miRNAs could be modified [40]. This approach has been shown to effectively deliver resistance against viruses including, most recently, the bipartite begomovirus ToLCNDV [41].

1.1.1.3 CRISPR-Cas9 against plant viruses

Plant viruses have relatively a small genome and by the use of next generation sequencing technology, it has been possible to precisely predict the targeted sequence for viral resistance but still the lack of durability of viral resistance is a draw back because of recombination ability of viral genomes and emergence of new species of plant viruses. Recently, the clustered regularly interspaced palindromic Repeats/CRISPR-associated 9 (CRISPR/Cas9) system has emerged as a promising technique of genome engineering which has been investigated against all pathogens at different level. It has become a simple, most user friendly and efficient, precise genome editing tool for development of genetically edited crops. The CRISPR/Cas9 system is a RNA guided programmable endonuclease based technology composed of 2 components, the Cas9 nuclease and an engineered guide RNA targeting any DNA sequence of the form used for novel genome editing applications in many organisms including plants. Recently many studies have been something is missing rep CRISPR/Cas9 mediated virus resistance development in the plants with promising resistance durability. The CRISPR/Cas9 mediated resistance strategies have been adopted to target the viral genes as well as the host factors which support the viral replication in the plant genome [42]. However, the success of virus resistance is limited to model plants yet. However, the researchers are quite hopeful in the sustainability of resistance in the field crops.

RNAi has become the technology of choice in efforts to develop transgenic resistance against phyto-pathogenic viruses. Numerous studies have shown this approach to potentially yield effective resistance against geminiviruses (reviewed by [43]. The work conducted here has shown for that this approach also has promise for engineering resistance against monopartite begomoviruses that interact with beta satellites.

1.2. Bacterial Pathogens

Bacterial pathogens can infect almost all the plants [44] . Until the late nineteenth century, there was no concept of bacterial diseases of plants but with the passage of time, famous botanist Dr. Antony de Bary explained first time about the rare occurrence of bacterial infections of plants which was reviewed by Smith in 1896. Bacteria are unicellular microorganism with more complex genotype as compared to plant viruses. They contain no nucleus and reproduce by dividing into two equal parts. Their mode of division is relatively fast and as a result there are chances of more mutations.

More than two hundred species of phytopathogenic bacteria have been identified so far and almost all of them are parasites within the plant either in soil or on the surface of plants. Among the ten top most reporting bacterial plant pathogens is *Pseudomoas syringae* causing serious economic losses to plants. *Ralstonia solanacearum* is most important pathogen of potato and banana plants. The specific transformation ability of *Agrobacterium tumefaciens* causes crown gall diseases to the plants and this ability of bacterium due to its binding factor has been adopted for scientific investigation of genes in host plants (Gelvin, 2003). *Agrobacterium* tumefaciens opened the new era of recombination DNA technology for molecular biologists. Various *Xanthomonas* species are standing on the fourth, fifth and sixth position of plant pathogens which have been categorized according to host range and pathogenicity. *Xanthomonas oryzae* infecting mostly to rice crop plants are standing on the position four and *Xanthomonas campestris* infect the wide range of host plants where as *Xanthomonas axonopodis* cause the bacterial blight in cassava lies on sixth positon. *Erwinia amylovora* is at the seventh positioncausing the fire blight disease in large range of bushes, ornamental and fruit plants. *Xylella* fastidiosa lies on the eighth position among the top ten bacterial pathogens and is claimed as first reported plant bacterial pathogens whose genome has been sequenced first after viruses. Two species of Dickeya named *Dickeya solani* and *Dickeya dandtii* are placed on the ninth and tenth position. It has been clear from the previous reports that Dickeya species cause important losses to the potato crop [45].

2. Resistance Strategies against Bacterial Plant Pathogens

Dissemination of bacteria can be accomplished by several means. Some bacteria can survive on inanimate objects, in water or inside insects. It is important to know the survival characteristics of bacteria for effective management strategy and intervention in dissemination.

Some species have the ability to move short distances in water on their own power by use of their flagella. Most bacteria, however, are disseminated by passive agents such as air and insects, water and soil movement, and to a lesser degree by humans, water and other animals. Infected seeds and transplants can also be a source of inoculums.

Among the plant pathogens, bacterial and fungal disease can be controlled by chemical agents to some extents but for the long lasting and broad spectrum resistance solution against the plant pathogens is development of transgenic plants which give resistance to the infectious DNA genomes at different level of replication, transcription and post transcription which have discussed in detail in section 1.2.

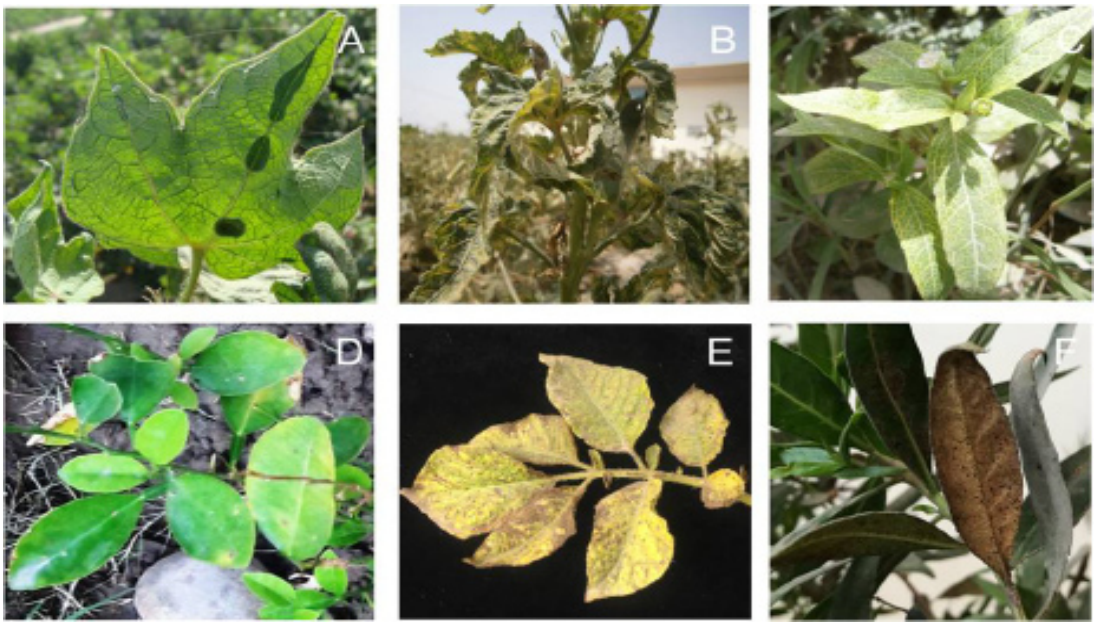

Figure 2: The viral infected plants of Cotton, Okra and Ageratum (A-C), bacterial attack on lemon (D) and fungal symptoms on potato plants (E-F).

2.1 Resistance strategies to control Bacterial pathogens

Bacteria are unicellular organisms. Inspite of not having well define nucleus, they contain many cellular structures which can be controlled by chemically as well as genetically means.

2.1.1 Chemical control of bacterial pathogens

Chemical control employs the use of chemical compounds for the treatment of pathogens on seed, vegetative organs, fruits, bulbs, corns or in soil. These chemical compounds although kill the infection but are very toxic. In the ideal condition, these compounds shouldnot disturb the microflora in soil and kill maximum pathogens without harming the plants and humans. These chemical agents are available as solutions, granules, dust or as emulsions. Plant protecting antibacterial compounds are delivered to sites where they closely contact the pathogens directly and absorbed by the plants and subsequently translocated to different tissues to make them toxic for bacterial pathogens. The efficient use of chemicals can minimize the wastage of chemicals and reduces the adverse effect of toxic compounds to plants and environment [46].

Antibiotics are most common substances used to control the microorganism that are capable to destroy the genome or destruction of bacterial cells. These are efficient and quickest control system where traditional protection measures have been failed. Various antibiotics are used in routine to control the bacterial pathogens. The choice of antibiotic is important according to bacterial infection. For example, Streptomycin is effective against various fruit pathogens including cankers and *P. syringae* but some bacteria has ability to develop resistance against one of the widely used antibiotics which should be limited for the urgent control of microbes for quarantine purposes only [47].

Use of essential oils as pesticides or fungicides is safer than chemicals, but it requires plenty of the plant to be effective. Hence, finding the effective compounds of essential oils and their synthesis decreases the problem of preparing the natural compound. The antibacterial effect of *Satureja hortensis* L., *Thymus vulgaris* L. essential oil, and their major constituents were determined using the disc diffusion method. These essential 0ils prevented *Erwinia amylovora* growth (that causes fire blight disease). The essential oils were fractionated using preparative column chromatography (Silica column) and all fractions were tested for their antibacterial activities on this bacterium. Effective fractions were analyzed by GC-MS. Results showed that carvacrol is the effective compound in *Satureja hortensis* essential oil and has strong antibacterial effect. The effective compounds in *Thymus vulgaris* essential oil are thymol and carvacrol. which also showed a strong antibacterial effect. These compounds prevented the growth of *E. amylovora* in sucrose and nutrient agar media [48].

2.1.2 Genetic control of bacterial pathogens

2.1.2.1 Induced host defense mechanism

Bacterial pathogens get support from different host components and by overcoming this assistance could result the bacterial resistance in the plants.The use of a bacterial gene (*bO*) encoding a proton pump (the bacterio-opsin protein) trigger an induction pathway similar to those induced by a pathogen infection,including the HR. Expression of the *bO* gene in transgenic tobacco led to an increased level of resistance to several viruses and complete resistance to *P. syringae* pv. *tabaci*.The transgenic plants also accumulated high levels of salicylic acid, which is a key chemical signal of a pathogen-induced SAR [49].

Use of breeding techniques to identify the resistant cultivars is another approach widely used by crop breeders along with expression of resistant R genes in the agronomically important crops.

Resistance genes of the non-RD pattern recognition receptor class typically confer long-lasting resistance because they recognize conserved microbial signatures, which, when mutated, cripple the virulence of the pathogen [50,51].

2.1.2.2 Transgenic approaches

Plant pathogens can cause significant reduction in crop yield. Due to these pathogens there is possible threat to wipe-out plant species. Therefore plant pathologists and biotechnologists trying their best to develop pathogen resistant plants against some diseases of economic importance caused by bacteria [52].

Many different genetic strategies has been proposed to engineer plant resistance to bacterial pathogens like *P. syringae*, these strategies include: including use of antibacterial proteins from different insect vectors and their transformation in plants for development of resistance [53] and inactivation of virlunence factors resulted the immunity of plants against the relevant bacterial species [54]. The resistance non bacterial genes can also be introduced by transgenic approaches for broad spectrum resistance against the devastating pathogens. The Shiva-1 is an antimicrobial protein obtained from the silk moth was introduced in the transgenic tobacco plants which were found resistant against *Ralstonia solanacearum* [55].

Lactoferrin is another iron-binding glycoprotein known to have antibacterial properties. The expression of a human lactoferrin gene in tobacco delayed the onset of symptoms caused by *R. solanacearum* from 5 to 25 days. This resistance appears to be due to the truncation of lactoferrin, resulting in a smaller peptide with strong antibacterial activity [56]. Recently, the use of RNAi has been emerged as important tool to counter the bacterial genome at transcriptional and post transcriptional level. siRNAs has proved effective against the crown gall disease in *Arabidopsis*, *Nicotiana* and *Lycopersicum* species caused by a pathogen *Agrobacterium tumefaciens* by transformation of inverted repeats of this pathogen genes ipt and iaaM to encode precursors of biosynthesis for two important phytochromes auxin and cytokinins [57].

Phenolic compounds (a group of secondary metabolites) are widely distributed in plants and have shown to possess antimicrobial properties. The anti-Xylella activity of 12 phenolic compounds, representing phenolic acid, coumarin, stilbene and flavonoid, was evaluated using an in vitro agar dilution assay. Overall, these phenolic compounds were effective in inhibiting *X. fastidiosa* growth, as indicated by low minimum inhibitory concentrations (MICs). In addition, phenolic compounds with different structural features exhibited different anti-Xylella capacities. Particularly, catechol, caffeic acid and resveratrol showed strong anti-Xylella activities. Differential response to phenolic compounds was observed among X. fastidiosa strains isolated from grape and almond. Elucidation of secondary metabolite-based host resistance to X. fastidiosa will have broad implication in combating X. fastidiosa-caused plant diseases. It will facilitate future production of plants with improved disease resistance properties through genetic engineering or traditional breeding approaches and will significantly improve crop yield [58].

3. Recent Strategies against Plant Pathogenic Fungi

Despite substantial advancements in plant protection strategies, global food production is still threatened by a multitude of pathogens including fungi. More than 8,000 fungal diseases exist in this universe which infect wide array of plant species to infect valuable plants. Premier management practices to overcome these diseases have been; use of chemical fungicides, development of disease resistant varieties and use of biocontrol agents and plant-based extracts. Chemical control is very effective for most of the diseases but is not to the acceptable level because of undesirable effects on human health, killing of beneficial organisms and environmental risks [59]. Breeding for disease resistance has been very impressive but faces certain limitations. Biotechnological interventions have not only broadened breeding possibilities by genome mapping and identification of resistance genes but have also helped to devise innovative strategies to combat fungal pathogens. Disease free seed is produced through *in vitro* techniques [60]. PCR (Polymerase Chain Reaction) and other diagnosis techniques have made possible early diagnosis of diseases as result epidemics are avoided. Advancements in omics (genomics, trancriptomics, proteomics, and metabolomics) have proved to be important in understanding molecular basis of plant-pathogen interaction and plant metabolic pathways, thus ultimately improved disease management measures. Developments in transgenic technology have made it possible to transfer genes across the species, to develop crop varieties with broad-spectrum resistance [61]. This section highlights notorious fungal pathogens as well as recent research in disease diagnosis and management.

3.1. An insight into disastrous fungal pathogens

Fungal diseases have emerged as a global problem having serious effects on crop yield and may even lead to complete crop failure. They not only infect edible plants but also damage timber trees, animals and humans. Fungi are classified into four major phyla including Zygomycota, Basidiomycota, Ascomycota and Oomycota. Some are less noxious whereas others may even be epidemic. A brief overview of the notorious fungal pathogens is given here.

3.1.1 Potato blight

Unluckily blight has the ability to evolve as quickly as the breeders' effort to outwit it, so has always been difficult to control through breeding. Potato blight is caused by *Phytophthora infestans,* an extremely virulent pathogen that has led to historic Irish famine. It appears in the form of small brown-black spots on the leaves, often surrounded by a pale halo, while underside of the leaves may take on a white, downy appearance in wet weather. These are the hyphae by which the fungus colonizes [62]. It can spread with an impressive speed, causing the complete collapse of the crop within a few days of infection in warm, humid conditions. Severe disease attack may even infect tubers, giving rise to brown rot and sunken patches. As a

result, secondary infection by other fungi and bacteria is increased and the prized potato turns into a mushy mess.

3.1.2 Red rot of sugarcane

Red rot*, the oldest known disease of sugarcane* is often referred as cancer of the cane and no effective method is available for *its control* yet. It is caused by *Glomerella tucumanensis*, earlier known as *Colletotrichum falcatum*. More than six races of this pathogen have been identified [63]. Disease may infect various plant parts but is usually known as stalk and seed set disease. Symptoms are never apparent during the early stage of infection but quite evident as the infection gets severe [64]. Appearance of elongated red lesions on leaf midrib, reddish patches or small spots on the leaf sheaths are other symptoms of this diseases. Advance stage of the disease results in breakdown of standing cane. The pathogen produces specialized structures known as acervuli, which support profuse sporulation whereas spore dissemination is dependent on rainfall.

3.1.3 Rust disease of wheat

Rusts are noxious foliar diseases of wheat. They may damage crop plants as stripe rust, stem rust and leaf rust. Leaf rust is comparatively least damaging as compared with others. It appears as orange-brown pustules on the upper leaf surface, so easy to diagnose. Leaf rust is caused by *Puccinia recondita* which reproduces asexually, hence requires a living host for its survival from one growing season to next. Stem rot results in formation of dark reddish brown pustules on stem, spikes and on both sides of leaves. Initially pustules are separate and scattered which coalesce with increase in infection. Primary infection develops from wind-borne urediospores which may travel long distances. As plants mature, masses of black teliospores may be produced. In addition to wheat, rust may also infect barley, triticale, and other related grasses whereas its alternative hosts are *Berberis* and *Mahonia*. Stripe rust is caused by *Puccinia striiformis* which results in the formation of pustules containing yellow to orange-yellow uredospores, in the form of narrow stripes on the leaf sheath, necks and glumes. The disease develops rapidly in the prevalence of free moisture [65]. Rust spores are wind-blown so can spread over large areas within no time and may even be epidemic in favorable climatic conditions.

3.1.4 Powdery Mildew

Diseased plants are more prominent in moist areas. Powdery mildew is very noticeable on the leaves as a white powdery mass which often covers the entire leaf blade. Later, the infected leaves turn yellow and die prematurely. Heavy attacks of powdery mildew cause plants to lodge and kernels to shrivel. In addition to crop plants, fungi may also infect lilac, roses, zinnias and English oak. Foliar fungicides are effective in controlling powdery mildew

even on newly discovered strains [66].

Root rot is another infectious disease caused by *Phytophthora*. It infects leaves, stem, bark and results in root decay ultimately leading to death of the plant. Smut appears in the form of blisters on the infected plant parts and results in discoloration as well. Its causal agent is *Ustilago maydis*. The infected kernels become huge, smelly and ultimately whole plant as well as fruit is destroyed.

Apple scab is caused by an air-borne fungus, *Venturia inaequalis*. Olive green-colored patches appear on the infected leaves whereas black or gray colored patches appear on the fruits eventually destroying it. Wilting is another infectious disease caused by *Fusarium oxysporum* (Fusarium wilt) and *Verticillium longisporum* (Verticillium wilt). The fungal invasions start in the roots which makes its way slowly into stem and plugs of vascular system. The pathogen infection results in complete destruction of the plant. A wide range of plants have been reported to be infected by wilt including cotton, tomatoes, potato and tobacco. *Rhizoctonia solani* infects underground stem and tubers of the potato plants severely and results in formation of lesions or sclerotia on tubers. Severe disease infection infects plant growth resulting in serious reduction in crop yield and quality. Decay is another disastrous disease caused by fungi. The pathogen infects living plant tissues and results in complete deterioration of invaded plant. A diverse range of mycoparasites (*Aphanomyces*, *Pythium* and *Phytophthora*) are involved in the decay of valuable crop plant species, fruit plants and timber trees. Likewise, anthracnose is also a devastating fungal disease caused by *Gloeosporium* and *Colletotrichum*. The pathogen becomes more infectious in warm and humid season and appears in the form of shrunken spots of different colors on stem, leaves, flowers and fruits [67]. These spots spread out to cover whole plant and ultimately causes its death. Downy mildew infects a wide range of plants and is caused by *Peronosporaceae*. Disease appears in the form of discolored blotches on the leaves. Pathogen infection results in retarded plant growth as a result productivity is seriously decreased.

3.2. Biotechnological interventions for the control of fungal plant pathogens

3.2.1. Detection and diagnosis of plant pathogenic fungi

Diagnosis is always critical as far as control of pathogens is concerned. They not only help in early detection and identification of the pathogen but also help to adopt remedial measures well in time. Conventional methods to identify infectious pathogens have been dependent upon morphological identification of cultured pathogens. These approaches have certain limitations i.e. require more time to culture and identify; few of the pathogens are not culturable; require knowledge of classical taxonomy for the identification of pathogens. Further, accurate quantification of pathogen is never possible [68]. Since the advent of molecular Biology, various methods have been developed by researchers for accurate detection and identification

of fungal pathogens. PCR technology has addressed all these limitations and has successfully been used for the detection of pathogen in mother plants, seeds and propagative material to minimize further spread of disease. It is more sensitive, accurate, reproducible and authentic as compared with conventional methods of identification. Advancements in PCR such as realtime PCR, multiplex PCR, nested PCR and loop-mediated isothermal amplification have helped in fast and accurate detection of plant pathogens. One of the critical aspect in this regard is selection of highly conserved sequences, to be used as target regions for the identification of particular fugal pathogen. Numerous target sequences have been explored in the fungal genome and the most promising ones are ribosomal DNA (rDNA). Internal transcribed spacer (ITS) region, intervening large ribosomal subunit (28S and 5.8S) and small ribosomal subunit (18S) are highly conserved among the fungal species and even genera [69] so have extensively been used for the identification of various pathogens.

The ITS region is ubiquitous in nature and is found in all eukaryotes. Further, higher copy number of rRNA genes in the fungal genome makes it more sensitive marker. As a result, ITS region of nuclear DNA has been proposed as a core barcoding marker for the identification of fungal isolates. More than hundred thousand ITS sequences have been deposited in the International Nucleotide Sequence Database and other databases. Additionally, several partial ITS sequences have also been submitted in public sequence databases which could be used to assess diversity among various species. So, ITS is the most desirable target region for the pathogen detection and has successfully been tested in various crop plants. Jeeva et al. (2010) developed PCR based identification of *Sclerotium rolfsii* infecting various crops. Similarly, Torres-Calzada et al. in 2011 [71] identified *Colletotrichum capsici* using ITS specific primers. Multiplex PCR was used for the simultaneous detection and discrimination of *Golovinomyces cichoracearum* and *Podosphaera xanthii* in sunflower [72]. Similarly, it was used for the detection of *Tapesia acuformis* and *T. yallundae* and for *Phytophthora lateralis* in water samples as well as cedar trees. Eleven different taxons of wood decay fungi (infecting hardwood trees) were distinguished using multiplex PCR [73]. Loop-Mediated Isothermal Amplification is another emerging technique that has appeared to be more suitable for field testing of pathogens. This technique has been used for the detection of *Fusarium graminearum* from wheat seeds [74] and for the detection of *Phytophthora kernoviae* and *P. ramorum* from field samples [75]. PCR-ELISA was used for the detection of different species of *Pythium* and *Phytophtora* as well as for the detection of *Didymella bryoniae* in cucurbits [76].

Nano-phytopathology is a cutting-edge science that has played pivotal role in integrated disease management at early stage of infection resulting in crop protection from epidemic diseases. Nanodiagnostics is the integration of molecular diagnostics with nanotechnology and has proved to be very promising for the identification of plant pathogens that has made possible fast detection of pathogen with extreme accuracy. Biosensors, nanoimaging and

nanopore DNA sequencing may provide high throughput analyses for pathogen detection and crop protection. Hence, nanodiagnostic is more cost effective, quicker and precise approach for on-site disease diagnosis with high degree of sensitivity. In addition to its critical role in diseases detection and identification, nanomaterials can be used for mycotoxin detection and detoxication. Further, different types of nanoparticles (Silica, silver ect) have potential to be used as antifungal agents thus may provide ecofriendly strategy to control fungal pathogens [77].

3.2.2. Control of plant pathogenic fungi by RNAi

RNAi is an RNA-dependent gene silencing process in eukaryotes. RNAse III enzyme acts as dicer and is involved in the cleavage of target dsRNA (double-stranded RNA) into small (20-25 nucleotides) RNA (siRNAs) with an overhang of two nucleotides at its 3′-end. Each of the siRNA is comprised of a sense and antisense strands. Then endocatalytic cleavage of target mRNA proceeds through catalytic component (argonaute protein) of the RISC complex. The target transcript is completely degraded, as a result, plants are secured from invading pathogens [78].

RNAi has emerged as a valuable tool for combating seriously challenging diseases caused by fungi [79]. It operates in lower as well as higher plants and uses double stranded RNA for the inhibition of transcription or translation of target mRNA. Discovery of small non-coding RNAs has particularly highlighted its significance. Desired knockouts may be developed; endogenous pathways may be explored for negative post-transcriptional regulation. Gene function can be studied, since hundreds of genes have been worked out by this technology [80]. It can silence a gene in specific tissue or in whole of the organism. Likewise, a gene can be partially silenced as well as may be completely turned off within an organism both under *in vivo* and *in vitro* conditions [81].

Owing to significance in pathogenicity, RNAi has emerged as a valuable ecofriendly tool to control plant pathogenic fungi. Besides manipulating host genes, RNAi technology has been used to target genes of invading pathogens or which are critical for virulence and disease progression, and toxin production in case of toxigenic plant pathogens [82]. Co-suppression, dsRNA or antisense techniques have been tested in *Magnaporthae oryzae*, *Cladosporium fulvum*, *Aspergillus nidulans*, *Fusarium graminearum* [83] and *Neurospora crassa*, *Venturia inaequalis* [84]. In addition, RNAi can also be employed to those plant pathogenic fungi as well which are polykaryotic and polyploid in nature. Researchers are of the view that this technology may be exploited for protecting fruits and vegetables from the fungal pathogens causing post-harvest diseases as well. Silencing of cgl2 gene was successfully attained in a tomato pathogen *Cladosporium fulvum* using the cgl2 hairpin construct [85]. Similarly, HCf-1 gene which encodes for a hydrophobin in *Cladosporium fulvum* was co-suppressed by ectopic

integration of homologous transgenes [86]. Transformation with a truncated copy of HCf-1 gene caused 30% co-suppression of hydrophobin synthesis in *C. fulvum*. Transcription rate of co-suppressed gene was higher in transformed isolates indicating that suppression was attained at post-transcriptional level rather than transcription. This was owing to ectopic expression of transgene at 3'-end of the promoter. Thus, resultant transformants had lower levels of mRNA as compared with wild type. Hairpin vector technology was used for the silencing of trihydroxynaphthalene reductase gene (THN) in *Venturia inaequalis*. Green Fluorescent Protein was used as marker to track silencing of THN gene involved in melanin biosynthesis. Likewise, multiple gene silencing has been achieved in plants using partial sense constructs [87] and in *Cryptococcus neoformans* using chimeric hairpin constructs [88]. Enhanced green florescent protein gene was used as a model to study systemic silencing in rice blast causal organism *Magnaporthe grisea*. In another study, a novel OsBRR1 (rice blast resistance-related gene) was identified by screening RNAi population of T0 rice plants.

3.2.3. Control of plant pathogenic fungi through transgenic technology

Recombinant DNA technology has uplifted disease diagnosis and treatment. One of the major milestones of the molecular biology is transgenic technology. This technology has got so much acceptance that more than 180 million hectares of the total cultivable land is under transgenic crops. Since no specie-barrier exists, so any of the transgene may be expressed across the species. Likewise pathogenesis related genes have ectopically been expressed in valuable crop plants to combat dissastrous mycoparasites. Co-expression of class I β-1,3-glucanase and thaumitin like proteins led to reduced fungal infection[89]. Expression of chitinase (ChiC), isolated from *Streptomyces griseus*, showed enhanced resistance against *Alternaria solani* whereas improved resistance to *Rhizoctonia solani*was demonstrated upon expression of mycoparasitic chitinase, glucanase enzymes. Similalry, hyperexpression of snakin-1 gene showed enhanced resistance to *Rhizoctonia solani* and *Erwinia carotovora*. Expression of *Phyllomedusa sauvagii* dermaseptin,*Nicotiana tabacum* AP24 osmotine and Gallus gallus lysozyme showed resistance development against bacterial and fungal pathogens. Antifungal protein (AFP) gene transformed into potato susceptible cultivar 'Shepody' to yield resistance against late blight [90]. A gene, *StoVe1*, derived from wild egg plant (*Solanum torvum*) demonstrated enhanced resistance to *Verticillium dahliae* infection. Expression of chitinase (*chiA*) andribosome inactivating protein (*rip30*) led to enhance resistance against *Rhizoctonia solani* in a greenhouse assay. Five unique thionin genes, from *Brassicaceae* species, yielded resistance against gray mold (*Botrytis cinerea*) in potato plants [91]. Literature reveiw suggests that broadspectrum resistance could be attained in valuable plant species through transgenic technology. Plant based hydrolytic enzymes (glucanase, chitinases, proteases, cellulases, kinase), antibiotics and thaumitin like proteins are shown to be very effective in engineering fungal resistance and against mycoparasitism[92]. Hence, ectopic expression of antifungal

proteins is a very effective strategy to combat fungal pathogens.

4. Summary

Various strategies have been adopted in the last two decades to control the plant diseases caused by viruses, bacteria and fungi. The viruses can't be controlled by the chemical methods. Therefore various genetic approaches have been adopted to produce the transgenic plants which are relatively less susceptible to the the phytopathogenic viruses, bacteria and fungi. During the late 20th century, the genes from different biological sources of plant, bacteria and fungi have been used to develop transgenic plants which were proved initially effective against different pathogens but with the passage of time, the pathovars cause symptoms in these plants by establishing resistance against these genes. Plant viruses have high recombination rate and can develop new species by sharing of genes between the species [93] and ultimately result the change of insect vector. The RNAi based strategy has not been much successful against the DNA viruses. Relying solely on a homology-based resistance, particularly in an area of high virus diversity such as south Asia would seem destined for failure [33]. The wisest course of action would be to stack (so called "pyramiding") resistances that act by distinct mechanism. So, for example, use the best available natural host plant resistance with an RNAi-based resistance and resistance based upon protein expression, either virus-derived or non-pathogen derived [94]. Further improvement in transgenic resistance against phloem limited viruses, such as many of the geminiviruses, can possibly be achieved by using tissue specific promoters for transgene expressions. This expression would also avoid accumulation of siRNAs in those tissues where viruses are absent [95]. A number of reviews have outlined the strategies that have been tried for obtaining resistance to geminiviruses [33]. Recently adopted amiRNA and CRISPR-Cas9 based strategies along with the selection of natural resistance can change the scenario of viral resistance with more reliable resistance against homologous and heterologous viruses.

The strategies of genome editing, TGS and PTGS have been widely investigated recently to find the long lasting and persistent mechanism against the closely related phytopathogenic bacterial species. The phytopathogenic fungi infecting large number of economically important crops. These fungal pathogens lower the defense system of host plant and reduces the yield and quality that amount to billions of US dollar losses over the globe annually. The chemical control of these fungal pathogens gives the fastest remedy with drawbacks such as resistance development and toxicity in the environment resulted the investigation of other molecular approaches for broad spectrum resistance against the fungi. Considerable developments have been made in the identification and isolation of genes involved in the host defense mechanism. With the help of contemporary molecular and bioinformatics tools, several resistance tools like antifungal peptides and proteins have been investigated and evaluated through in vitro bioassays. Various strategies like enhancement of plant structural defense, ubiquitous defense mechanism

based on RNAi, neutralization of fungal toxins and exploitation of antifungal genes from non-plant sources, have been used widely in transgenic plants to develop resistance. Successful execution of these approaches has led to significant reduction in different fungal diseases in transgenic plants. However, pyramiding multiple resistance genes rather than single gene and use of inducible promoters instead of constitutive ones could produce superior performance in transgenic plants. On the basis of all the studies discussed here, it can be predicted that global food security can be secured by developing resistance sources against phytopathogens.

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