Chapter

The Trends in the Evaluation of Fusarium Wilt of Chickpea

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Abstract

Chickpea (*Cicer arietinum* L.) is one of the important annual legume crops, cultivated throughout the India since ancient time. It is also grown in many countries of the world. The crop has been facing numerous biotic and abiotic constraints. Among biotic constraint crop affected adversely by diseases, caused by many pathogens. Ever since 1918 when for the first time wilt disease of chickpea was reported and *Fusarium oxysporum* f. sp. *ciceri* was the causal organism many strategies have been adopted to control the wilt disease. The controlling methods included conventional as well as modern one. However, more and more emphasis was given on biological control agents such as AM fungi and *Trichoderma*. The role of AM fungi have been evaluated for controlling the wilt disease similarly role of *Trichoderma* is thoroughly established biological control agent against Fusarium wilt. With the advent of modern tools and techniques developing markers, resistant varieties, all such sources enable us to reduce the effect of pathogens. Here an attempted has been made to acknowledge the trend of disease management and evaluation strategies of Fusarium wilt of chickpea for getting better yields of the crop.

Keywords: pathogenesis, pathotype, ITS, PCR-based diagnosis, chickpea

1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the major legume crops grown in the cool season, mostly in dryland [1]. India is the largest chickpea producer as well as consumers in the world [2]. Chickpea is also known as garbanzo bean or Bengal gram, is a self-pollinated, annual diploid (2n = 2x = 16) species with a genome size of 738 Mb [3] belongs to the family of Fabaceae (Leguminoseae) sub-family Faboideae (*Papilionaceae*) and tribe *Cicereae* it has 9 annual and about 34 perennial wild species. Among 9 annual species, chickpea is the only cultivated species worldwide [4, 5]. Chickpea served as a major dietary protein for humans at the cheapest cost compare to other sources of protein. Like other crops, chickpea is subjected to many abiotic and biotic stress, which, causes limited production as per the theoretical potential. The production of chickpea in the Indian subcontinent and Asian countries are severely affected by the pathogenic fungus, bacteria, virus, and nematodes which causes disease like *Fusarium* wilt, dry root rot, *Ascochyta* blight, Collar rot, Bacterial blight, Filiform virus and Root nematode [2, 6]. The wilt disease caused by FOC was more hazardous and the impact was so severe, that sometimes no yield is recorded, however, the percentage of loss depends upon agroclimatic conditions of the region [7]. FOC is a soil-borne disease and the mode

of dissemination are many such as infected plant debris (root, leaf, and stem), soil and seed as mycelium, microconidia, macroconidia, and, most commonly, as chlamydospores [8–10]. Pathogenic variability has been observed on the basis of symptoms causes to the host, FOC subdivided into two pathotypes i.e. wilting pathotype and yellowing pathotype, FOC has eight races 0, 1A, 1B/C, 2, 3, 4, 5, and 6 that have been identified till now [8, 10–13]. The Races 0 and 1B/C are of the yellowing pathotypes, while races 1A and 2,3,4,5, and 6 belong to the wilting pathotypes, and caters are known for their economic loss to the farmer [14]. The variability in the pathogenicity makes it more complex and difficult to identify them. Therefore the proper identification of the pathogens is the most important part of the management of the disease and in developing resistance variety of the crop against the pathogen. However, to identify the causal organism applying molecular tools such as PCR techniques, specific primers, and PCR assays have been developed [9, 11, 12, 15, 16]. The evaluation of *Fusarium* wilt is involved in many stages, which has been discussed in this chapter and also will list out the troubleshoots in the diagnosis and the identification protocols both morphological and molecular method.

2. Pathogenesis and pathophysiology

The FOC shows variableness inside their populations also based on their severity index their virulence is decided, it was discussed in the above sections that FOC has eight pathotypes i.e. yellow pathotypes and wilting pathotypes. The yellow pathotypes races cause progressive foliar yellowing accompanied by vascular discoloration and late plant death while the wilting pathotypes cause fast and extreme chlorosis flaccidity, vascular discoloration, and early plant death, the life cycle of the pathogen is of two-phase; the parasitic phase and the saprophytic phase (**Figure 1**; [17]). At the parasitic phase, the fungus gets to enter or invade the host through penetration by crack, wounds, roots hairs root cap, or root branches, the penetration process is likely to be enhanced by the hydrolytic enzymes, after the establishment of the pathogen into the roots, the cortical region is colonized by the emerging mycelia and slowly and gradually enters into the vascular stream and distributed throughout the plants and the gradual blockage of the vessel happens by the mycelium and the spores that were reflected by the appearance of the symptoms on the host [18–20]. It does not matter how the infected plant died either by wilting

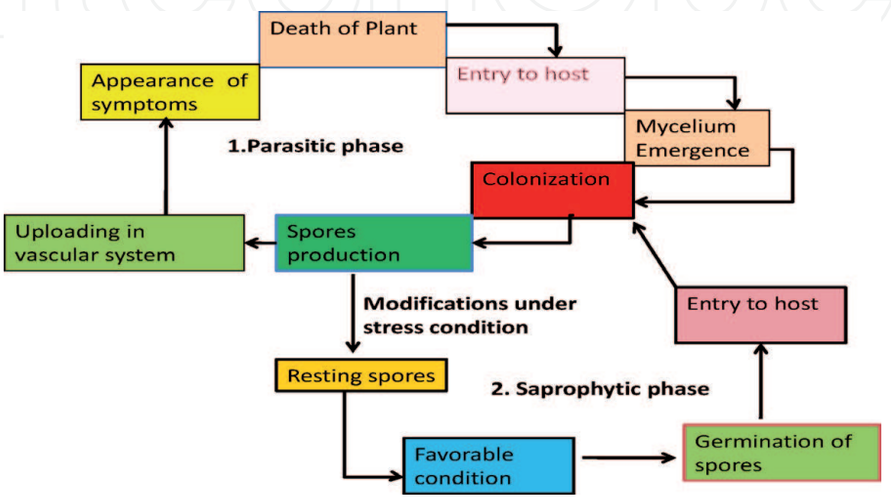

Figure 1.
Illustration showing the parasitic and saprophytic phase of Fusarium oxysporum *f. sp.* ciceri on *Chickpea.*

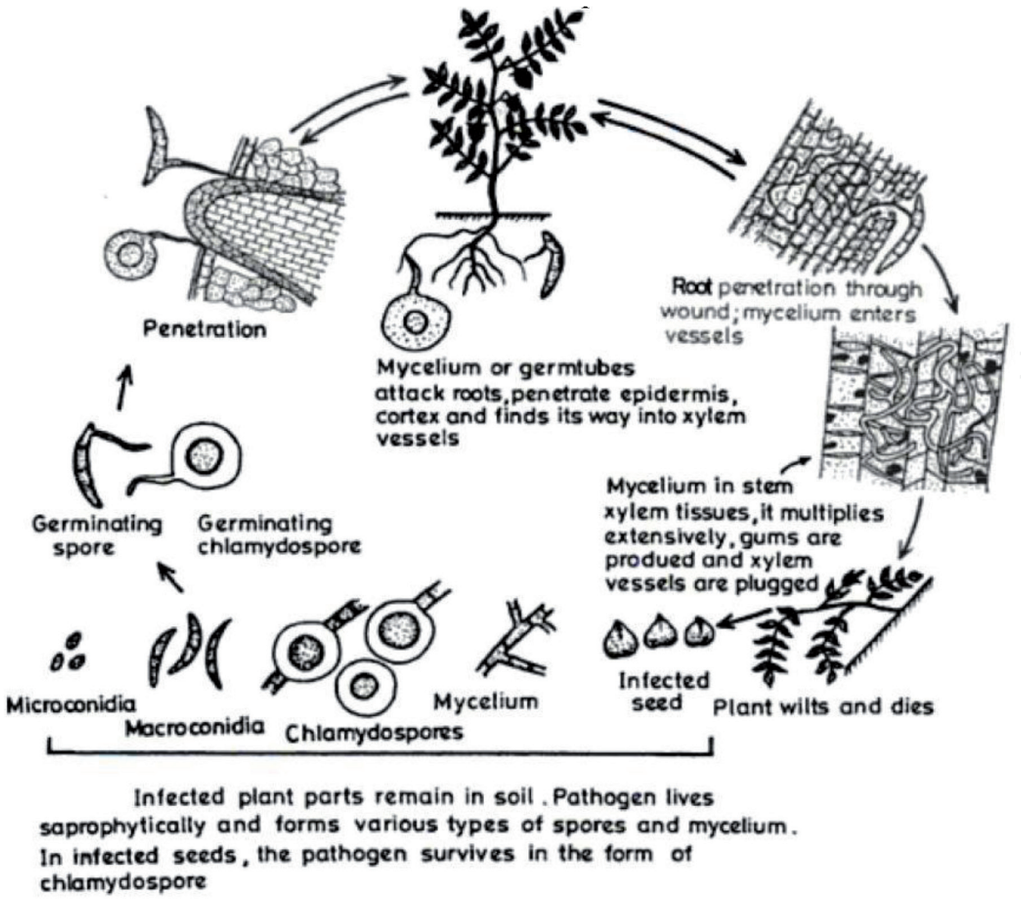

Figure 2.
Disease cycle of F. oxysporum *f. sp.* ciceris *(source: Jalali and Chand, 1992) [21].*

or chlorosis the important thing is that the pathogen survive through resting spores, the importance of the present monograph lies in how *Fusarium* causes wilt disease in chickpea and what measure has been used to control the disease is discussed in length (**Figure 2**).

3. Pathogenic variability in *Fusarium. oxysporum* f. sp. *ciceris*

The FOC shows high variability with respect to its morphology (**Figure 3A** and **B**), cultural characteristics, and virulence, In the number of studies that have been carried out suggested high pathogenic variability among the isolates of different regions [22]. In Mexico PCR based study on 355 isolates reported that 161 strain are positive for the FOC and has shown morphological variability in FOCs and also revealed that it is not a function of the physical and chemical properties of the soil, nor the geographic location of the crop fields rather it is a race of the FOC [23]. In a laboratory study carried out in Bangladesh, FOC isolates showed significant variability in their cultural, morphological, and physiological traits, i.e. colony color, shape, margin, and texture; mycelial radial growth, and spore production [24] considering the above facts it can be established that FOC has significant variability despite of their occurrence. Variance in the isolates of FOC has led to the designation of pathogenic races, and the different races of FOC can be identified by their severity on a different set of differential chickpea cultivars [13]. Pathogenic races of FOC differ in geographic distribution [17]. Races 0, 1B/C, 5, and 6 are found mainly in the Mediterranean Basin and California (USA) Race 1A has been reported in India, United States, and the Mediterranean regions [13, 19, 25], whereas races 2 and 3 have been reported in

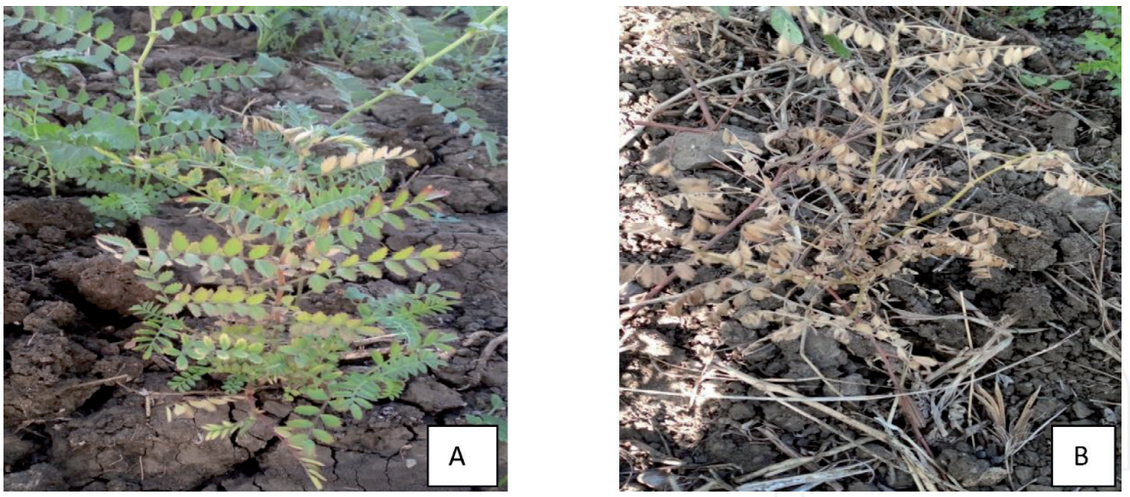

Figure 3.
Showing yellowing symptom (a) and wilting symptom (B).

Ethiopia, India, and Turkey and race 4 has been reported in Ethiopia, India, and Iraq [13, 26]. In short, it can be said that FOC showed dynamic variability respective to geographical distribution. One must consider races for the evaluation and management of diseases. Some diagnostic trends have been discussed here.

4. Pathogenicity test for FOC

Confirmation of the pathogen is commonly conducted by the methods of Koch Postulations, for pathogenicity test in the chickpea caused by the FOC are added here:

a. Blotter paper technique:

In this method, the Pure culture of pathogens is obtained from the infected host, the obtained fungus is then multiplied in potato-dextrose broth and after incubation, the mycelium mat were collected and macerated in distilled water then stored, simultaneously the host seedling of 5 days old grown on riverbed sand soil was uprooted, the roots were washed with running tap water and these roots were dipped in the solution of inoculum by touching the edge of the beaker then after the seedling of the test line are placed side by side on a blotter paper (size 45 cm × 25 cm with one fold.) so that the roots are covered and the green part remains outside of this paper. Folded blotters were kept, one on top of the other, in heaps of 10 in a tray. The trays were placed in an incubator at 25° C with 12-hr artificial light. After 8 days of incubation, blotters were moistened adequately every day. The seedlings were examined for the extent of root damage and a score of the disease [27]. Nene et al. [28] also recommended this technique for screening of chickpea resistant variety against FOC.

b. Soil Inoculation method/Pot Screening:

To find out the variability among the different isolates of pathogens, the investigation on soil inoculation by FOC is a common method. In the soil inoculation method, different isolates of FOC were multiplied individually on sand and soil (3:1) medium and filled in plastic pots. The high inoculum level of 20% w/w is used for the maximum wilting. The inoculated and un-inoculated control pots

are maintained under controlled environmental conditions. Observations on wilt incidence in seedlings were recorded from 10 days after sowing (DAS) to 30 DAS.

c. Sick plot method:

The sick plot method is a method used to check the pathogenicity and screening of chickpea cultivar lines against FOC. This method is conducted in field conditions. A suitable size plot is developed into a sick plot, about six-years required to develop a sick plot by the pathogen inoculums [28] following steps are given below.

1. Select a plot of adequate size and ensure that it is isolated from other chickpea fields to avoid the spread of the fungus inoculums from this plot to others. The selected plot should have been harvested in the previous year with chickpea, and at least traces of wilt incidence should have been observed.

2. Wilted plants from other fields were collected in bulk, chop into small pieces, and incorporate uniformly in the surface soil of the plot.

3. Susceptible cultivars were grown in the selected plot. Make sure a good plant population and maintain normal agronomic operations.

4. At the end of the season, the cultivar shows 20% wilting symptoms, then all the debris of the cultivar were scattered after harvesting and threshing uniformly all over the plot and incorporate it by discing. Step II may be repeated this will help in increasing the level of the inoculums to make the soil "sick."

5. Steps III and IV were repeated in the next season. More than 90% wilt incidence in the selected plot should be observed. If the incidence is less than 70%, then steps III and IV are repeated.

6. Screening should be initiated in the next season. Sow a susceptible cultivar after every two test rows in the whole field, these rows will serve to checks in monitoring and maintaining the wilt sickness of the plot. Now the susceptible check rows must show more than 90% wilt.

7. From the 4th or 5th year onwards, plant every fifth row as a susceptible check. This will provide for more breeding material and at the same time maintain the level of sickness.

8. Planting any other crop in this plot is not recommended.

Sometimes even taking all the care, a sick plot which is predominated by FOC also provides room for other soil-borne pathogens.

5. Isolation and maintenance of pathogens

The pathogens can be isolated either from an infected host or from the soil. To isolate the pathogen from the infected plant parts, the plant sample collected randomly, basically, the root and stem of the plant is equally used to isolate the pathogen. The obtained plant sample with 1:1000 mercuric chloride solutions for one minute and washed twice with sterile water after cleaning the roots or stem. Cut the section of the root or the stem then placed it on the PDA medium and incubate

at 28-30°C to get the fungal colony. The colony obtained on the Petri plate of PDA medium is then purified by using the single spore culture method in PDA slant. If the pathogen has to be isolated from soil then the soil sample must be obtained from the infected crop field and using serial dilution methods, the culture is made on the Petri Plate of PDA (10^{-6}–10^{-8} dilution is appropriate dilution for the culture) to obtain the colony followed by the single spore isolation method in slant for pure culture. The fungus slant can be store for further study.

6. Diagnostic analysis of FOC

Diagnosis is an integral part to analyze any disease and the approach to establish host and pathogen interactions. The approach could be conventional or advanced. Methods have been discussed in details see below.

6.1 Diagnosis at morphological and at the anatomical level

The morphological study includes observation of the cultivar in the field and the symptom appearance; it was discussed in the section (1.2) that the FOC consist of two pathotypes i.e. yellow pathotypes causing yellowing of the foliar (**Figure 3A**) and gradually the death of the plants and other is wilting (**Figure 3B**) where the chickpea infested by the FOC wilted and the cultivar dies faster and led to the destruction of the crops. The chickpea infected by FOC has a formation of root nodule or has a trace of root nodule (**Figure 4A** and **B**.).

Under anatomical studies, the fungal mycelium present in the vessel lumen of infected crops, entry of mycelium follows an intracellular path to the cortex and enters xylem vessels through the pits, the mycelium in xylem branches and produce microconidia, these microconidia detached and carried upward by the vascular system and the lateral movements of microconidia between the vessels are through the pit, as a result, the water economy of infected plants is eventually severely compromised by blockage of vessels, resulting in stomatal closure, wilting and death of leaves, often followed by the death of the whole plant [9, 25]. *Fusarium oxysporum* f. sp. *ciceris* can survive as mycelium and chlamydospores in seed and soil, and also on infected crop residues, roots and stem tissue buried in the soil for up to six years [5, 20, 29]. Different isolates of FOC have cultural variation, when isolates of FOC grown on PDA shows fluffy, partial fluffy, and white cottony mycelium growth having different pigmentations like

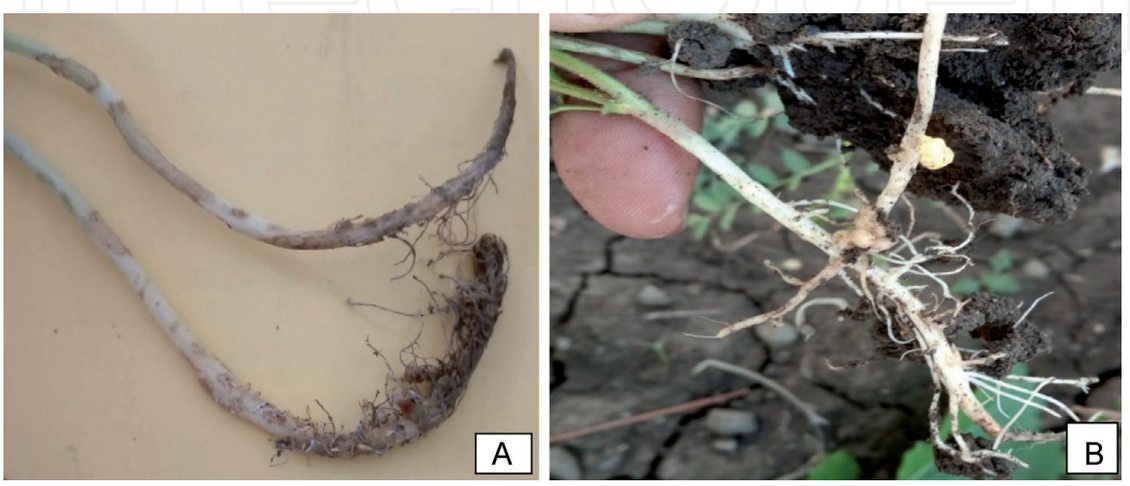

Figure 4.
Figure showing the root nodulation in infected and healthy chickpea; A- No or trace nodule, B- root nodule in health chickpea.

gray purple, orange group, and gray-brown [5], one must undergo an in-vitro study for the details cultural description of the FOC since it has a high rate of variability.

6.2 Diagnosis at the molecular level

The identification of the Pathogenic race of FOC is predominantly based on the differential reactions on the host genotypes they infected [30]. Soon after the development of PCR and other biotechnological approaches like Random Amplified polymorphic DNA (RAPD), Amplified fragment Length Polymorphism (AFLP), Simple sequence repeat (SSR), and inter-simple sequence repeat (ISSR) are being used to study the pathogenic race and the pathogenic variability [30]. To study the pathogen colonization and progression in wilt susceptible and resistant variety of the chickpea, insertion of GPF gene in the pathogenic race using the molecular tool helps to visualize the colonization and progression under confocal microscopy, moreover, quantitative PCR (qPCR) is helpful in estimation of the pathogen load and the progression of the diseases across various tissue in both the cultivar during the course of the disease [31]. The synergistic gene-specific markers, ITS-RFLP, ISSR, and AFLP are used in distinguishing *F. oxysporum* f. sp. *ciceris* races, the use of oligonucleotides designed from the conserved region of Hop78 transposon helps in distinguishing the FOC races 1,2 and 4 from race 3 by PCR amplification methods [32]. Moreover, the genetic variability of different isolates of FOC race can also be established by using the molecular marker, RAPDs, and AFLP, and help in identifying the different clusters of the gene that represent the races of the pathogens from the coordinate analysis of the similarity index data generated from the molecular marker studies [33]. The population of pathogens is genetically as well as pathologically distinct from those in other countries which can be reported from DNA fingerprinting studies [34].

7. Development of *Fusarium* wilts in relation to environment

Agro-climatic factors are solely related to crop yield and the virulence of plant pathogens. Changes in climatic factors like temperature, carbon dioxide, and the frequency and intensity of extreme weather could have a significant impact on crop yield. The effects of increased temperature on any crops depend on the crop's optimal temperature for growth and reproduction. Warmer and moist weather favor many pests, weeds, and fungi to thrive. The climatic change affects the multitrophic interaction in the soil, which is primacy for the plants and other functional domains of the soil ecosystem; the soil food web is highly interlinked to all the functional domains of the soil [35]. Soil food web sequesters carbon, helps in cycle nutrients, maintain soil health to suppress pathogens, helps plant tolerates abiotic and biotic stress, and maintain ecosystem resilience and sustainability. Temperature plays an important role in the resistance of the chickpea against the *Fusarium* wilt, the effect of temperature on-resistance of the chickpea cultivars to *Fusarium* wilt that caused by various races of *Fusarium oxysporum f.sp. ciceris* (FOC) when temperature increased by 2-3°C from the normal temperature, the cultivar became more susceptible to pathogens [36]. To combat the effect of the global change in temperature on chickpea production different workers are evaluating for the validation of the chickpea resistance in different environmental constraints. Now to mitigate the different environmental constraints a new approach or model is the demand of today agriculture practices, Anuga and Gordon [37] in this regard has thrown light over the Climate-Smart (CS) weather practices/strategies to mitigate climate-induced agriculture impacts but it would be very imperative to understand the CS weather practices that are effective in influencing farmers productivity positively.

8. Evaluation methods of fungicidal/bioagents on FOC

The antifungal substances obtain the substance that inhibits the growth of the fungus or completely restrict fungal growth. The antifungal activities are done basically *in vitro* or *in vivo* conditions. The methods are described below.

a) Cavity slide test method

The effect of the antifungal substance on the spore germination of the pathogens conducted by using the cavity slide method, For this the spore suspension at the density 10^6 spores/mL was placed on the cavity slide along with the antifungal substance and incubated under mist-chamber at 28–30^0 C. The percentage rate of the spore germination is recorded after 24 hours under a light microscope.

b) Poisoned Food Technique

Food poisoned techniques represent the method of poisoning fungal growth medium by using the antifungal substance or the agent. The reduction of the fungal growth on the medium was recorded, the reduction of the fungal growth indicates that the inhibition was caused by the antifungal substances. For this, the PDA medium is prepared and amended with the desired concentration of the substances, and the mycelial discs of the FOC are placed at the center of the Petri Plate and then incubated at 28-30°C for ten days and the radial mycelium growth are measured. The percent reduction of mycelial growth over control is calculated employing the following formula:

$$\text{Percent decrease over control} = \frac{Dc - Dt}{Dc} \times 100 \quad (1)$$

where Dc - Average diameter of fungal growth in control; Dt - Average diameter of fungal growth in treatment.

c) Well diffusion method

The well diffusion method is employed to assess the antimicrobic ability of the substance against the FOC, PDA medium was prepared on plates and the sub-cultured pathogen on sterile Potato dextrose broth is swabbed on the plates, then after using a sterile cork borer well are cuts, and are filled with the antifungal substance. The FOC pathogen is allowed to grow under optimal states with a positive and negative control. The diameter of the inhibition zone is recorded for the fungicidal substance.

d) Blotter method

This method is suitable to check the infection rate in the seeds of chickpea by the FOC pathogen, in this method the spore suspension is made and the required number of the seeds are soaked in the fungal spore suspension, and then after seeds are submerged in the fungicidal substance of desired concentration. The treated seeds are placed on the moist blotter paper in a Petri plate and incubated (20–22°C) for seven days, the seeds are examined for the growth of seed-borne pathogens. The result is expressed in the percentage of disease reduction as compared to control.

9. Differences between *Fusarium* and *Verticillium* wilt symptoms

To conduct any successful evaluation of a disease, the critical analysis of the symptom represents a crucial part, therefore the *Fusarium* wilt of chickpea, are frequently confused with *Verticillium* wilt so the difference between them must be highlighted to resolve the confusion between the symptoms they caused, *F. oxysporum* causes vascular discoloration (brown staining of stem tissue) which is darker and continuous whereas *Verticillium* causes more of a "flecking" or "spotty" type

of strain. The vascular discoloration in plants infected by *Fusarium* is low in the lower part of the stem than the plant infected with *Verticillium* (more in the lower stem, below the cotyledonary node and upper taproot in *Fusarium*). Another most interesting difference fact is that *Fusarium* wilt is favored by warm temperatures while *Verticillium* wilt is favored by cool temperatures it can be stated that the *Fusarium* became more active in warmer weather and *Verticillium* in the cooler weather [1, 17].

10. Managements of fusarium wilt of Chickpea

A successful evaluation of disease is always followed by management strategies to combat the disease. Management of *Fusarium* wilt of chickpea is difficult to achieve and no single control measure is sufficiently effective [36]. *Fusarium* wilt of chickpea is a monocyclic disease in which development is driven by the pathogen's primary inoculums [13]. *Fusarium* wilt is difficult to control with cultural practices and chemical control is one of the most used approaches. Biological control and plant resistance, however, provide an environmentally and economically appropriate means for disease control that can be easily included within an integrated disease management strategy [38]. In fact, the use of natural resistance for the management of fungal diseases in chickpea may be enhanced through biological control using either bacterial or fungal antagonists [7, 39–42]. The use of arbuscular mycorrhizal consortium to control biologically *Fusarium* wilt is found to be an effective measure to manage wilt disease of chickpea [7, 43]. Different biocontrol agents, including bacteria belonging to the genera *Bacillus*, *Pseudomonas,* and *Rhizobium* and fungi such as nonpathogenic and non-host *Fusarium* species, have been used successfully and resulted in a significant reduction in both pathogenic fungal growth in vitro and disease development in the plant [44–47]. Biofumigation using brassica may be included in alternative strategies for the management of *Fusarium* wilt of chickpea [48]. Induced resistance through the accumulation of various phenolic compounds and phytoalexins, as well as the activation of peroxidases, polyphenoloxidases, and key enzymes in phenylpropanoid and isoflavonoid pathways, play a crucial role in the biological control and resistance of chickpea to pathogenic attacks [49–52]. For instance, recent investigations revealed that the pre-treatment of chickpea seedlings with selected *Rhizobium* isolates before challenging with FOC, increased significantly the levels of total phenolics and the constitutive isoflavonoids, formononetin, and biochanin A [46]. Protection of chickpea against *Fusarium* wilt by the nonpathogenic and non-host *Fusarium* species shown to be associated with the induction of the synthesis of the phytoalexins medicarpin and maachiain and the related isoflavones formononetin and biochanin A [53]. Maakiain and medicarpin exhibited potent antifungal activity towards *Fusarium* spores, by inhibiting their germination and hyphal growth. Studies on phytoalexin tolerance in chickpea pathogenic fungi have also shown a relationship between virulence and the ability of these fungi to detoxify phytoalexins [54]. This can be exemplified by the fact that the fungus *Nectria heamatococca* can metabolize and detoxify maakiain and medicarpin and that these reactions are required for pathogenesis by this fungus on chickpea. Among the other phenolic compounds studied, gallic, cinnamic, ferulic, and chlorogenic acids are also associated with the protection of chickpea from fungal attacks through induced resistance. Of the induced defenses, phenolics and phytoalexin production have received particular attention in chickpea. However, efforts must be made in providing good evidence that these compounds accumulate at the right time, concentration, and location and to elucidate the regulatory genes involved in their rapid and coordinated induction in response to fungal attack [53].

10.1 Use of resistant cultivars

The use of resistant cultivar endure the most convenient and economically efficient way to control measures for the administration of Fusarium wilt of chickpea. Resistant cultivars of chickpea display the key component in integrated disease management programs. Resistance to FOC races has been only recognized in the desi germplasm and lesser extent in Kabuli chickpea as well as in some wild *Cicer spp*. The use of resistant cultivars receives some critical issues due to some undesirable agronomic characteristics. Furthermore, the high pathogenic variability in FOC populations limits the effectiveness and extensive use of available resistance variety [13].

10.2 AMF based fusarium wilt control

The rising concern for community health and the environment due to use of chemicals has fulled the investigation on the biological control methods. The key to achieving successful biological control depends on the knowledge of ecological interaction taking place in the soil and the root atmosphere. AMF fungi are one of the potential biological agents found to possess significant control over the FOC. The introduction of AMF to chickpea suppresses the effects of pathogens in rhizoplane as well as in the rhizosphere [7]. AMF helps in the nodule formation, Species of AMF like, *Glomus ambisporum*, *G. mosseae*, *Acaulospora nicolsonii*, *A. spinosa*, *Glomus fasciculatum,* etc., is reported as best phytoprotection. The percentage of root colonization by AMF depends on different factors, AMF is a strong candidate for controlling pathogens biologically through competition for space by virtue of their ecological obligate association with roots. It is imperative to use native AMF as it has good potential to protect the plant from FOC and not only they protected the host plant but also they influenced their developing nodules and percent recovery of yield loss [55].

11. Conclusion

Fusarium wilt of chickpea undoubtedly remains the grave threat in the consistent production of the chickpea worldwide. This chapter has been considered to discuss the cause of wilt disease and its controlling measures and standard protocol of its extensive evaluation. Since chickpea is a staple crop and to adequately maintain its high yields instantly a synergetic approach is required because in complex nature one preferred method is not sufficient to combat the prevalent disease, therefore, possible attempt has been wisely made to discuss traditional and modern techniques. Biocontrol agents and beneficial AM fungi showed potential to not merely reduce the wilt but also remediate the soil.

DOI: http://dx.doi.org/10.5772/intechopen.95612

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