







Review

Genetics and Genomics of Fusarium Wilt of Chilies: A Review

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Abstract: Hot pepper (*Capsicum annum* L.) is a major spice crop and is used worldwide for its nutritional value. In the field, its plant is susceptible to various fungal diseases, including fusarium wilt, caused by soil-borne fungus *Fusarium oxysporum* f. sp. *capsici*, which can survive in the soil for several years. The infected plant can be recognized by the yellowing of older leaves and downward curling of apical shoots, followed by plant wilting and ultimately the death of the plant. The resistance mechanism in plants is controlled by a single dominant gene, and conventional plant breeding techniques are used to develop a wilt-resistant germplasm. Non-conventional techniques such as gene pyramiding and expression enhancement of antifungal genes could be used to shorten the time to develop resistance against fusarium wilt in hot peppers. In this review, we discuss different aspects of the disease and the molecular basis of resistance in chili/hot pepper plants. Furthermore, this review covers the scope of conventional and non-conventional breeding strategies and different management approaches used to tackle the disease.

Keywords: *Fusarium oxysporum* f. sp. *capsici*; hot pepper; genetics; genomics; resistance breeding; GMOs

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1. Introduction

Chilies or hot peppers (*Capsicum annum* L.) are a major spice vegetable belonging to the family *Solanaceae* [1]. It is famous for its nutrition: its abundance of total soluble phenolics and vitamin C [2]. Chili fruit contains a significant amount of essential vitamins (A, B, and C), providing sufficient ascorbic acid and carotene (contributor of vitamin A) to poor people in India [3]. Capsaicinoid compounds present in the *capsicum* genus provide a pungent taste in addition to antioxidant activity [4]. The presence of vitamin C (111 mg/100 g), carotene (17 ug/100 g), niacin (0.900 g/100 g), thiamine (0.190 g/100 g), and riboflavin (0.190 mg/100 g) make it more nutritious [5] and increase its significance in food. The existence of bioactive molecules such as fatty acids, volatile oils, capsaicinoids, and carotenoids increase its importance in a healthy diet, due to their anti-inflammatory and antioxidant properties [6,7]. Aside from their edible properties, capsaicinoids are alkaloids that are useful in pharmaceutical industries [8].

The South American tropical region is considered as the primary center of origin of chilies, from where it was introduced into the Indo-Pak subcontinent before 1585 by the Portuguese [9]. The total worldwide production of chilies in 2019 was 4.3 metric tons. Out of the total world production, India produced 40.6%, followed by Thailand (8.18%) and China (7.66%). Pakistan ranks 10th in the world for chili production and has a share of 2.39% (FAO, 2019 chilies and pepper dry data code, 0689). Chili production is significantly affected by both biotic and abiotic stresses and, being a sessile plant, it adopts different mechanisms to cope with stresses [10]. In terms of biotic stresses, the chili plant faces viral, bacterial, and fungal diseases. Among these diseases, chili leaf curl virus, murda complex, leaf spots, anthracnose, powdery mildew, and wilt are highlighted [11]. Abiotic stresses mainly include drought, salinity, heat, heavy metals, and cold.

To combat the abiotic stresses (salinity and cold stresses), seed priming may be used as a primary solution, and this has been found to be effective against salt (NaCl) and cold stresses [12]. Seed priming needs to be repeated every time, and it is laborious. Genetic transformation can be used in chili plants to counter abiotic stresses, and has yielded positive results in the past [13,14]. Genetic transformation is more reliable than seed priming because transformed objects become part of the genome. In the past, several transformations have been attempted to mimic the fungal attack on plants [15–17].

Fusarium wilt causes a significant reduction in yield [18–20]. *F. oxysporum*, *F. proliferatum*, *F. solani*, *F. moliniforme*, *F. anthophilum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Pythium aphanidermatum* were found to be associated with wilt disease symptoms in Pakistan [21], whereas in India, fusarium wilt disease is commonly caused by two species of fusarium, *F. oxysporum* and *F. solani* [22]. Among *Fusarium* spp. it is mostly associated with *F. oxysporum* [23] (Table 1). In this review, we will discuss *F. oxysporum* as the causal agent of this disease. Moreover, this review will cover the brief genetics of this pathogen and the genes responsible for causing the disease. In addition, we have summarized the genetics for resistance against fusarium wilt in chili plants, which have not been summarized in any previous review.

Table 1. Reported pathogens associated with wilt disease in chilies.

Serial No.	Pathogens Associated with Fusarium Wilt Disease in Chilies	References
1.	<i>F. solani</i>	[21,24,25]
2.	<i>F. oxysporum</i> , <i>F. proliferatum</i> , <i>F. solani</i> , <i>F. moliniforme</i> , <i>F. anthophilum</i> , <i>Macrophomina phaseolina</i> , <i>Rhizoctonia solani</i> , and <i>Pythium aphanidermatum</i>	[21]
3.	<i>F. oxysporum</i> and <i>F. solani</i>	[22]
4.	<i>F. oxysporum</i>	[5,23,26]
5.	<i>F. pallidoroseum</i>	[27]

The *Fusarium* genus encompasses a variety of soil-borne pathogens [28], and recently this pathogen has also been reported to be a human pathogen. *F. oxysporum* Schlecht has been classified into 120 different formae speciales based on host specificity [29], and the pathogen of the present study is known as *Fusarium oxysporum* f. sp. *capsica*.

F. oxysporum is the most common species of this genus that causes wilt disease in numerous crops, i.e., chilies, tomatoes, and other members of the *Solanaceae* family [30]. *F. oxysporum* is classified based on morphological structures, shape, formation, and structure of micro-conidiophores. Furthermore, *Fusarium* species are grouped into two categories based on their pathogenicity, i.e., pathogenic and non-pathogenic races.

The survival of chlamydospores inside the soil initiate the life cycle of *F. oxysporum*, and this stage is referred to as the saprophytic phase [31]. Chlamydospores remain inactive in the residuals of decomposed plants, and start germinating after acquiring nutrients from the roots of host plants [31,32]. After germination, conidia and chlamydospores emerge from thalli within 8 h and 3 days respectively, under optimum conditions [33].

The development of the pathogenic strain of *F. oxysporum* chlamydospores often takes place in the hyphae inside the damaged tissue of the host plant, and in an alternative way they might be developed from macroconidia that emerge from the sporodochia present on lesions at the surface level of the soil [34,35]. The development of chlamydospores is directly proportional to the availability of nutrients; the germination of chlamydospores does not commence until the roots of the decayed plant do not release any sort of carbohydrate [36].

Usually, the pathogenic strain attacks the plant while non-pathogenic strains are used to control the pathogenic strain. Three classes of pathogenic races of *F. oxysporum* have been defined as obligate, opportunistic, and true pathogens [37]. Obligate pathogens grow inside the host plant using their metabolism and result in altered plant growth. Opportunistic ones enter through damaged portions and attack weakened plants. These are characterized by a wide host range and reduced virulence. True pathogens possess high virulence and are able to live outside the host, but require host tissues to grow [38].

2. Genomics of *F. oxysporum*

The genome size of *Fusarium* ranges between 18.1 to 51.5 Mb, with 7 to 14 sets of chromosomes [39]. Of the total genome, 5% is occupied by different types of transposable elements (TEs) [40]. Among these TEs, eight families have been studied through different techniques [41].

F. oxysporum has a genome size of 61 Mb and possesses 15 chromosomes and ~17,735 genes. This genome has 16.83 Mb and 3.98% TEs and repetitive regions, respectively [42]. The genome of *F. oxysporum* is categorized into two parts, i.e., the core genome and the adaptive genome. The core genome takes part in reproduction and primary metabolism whereas pathogen virulence is carried out by the adaptive genome. Genes for host specification and virulence are present in lineage-specific chromosomes (LS) [43].

Host specificity is largely dependent on LS chromosomes, and upon transfer to a non-pathogenic strain pathogenicity can be conformed in a host [42,44]. Structural variations in LS chromosomes are present among different speciales. The authors of [45] compared the LS chromosomes (3, 6, 14, and 15) of *F. oxysporum* f. sp. *niveum* with the reference genome assembly of *F. oxysporum* f. sp. *lyopersici* (Fol 4287, strain number) and found significant variation among LS chromosomes. Moreover, in syntenic analysis, no clear chromosome was found in *F. oxysporum* f. sp. *niveum* that corresponded to chromosome 15 of tomato speciales. It is worth noticing that a 106 scaffold with a length of one million base pairs was found in a pathogenic isolate. The presence of this scaffold in a pathogenic isolate indicates that it was chromosome 15, but its divergence causes it to be unidentifiable in the reference genome [45].

Core chromosomes harbor housekeeping genes, while accessory chromosomes contain bunches of TEs. The authors of [45] sequenced pathogenic and non-pathogenic strains and found that core chromosomes are conserved in both strains. Moreover, these chromosomes are associated with basic cellular and metabolic functions [45].

Accessory chromosomes also carry effectors which participate in causing disease [42]. These effectors have gone through evolutionary processes on chromosomal regions and have been termed as CDCs (conditionally dispensable chromosomes, mini-chromosomes, B chromosomes, lineage-specific chromosomes, and accessory chromosomes). Regions of LS chromosomes are characterized with localized regions that directly contribute to pathogenicity, whereas the genes in core chromosomal regions are conserved across several generations [42,46].

Scientists have attempted genome sequencing of several speciales of *F. oxysporum* [47–50], but the most accurate genome sequencing of *F. oxysporum* has been performed in *F. oxysporum* f. sp. *lyopersici*, which has been assembled from whole-genome shotgun fragments. LS regions of chromosomes, which include chromosome no. 15, 14, 6, and 3, as well as the parts of chromosomes 1 and 2, also fell in this category [42]. However, chromosome 14 of watermelon speciales of *F. oxysporum* was devoid of an avirulence gene (SIX6) [51].

Syntenic analysis could be used to differentiate the LS chromosomes by quantifying the similarities between genomes [52].

LS chromosomes are characterized by transposon-enriched regions and contain SIX (secreted in xylem) genes as well. SIX gene families are effectors, and are characterized by cysteine-rich proteins, and act inside the host plant. These effectors act on plant cell walls and affect host immune responses [53]. On the other hand, Gawehns and Houterman [54] stated that SIX genes are not a family, since they lack any identifiable motifs and conserved domains.

Through numerous molecular techniques, 14 SIX genes have been recognized in *F. oxysporum* f. sp. *lycopersici* [42,53,55–57]. SIX proteins have been reported in other formae speciales as well [54]. Other than pathogenic strains, the presence of SIX genes has also been reported in supposed non-pathogenic strains of *F. oxysporum* at low levels [58–60]. An extensive study of *F. oxysporum* genomics was performed on *Lycopersicon* species, and no comprehensive study has yet been reported on *C. annuum* L.

2.1. Genetics of Disease Development

F. oxysporum, in a similar manner to other fungi, requires cell-wall-degrading enzymes (CWDEs) in order to enter a plant cell, and these enzymes are produced by various genes. In the past, genes of these enzymes were identified, cloned, transformed, and silenced to check their contribution towards pathogenicity. The mode of infection decides the genes required for pathogenesis as the fungus has various infection processes, i.e., degradation of the cell wall or entry through a damaged portion/natural opening [61]. The 79 genes were listed and classified by Idnurm and Howlett [62] based on their CWDE, participation in the formation of specialized structures required for fungal infection, overcoming the host defense system, signal cascades, and in the biosynthesis of toxins. Ma et al., 2010 [42] identified LS genomic regions which consist of four complete chromosomes (Ch No. 3, 5, 6, and 14), which are about one-fourth of the total genome. These regions contain several genes and transposons which are related to pathogenicity (Table 2).

Table 2. Transposon elements (TEs) in *F. oxysporum* genome by Ma et al., 2010 [42].

	SINE	LTRs	Total	Pogo	hAT	Helitron	MITEs	Impala	Others	Total
<i>Fo</i>	159,408	274,097	433,505	491,352	6,294,444	163,307	21,180	14,563	342,368	1,506,939
<i>Fo Cons</i>	46.01%	50.98%	49.15%	20.78%	18.54%	29.84%	5.38%	0%	22.76%	5.19%
<i>Fo Ls</i>	53.99%	49.02%	50.85%	799.22%	81.46%	70.16%	94.62%	100%	77.24%	94.81%

The first row contains the TE of each type in the whole genome of *F. oxysporum*, while the second and third rows contain the TE in the conserved and LS portion of the pathogen, respectively.

Virulence is a quantitative character controlled by a number of genes. These genes regulate the transcriptional regulators, which later contribute to pathogenicity. However, to the best of our knowledge, the major transcriptional regulators responsible for virulence in *F. oxysporum* are listed in Table 3.

Table 3. Seven major transcriptional factors responsible for pathogenicity in *F. oxysporum*.

Serial No.	Names	Function	References
1	<i>Con7-1</i>	Cell division, cell wall biogenesis, and nuclear localization gene	[63]
2	<i>Sge1</i>	Parasitic growth, regulation of SIX gene expression, and role in colonization	[64]
3	<i>Ftf1</i>	Virulence at initial stage of disease development	[65]
4	<i>Ste12</i>	Invasive fungal growth	[66]

Table 3. Cont.

Serial No.	Names	Function	References
5	<i>XlnR</i>	Transcriptional activators of xylanase genes	[67]
6	<i>pacC</i>	Negative regulator of virulence	[68]

Before establishing infection in a plant, the pathogen colonizes the vascular tissue which is regulated by MCPs (mitochondrial carrier proteins), produced by the FOW1 gene (see Table 4). Homogalacturonan, abundantly found in the plant cell wall, is depolymerized by endoPGs coded by the *pg1* gene. The role of the *pg1* gene in disease development is further confirmed by RT-PCR (reverse transcription-polymerase chain reaction), which revealed its expression in the lower stem and root zone of infected plants [69]. *Xyl2* and *-3* genes from *F. oxysporum* encode family-10 xylanases involved in the breakage of the xylan backbone. Ruiz-Roldán et al., 1999 [70] isolated and cloned these genes from pathogens and disclosed that a notable homology was found between the sequences of predicted amino acids of these genes and other xylanase families. Expression analysis suggested that *Xyl2* was expressed during the final phase of disease only, whereas *Xyl3* was expressed in the lower stems and root zone of infected plants during the whole cycle. In another study, a gene, namely *Xyl4*, encoded family-11 xylanases that were isolated from the pathogen, and this gene was expressed during the entire disease cycle [71]. Pectate lyase (Pl1)-encoded enzymes are necessary in infection establishment in plants since it reacts to a major component of the plant cell wall and middle lamella.

The transcription of Pl1 gene is seen in vascular tissues under polygalacturonic acid sodium salt. Moreover, an expression analysis revealed its expression in the roots and stem portion of an infected plant [72]. Expression analysis of most genes involved in CWDE production showed that these genes are being expressed in the stem and root zone. Strengthening this portion of the plant may lead to fruitful results in terms of resistance against this destructive pathogen.

Table 4. List of genes related to pathogenicity.

Serial No.	Gene(s) Name	Function	References
1	<i>FOW1</i>	Plant colonization	[73]
2	<i>Fmk1</i>	Root penetration and cortex invading	[74]
3	<i>ARG1</i>	Production of argininosuccinate lyase	[75]
4	<i>Pg1</i>	Depolymerization of homogalacturonan	[69]
5	<i>Xyl2</i> and <i>Xyl3</i> (family-10 xylanases)	Breakage of xylan backbone	[70]
6	<i>Xyl4</i> (family-11 xylanases)	Breakage of xylan backbone	[71]
7	<i>Pl1</i>	Trans-elimination of pectate	[72]
8	<i>Pgx4</i>	Production of pectinolytic enzymes	[76]

2.2. Role of Cell-Wall-Degrading Enzymes in Pathogenicity

The ability of a pathogen to develop an infection in any host depends on its ability to avoid the host's genes responsible for producing defense proteins. *F. oxysporum* contains genes that can avoid and detoxify the host defense proteins. Biomolecules of the pathogen which involve CWDEs are responsible for the virulence and identification of pathogenic genes, which code extracellular enzymes. *F. oxysporum* produces a variety of host toxins or CWDEs after entering to endosperm followed by the perforation of host cell walls [77].

These enzymes facilitate the entrance of fungal infection into the plant cell by degrading the components of the cell wall. The digestion of cell wall polymers to secure the

nutrient source followed by the degradation of the cell wall leads to fungal entrance into plant cell wall, which later spread through plant tissue [78]. The entrance of *F. oxysporum* into the host cell requires CWDEs, which mainly include proteases, cellulases, xylanases, pectate lyases, and endo- and exopolygalacturonases (PGs), which are produced in enzymatic pathways regulated by various genes. Enzymes produced in these pathways can overcome the barriers constituted by the plant cell wall [76]. Pectate lyases are enzymes that have been extensively studied, which depolymerize the pectin followed by trans-elimination, a major component of lamellae and primary cell walls [29]. PGs which are further classified into two categories, i.e., exo- and endopolygalacturonases, have been successfully isolated from numerous pathogens. EndoPGs are focused among these enzymes, as these enzymes depolymerize the homogalacturonan, which results in the maceration of plant tissues [79]. PG1 is the most abundantly produced endoPG in *F. oxysporum* [80]. The role of xylanase enzymes has been described [71] in the development of wilt disease in plants, and this enzyme may play a key role in infection as xylan is one of the most abundantly found polysaccharide components of the plant cell wall. Endo 1,4- xylanases act on the xylan backbone, cause the depolymerization of xylan, and are classified into two families, i.e., family 10 and 11 [81,82]. Christakopoulos and Nerinckx [83] disclosed that a variety of xylanases are produced by *F. oxysporum*. Two of these enzymes, one with basic and the second with acidic pH are secreted during the growth of fungi on vascular tissue [84].

3. Disease Symptoms

3.1. Morphological Changes in Plants

The indication of any disease due to the exhibition of physiological reactions of the plants caused by a pathogen are called symptoms. Disease symptoms are usually shown at a later stage of growth and can be observed on older leaves first, and then the younger leaves start being affected as the result of the progress of disease; in the end, the plant dies. The symptoms of this disease include the yellowing of older leaves, vein clearing, stunting of growth, and epinasty [23]. The disease is first visible at the external part of newer leaves as modest vein clearing, followed by the downward turning (epinasty) of older leaves [85]. At an earlier stage of the plant, infected plants may wilt and die shortly after the appearance of symptoms. In older plants, downward turning frequently brings up the formation of adventitious roots, wilting of stems and leaves, defoliation, necrosis at the margins of remaining leaves, and ends with the death of the whole plant [23]. At an earlier stage of the plant, the fungus grows in seed tissues and enhances inoculum in order to invade the seedlings through an infected portion of tissue [86].

It changes the plant color to brown and causes discoloration; at the base the girdling of cankers can be seen [87]. The chili wilt causes the death of plants followed by the downward curling of apical shoots and the absence of green pigment from foliage [88]. Infection in plants at a mature stage results in the abnormal production of flowers and fruits. Wilting is not a primary symptom, as it comes after browning of the basal stem, and can be seen only by removing the upper layer of the stem [89].

3.2. Biochemical Changes in Plants

Plants have two types of counter defense mechanisms: one is general and the second is specific [90]. The first type of mechanism includes the initiation of signaling and defense pathways and the creation of antifungal proteins. In second type, products of defense-related genes identify the pathogen effectors and the detoxification of toxins occurs [91]. The growth of the pathogen's mycelia in vascular tissues causes a water shortage which occurs after vessel blockage, and thus wilt symptoms arise. Plants respond to stress by producing gels, i.e., polymers, tylose, and parenchyma cells, as well as defense factors which result in further vessel blockage [92]. Resistant plants produce antifungal compounds such as gums, tylose, and gels which restrict fungal growth. While in susceptible plants, symptoms develop more rapidly due to the slow antifungal production. Plants may survive without

symptoms, if the plant produces antifungal compounds prior to fungal arrival in stem vessels [93].

4. Disease Incidence

Wilt caused by *fusarium* is considered a serious threat to chili cultivation and has caused serious damages of up to 40% of total chili production. In some areas of India, the highest mortality rate was recorded before the first picking [11]. In Pakistan, the disease incidence was 21.9%, out of which 16.6% was recorded in chilies [9], while in the rest of the world the prevalence of diseases was 48%.

The multiplication of spores causes a reduction in growth parameters of plants such as the plant fresh and dry weight, total yield per plant, and plant length [94]. Baba and Padder [95] reported that the prevalence of fusarium wilt in the Kashmir valley was 6% at transplanting and 40% at the fruiting/flowering stage. In a survey, fusarium wilt caused up to 79% of losses in the chili plant population in Thailand [26]. Plants show damping off and vascular wilt symptoms affected with *F. oxysporum* artificially, and extracts of pathogens obtained from chilies can promote damping off in *Lycopersicon esculentum* and *Datura stramonium Physalis* sp. [96]. In Egypt, *F. oxysporum* destroyed 56% of chili seedlings after 30 days of its infestation in soil [97]. High temperature and moist soil enhanced the development of the pathogen. Moreover, it can cause the death of leaves followed by the upward and downward rolling of leaves [9]. This disease can be observed throughout the year, but in India the frequency is found to be high in the months of November–December compared to the rest of the year [98]. India is one of the largest producers of chilies, and in recent years fusarium wilt has significantly reduced yield, which highlights the need of resistance integration through proper conventional/non-conventional breeding techniques in chili plants.

5. Mode of Damage

F. oxysporum is the predominant pathogen that induces destructive wilt in more than 100 plants, and is ranked fifth out of the ten most lethal plant pathogens [99]. Plant fungi have advanced their strategies to invade host plant tissues, followed by the colonization, growth, and disease establishment in the host plant. The pathogen must receive signals from the host plant to respond with changes, i.e., morphogenetic and metabolic changes essential for disease development. The secretion of toxins, formation of specialized structures, and hyphal growth are some of the important changes the pathogen adopts prior to invading the host tissue [100]. Fungal pathogens release effectors that repress the host defense mechanisms and support invasion [101]. Furthermore, numerous biochemical and morphological changes occur as a result of fungal attack on the host plant [102]. These changes are largely dependent on the activity of genes (specific product/protein), signaling pathways which involve cAMP signaling [103], MAPK (mitogen-activated protein kinase) [104], and activation of G proteins [105]. The fungus colonizes the host plant vascular tissue prior to establishing infection. The FOW1 gene produces the mitochondrial carrier protein (MCP) required by the fungus to colonize the plant tissue [73].

The soil-borne fungus enters the stele via the cortex and starts growing inside, which leads to the wilting of vascular tissues [29,106]. It starts its growth in the vascular tissues of the roots and stems, which facilitates the movement of conidia in the transpiration pathway. Moreover, the pathogenic strain of *F. oxysporum* penetrates the roots, causing tracheomycosis or root rot when it enters the vascular system, while the other strains can enter the roots but are unable to cause disease [107]. The fungus can enter the plant roots by using its mycelium or sporangial germ and the infection may be initiated from the root tip, wind, mechanical damage, or wounds caused by hail. The mycelium starts growing through the root cortex inside the cellular layers after the entrance of pathogens. After the mycelium reaches the xylem, it occupies the vessels via xylem pits, and plant water as well as nutrient supply is significantly reduced due to the internal growth of fungus in

plant vascular tissues; in the end, the plant dies from the wilting of leaves and closing of stomatal apparatus due to the lack of water [23].

F. oxysporum at first enters the plant without showing symptoms, and later occupies the vascular tissues and initiates heavy wilting and chlorosis of the stem parts of a plant [42]. The pathogen can cause the death of an infected plant because it has a systematic reaction within a plant [108]. It bypasses the non-self-recognition and defense system of plants while penetrating and recognizing physical and chemical signals from host plants, which are countered with proper morphogenetic and metabolic changes required for any pathogenicity [109].

6. Management Approaches

6.1. Physical and Chemical Measures of Control of Fusarium Wilt

Since this pathogen causes significant yield losses in chilies, several methods have been developed to control this disease and tested to check their efficiency. As this disease is soil-borne and moisture in soil plays a key role in the development of this disease, lowering the soil moisture may result in a reduction in this disease and, if economically feasible, soil solarization could be used to inhibit pathogen population. Ridge sowing with an optimum level of irrigation can reduce the disease incidence successfully in field conditions, as more irrigation causes a high moisture level which facilitates the development of the disease in soil. Increasing the pH level of soil inhibited the growth of *Fusarium* species in soil; to enhance the pH level, hydrated lime could be used [110].

Combinations of different fungicides showed that mancozeb + carbendazim was effective under laboratory conditions to inhibit the mycelial growth up to 93.6%, while 92.4% inhibition was recorded by employing carbendazim alone. In field conditions, the same combinations of fungicides resulted a reduction in the pathogen population [19]. Wilt incidence on the plant can be reduced up to a significant level by spraying fungicides on the crown parts of the plants, as both seed and seedling treatment are insufficient to control the disease. Moreover, a 59.8% reduction in disease incidence was observed by combining the treatment of seed + seedling along with the spraying of carbendazim and metalaxyl [111]. Apart from these methods, integrated pest management strategies can also be adapted to control the disease development (Table 5). Since chilies are an edible crop and too much spray may leave residual effects on crops, crops receiving more fungicide spray than the threshold level are not accepted in both the local and international market.

Table 5. Integrated disease management to control the wilt disease of chilies.

Methods	Treatments/Practices	References
Natural Extracts	1. Extracts of neem and clove with the concentrations of 90 and 70% oil, respectively, along with mustard (mustard essential oil) and cassia suppressed disease development (80 to 100% healthy plants).	[112]
	2. Disease suppression of 85% by using the leaf extracts of <i>Eucalyptus Citriodora</i> .	
	3. Chitosan at a concentration of 4.5 g/L causes a complete 100% reduction in fungal growth.	[18]
	4. A 100% reduction in fungal activity can be achieved by using natural oils (mint oils, thyme, peppermint, and lemongrass) at a concentration of 6%.	[97]
	5. <i>Allium sativum</i> at a concentration of 10% reduced the fungal activity of pathogens up to 57% followed by <i>Azadirachta indica</i> (47.7%).	[113]
	6. Phenolic compounds (Phenols ortho-dihydroxy phenols) and their enzyme activity were higher in resistant genotypes and hybrids.	[114]
	7. Extracts of rice, corn, hot pepper, and soybean inhibited mycelial growth at concentrations of 0.04 g L ⁻¹ .	[115]
	8. Salicylic, propylgalate, citric, and coumaric acid at concentrations of 200 ppm reduced disease incidence.	[94]
Antioxidants		

Table 5. Cont.

Methods	Treatments/Practices	References
Biological Control	1. Chitinolytic bacteria could be used to inhibit disease development; BK-08 found to be the most effective biocontrol agent.	[116]
	2. <i>Trichoderma harzianum</i> successfully inhibited the growth of <i>F. Solani</i> , and TR-8 inhibited the growth of the pathogen by up to 89%.	[95]
	3. <i>T. viride</i> , <i>Bacillus subtilis</i> , and <i>Pseudo-monas fluorescens</i> proved inhibitory effects against the growth of pathogenic fungi.	[97]
	4. Non-pathogenic strains of <i>F. oxysporum</i> can suppress the disease development in soil, as it affects the germination of chlamydospores of the pathogen and can switch on the plant's defense mechanism too by competing with the pathogen at infection sites.	[117]
	5. <i>T.viride</i> and <i>T. aureoviride</i> suppressed 62% and 36% of disease development, respectively.	[118]
Chemical Control	1. Topsin M fungicide stops the linear growth of the pathogen.	[97]
	2. Mancozeb + carbendazim and carbendazim alone.	[119]
	3. Carbendazim and metalaxyl.	[111]
Cultural Control	1. Organic cow manure at a concentration of 2% along with sandy loam soil.	[120]
	2. Vermicompost	[119]
	3. Ridge sowing with a low irrigation level.	[110]
	Use soil with a higher pH; hydrated lime could be used to enhance the soil pH. Soil solarization.	

According to the European Union, only 5 µg kg⁻¹ and 10 µg kg⁻¹ of aflatoxin B1 and total aflatoxin, respectively, are allowed to be used for chilies. These are the two main restraints for chili exports, and in the past chili exports were restricted from entering Europe and Japan due to the presence of these toxic elements [121]. Apart from residual effects, excessive use of pesticides and fungicides affect our ecological system. To minimize these risks, a biological mode of control is preferred over chemical control.

6.2. Biological Control of Pathogen

At the present point in time, fungicides and soil fumigation are used to control the chili diseases caused by soil-borne pathogens, especially *F. oxysporum*, on economically important crops. Due to it being soil-borne in nature, it becomes difficult to control this disease through a chemical mode of control without any side effects on both the soil and environment [122]. Methyl bromide, which is widely used to control the soil-borne pathogen, has been declared as a hazardous chemical by the U.S. Clean Air Act (section 605), because it causes the depletion of the ozone layer [112]. Hydrolytic enzymes are involved in the degradation of fungal cell wall. Chitinases play an undeniable role in cell wall lysis and are considered as key factors in biological control [123]. For biological control in agriculture, micro-organisms or enzymes (chitinolytic) that are capable of chitin degradation have been used [124,125]. Chitinolytic bacteria produced promising result in regard to controlling fusarium wilt in chilies; they have the ability to suppress the disease without affecting seedling length [116]. Non-pathogenic strains can also be used as a competitor in nutrients which results in the decline of chlamydospore germination. These strains switch on the plant defense system by competing at attacking sites in roots [117]. Different *Trichoderma* species were studied to test their antagonistic efficiency against pathogenic strains of *F. oxysporum*; among all tested species, *T. viride* was the best biological controller of *F. oxysporum* and caused a 62% reduction in colony growth of pathogenic fungi [118]. A study was conducted to check the antagonistic effect of different micro-organisms against wilt disease viz., *T. harzianum*, *T. aureoviride*, *T. viride*, *B.subtilis*, and *Pseudo-monas fluorescens*, and *T. viride*, *B. subtilis*, and *P. fluorescens* showed maximum inhibitory effects against the growth of the causal pathogen [97]. Although disease reduction can be obtained through

biological control, this mode is quite slow and total disease inhibition is not an easy task. To minimize disease incidence, there is a need to breed a genotype against specific biotic and abiotic stresses. The breeding of any genotype of any crop requires some pre-requisite.

7. Conventional Breeding Strategies

7.1. Breeding and Genetics of Resistance of Fusarium Wilt in Host Plant

The breeding of any crop plant to improve a particular trait is entirely dependent on the genetic nature of the trait; either it is controlled by one (mono/oligogenic) or more than one gene (polygenic) as well as its inheritance process [1]. To breed against any biotic stress (fusarium wilt), a breeder must come across several steps, i.e., collection of material both resistant and susceptible, screening of germplasms under natural and artificial conditions (artificial inoculum), and then transformation of these resistant resources by a suitable breeding technique. Hybridization, single-seed descent, backcross breeding, mass selection, and recurrent selection are the most frequent breeding methods used in chili breeding [126]. Among all the suitable methods, backcross is considered as the best method to introduce a resistant gene(s) in any cultivated variety [1].

For a balanced agricultural system, resistant resources must be present, as they play an undeniable role in the survival of crop plants against any sort of stress. The resistance mechanism is of two types; either it is qualitative (controlled by one/few genes) or quantitative (controlled by many genes) [127]. In the first type of inheritance, genes show complete resistance against the pathogen, as genes are only active against specific races of the pathogen and exhibit interaction with the genes of the pathogen. The second type of inheritance process of disease development is comparatively slow due to the several factors in which epidemic parameters and latency periods are the most important [1]. A study was conducted to reduce the expenditure of fungicides to control fusarium wilt, and they studied SNK \times P3, KA2 \times P3, and JAJPUT \times P3 crosses and found a qualitative type of resistance controlled by only a single gene [3]. High-yielding and susceptible varieties were crossed with moderately resistant genotypes under both natural and controlled conditions, and it was found that the inheritance pattern of fusarium wilt was governed by a dominant, single gene [114].

7.2. Biochemical and Molecular Basis of Resistance for Fusarium Wilt in Host Plants

Plant-based phytochemicals play their role in plant defense and metabolism [128]. The presence of these compounds inhibit the pathogen (bacteria, insects, viruses, fungi, and herbivores) attack [129]. Phenolic compounds are involved in the resistance mechanism. Resistant hybrids have a constantly high activity of enzymes and ortho-dihydroxy phenols. Moreover, a positive association between phenolic compounds and enzyme activity in resistant plants to fusarium wilt was observed [130]. Enhanced activity of peroxidase and PPO is the sign of phenolic oxidation, which leads to the emergence of oxidative products and toxic quinones. These products could serve as the leading factor to suppress the pathogen attack in host plants [114]. Wongpia and Lomthaisong [26] studied changes in proteins in resistant and susceptible genotypes through a two-dimensional electrophoresis technique. Proteins of ROS detoxification were highly expressed in resistant ones. Furthermore, expression of non-inducible immunity 1 protein was associated with the ability of the plant to combat fusarium disease.

Resistance against fusarium wilt is also associated with the expression of a protein known as class II chitinase, belonging to the class of defensive proteins (PR proteins) and coded by the gene *CaChi2*. Chitinase enzymes (EC 3.3.14) act on chitin and cause its hydrolysis [131]. These types of enzymes are produced in plants under pathogen attack, gather at infection sites, and reduce plants' susceptibility by acting on infection sites [132]. The role of chitinases in fungal inhibition has been explained through isolation from plants [133]. Isozyme patterns of chitinases showed that the expression of ~70 kDa chitinase in intercellular fluid is associated with fusarium resistance, and this could also be used in molecular breeding to identify resistant/tolerant lines [134].

In a resistant variety (Dandicut), defense-related enzymes were examined and a reasonable amount of peroxidase, polyphenol oxidase, and phenyl ammonia lyase were found in the resistant genotype. The salicylic acid pathway and signal pathway showed a positive association, which indicates that resistance is due to salicylic acid [135].

The production of pathogenesis-related (PR) proteins is an important mechanism, i.e., the production of chitinases in response to pathogenic attack [136]. Chitinase genes code a chemical defense of chitinase and one of their members is *CaChi2*, present in chilies (see Table 6). It degrades the fungal cell wall by acting as a PR protein [137]. The role of *CaChi2* gene in resistance against fusarium wilt was further confirmed through qRT-PCR. Expression was comparatively higher in resistant plants [89]. Chitinases also release an endogenous elicitor which assists the plant to provoke systemic acquired resistance (SAR) in plants [138]. Chitinases are classified into class I to class V. *CaChi2* is one of the class II chitinase genes with almost 250 amino acids without any cysteine-rich domain [139]. The enhanced expression of chitinases forbids pathogen infection in acacia and grape [140], but this practice is not yet performed in chili plants. Plant defense expression requires the *CaChi2* gene to synergistically work with another gene. The activation of chili defense against fungal attack requires the co-expression of the *CaChi2* gene with *CaBPR1* (class 1 pathogenesis-related protein) and *CaBglu* (glucanase) [137]. In peas, both glucanase and chitinase activate the defense system [141]. An investigation on the expression of chitinase genes of chili crops showed that *CaChi2* gene's expression increases under the attack of *Xanthomonas campestris* pv. *Vesicatoria*. Expression alone is unable to stimulate defense, and requires PIK1 (cytoplasmic kinase protein). Hence, PIK1 is the key gene involved in resistance and the chitinase gene acts as an enhancer [142]. A higher expression rate of genes in a resistant (Dandicut) variety, such as acidic chitinase 3, acidic glucanase, metallothionein 2b, and osmotin-like PR-5, confirmed its resistance towards wilt disease, which indicated the involvement of these genes in plant defense [135].

Table 6. Genes involved in plant defense mechanisms against fusarium wilt in chilies.

Serial No.	Gene Name	Function	References
1	<i>CaChi2</i>	Fungal cell wall degradation Releases endogenous elicitor	[137] [138]
2	<i>CaBglu</i>	Activates the plant defense	[137]
3	<i>PIK1</i>	Key gene in resistance and plant defense stimulation	[142]
4	<i>Acidic chitinase 3, acidic glucanase, metallothionein 2b, and osmotin-like PR-5</i>	Activation of defense	[135]

8. Non-Conventional Crop Breeding Strategies

Since this disease is soil-borne, conventional approaches are used to mimic the effect of this disease. A conventional breeding program is not applicable for existing germplasms except through extensive backcrossing. Screening of germplasms is a pre-requisite of conventional breeding. The development of resistant varieties through a conventional approach requires 8–10 years. Non-conventional techniques are applicable on existing germplasms and could produce promising results in a short period of time. *F. oxysporum* starts its attack from the lower part of a plant and invades the vascular tissue through the root zone; therefore, strengthening this portion of the plant may lead to the development of a tolerance mechanism in crop plants against this destructive pathogen. Plants contain several genes that are related to defense mechanisms and could be utilized against pathogens to protect from pathogenic attack. Such a type of gene has been identified in tomato mutants and designated as Solyc08g075770, expressed in the root zone. Further roles of this gene towards tolerance have been examined through knock-out and gene complementation. Knock-out transgenic lines showed susceptibility, while the comple-

mentation lines depicted tolerance against fusarium wilt. Transformation of this gene could also lead to the development of tolerance in chilies, as to the best of our knowledge, no transformation has been attempted in chilies [16]. Another transformation has been attempted in bananas against *F. oxysporum* to maximize antifungal activity. *Ace-AMP1* and *pflp* genes presented in an embryogenic cell were transformed through agrobacterium transformation. Transformant lines with stacked genes showed tolerance against oxidative stress induced by wilt disease by producing peroxidase and oxide dismutase [143]. Such types of transformations are needed in chilies to prevent yield losses due to fusarium wilt. The role of plant defensins cannot be denied in plant defense mechanisms. The *MsDef1* defensin gene from *Medicago sativa* was transformed and expressed under the CaMV 35S promoter in tomatoes against *F. oxysporum*, and it yielded positive results [15]. To the best of our knowledge, no transformation of such a type has been reported in chili plants against fusarium wilt disease despite its serious threat to chili production. Such a transformation must be adopted to protect chilies from fusarium wilt, as these practices benefited tomato and banana plants substantially.

Other genetic engineering techniques such as the enhancement of gene expression through promoter designing of the *CaChi2* gene or conditional gene regulation of genes involved in resistance would provide fruitful results against fusarium wilt, since the expression of these genes is positively correlated with disease control.

However, to the best of our knowledge, there is no practice of increasing or integrating phenolics in chilies through any technique. Such a practice has been reported in the *Solanaceae* family (tomato plants) against the attack of the same pathogen, in which *Pseudomonas fluorescens*, a non-pathogenic bacterial isolate, Pf-1, was used to integrate defense proteins. After the fifth day of bacterial treatment on infected plants, a higher accumulation of phenolics and other defense gene products, such as polyphenol oxidase (PPO) and peroxidase (PO) was seen. This catalyzes the origination of phenylalanine ammonia-lyase (PAL), which is involved in the synthesis of phenolics and phytoalexins [17]. Non-conventional techniques, in contrast to conventional techniques, resulted in rapid progress against biotic stresses since conventional techniques consumed plenty of time to improve a plant against any stress.

RNAi Host-Induced Resistance against *F. oxysporum*

Commonly, fusarium wilt is controlled through breeding strategies, which are largely dependent on the availability of resistant germplasms. The evolution of pathogenic races is one of the major hurdles in controlling the spreading of wilt disease through conventional crop breeding techniques [144]. Fungicides are also used to control the fungal infection, but these fungicides are non-specific, causing serious threats to the environment. Transgenic plants containing anti-fungal genes have also been produced, but [145] these plants did not demonstrate a high resistance against fungal diseases [146]. RNAi has produced promising results in producing disease-resistant plants [147–150]. Singh et al. (2020) [151] targeted the polyamine biosynthesis gene and ornithine decarboxylase (ODC), essential for the normal growth and development of *F. oxysporum*, and silencing these genes did not affect plant polyamine levels, morphology, and ODC gene expression. Moreover, silencing of the *ODC* gene may provide fruitful results in controlling other phyto-pathogenic fungi [151]. The *FOW2* and *chsV* genes of *F. oxysporum*, which are secreted constantly were identified through semi-quantitative PCR and also silenced by using the RNAi technique. Silencing of these genes resulted in reduced mRNA expression along with less virulence and altered physiological characteristics, including growth and sporulation on growing media [152]. Moreover, RNAi, through in planta transformation, has also been used in tomatoes against fusarium wilt for *Fmk1*, *Pbs* MAP kinase signaling genes, and the *Hog1* gene. Different results were obtained for the silencing of each gene. Hypovirulence, reduced invasive growth, and reduced surface hydrophobicity were observed in *Fmk1* RNAi fungal transformants. *Pbs2* and *Hog1* transformants depicted reduced invasive growth and pathogenesis [153].

However, no similar transgenic practice has been performed in chilies. Such a type of transformation must be performed in chilies to induce resistance.

9. Conclusions and Future Prospectus

Fusarium wilt is promoted in moist soil combined with high temperature, and it is most destructive in November and December, as the levels of moisture in soils become higher in these months. Apart from chili, this disease can also appear on tomato and eggplants, which is a sign of real threat because all these crops have overlapping growing seasons in Pakistan and the pathogen can be transferred from one crop to another. The seedling is the most crucial stage for this disease, and disease incidence can be minimized to a significant limit by adopting safety measures at this stage. Numerous methods have been developed to control this disease in which biological control and natural extracts have been highlighted and studied due to the efficiency of these methods to mimic the disease. Different fungicides have also shown promising results against the pathogen, but several factors must be studied before using these types of fungicides, including economic threshold level, the quantity of fungicides (up to safe limits), and the stage of plants to be sprayed. Establishment of genetic protocols is now essential, as transformations gave positive results against *F. oxysporum* in the *Solanaceae* family and other crop plants, such as bananas. Advanced biotechnological techniques such as gene pyramiding, gene stacking, and expression enhancement of antifungal genes have not yet been practiced in chili plants. Scientist should give more attention to this crop.

As we are living in the modern era and the population is increasing rapidly, especially in South Asian countries, in the near future food demands will be higher than nowadays. To fulfill the food demands, chilies have a significant role, and this can only be achieved by increasing its production, which is largely dependent on limiting factors; fungal diseases are one of the most destructive factors which limit its production in Asia as well as in the whole world. Growing resistant genotypes is the only solution to combat this issue and resistance in plants can be generated by crossing resistant parents, and more attention should be given to the germplasm which have a CaChi2 gene, as this produces proteins responsible for resistance against the causal pathogen. Moreover, more work should be done on wild resistant species which can yield better results against fusarium wilt. Integrated disease management approaches can be adopted to control this destructive disease.

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