J. Plantarum., 6(2): 43-54

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

CHARACTERIZATION OF *FUSARIUM OXYSPORUM* ISOLATED AS LEAF SPOT PATHOGEN FROM *CAPSICUM ANNUUM* L.

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Received on: 22-01-24; Reviewed on: 24-04-24; Accepted on: 12-06-2024; Published on: 10-12-2024

Abstract

Chili (*Capsicum annuum* L.) is a popular commercial crop that is used as both a spice and a medicinal herb. During the previous years, fungal wilting infections were found very common in Chili, resulting in a global yield loss of about 10-80% in the annual production of chili. Presently, from September to November 2021, a field survey was carried out to gather diseased samples of chili plants exhibiting wilt symptoms, in several villages specifically 139 6/r, 166 7/r, 138 6/r, and 425 6/r in Tehsil Haroon Abad, situated in the district of Bahawalnagar. The wilting disease was shown to be present in 55 and 60 percent of *C. annuum* plants. *Fusarium oxysporum* was identified as the wilt disease-causing agent of chili plants through fungal pathogen isolation and morphological identification. *Fusarium* pathogenicity was proven in Koch's pathogenicity trials using the detached leaf method and pot assays. The pathogen could result in a serious economic impact on the chili crop or might be of other spices and herbs if not managed in time.

Keywords: *Capsicum annuum, Fusarium oxysporum,* Isolation, Pathogenicity, Wilting.

Introduction

Chili (*Capsicum annuum* L.) is a widely grown, commercial crop that is utilized as a spicy and an important medicinal plant (Arin, 2019). Red pepper, also referred to as chili, is a plant in the Solanaceae family that grows best in warm, sunny semi-tropical and tropical climates (Vaughan and Geissler, 2009). It contains considerable amounts of minerals such as molybdenum, manganese, thiamin, foliate, copper, and potassium as well as vitamins A, B, C, and E (Olatunji and Afolayan, 2018). India produces the most chilies in the world. According to FAO, India exported 21% of its total dried chili output (Ferdouse *et al*., 2018). Chili was grown on 47,349 hectares in Pakistan in FY2018-19, yielding 2.68 tons per hectare (1.072 tons per acre) and generating 126,943 tons per year. It is used as a spice, vegetable, pickle, condiment, and sauce (García-Casal *et al*., 2016). Green chilies are reported to be significantly higher in vitamin C than red chilies (VanderWeele and Ding, 2017). Green chilies are renowned for their high content of protein, minerals, and vitamins A and C, while dry chilies are abundant in vitamins A and B (Ferdouse *et al*., 2018). The three fruit ingredients of capsicum are capsanthin, capsaicin, and oleoresin. In addition to having anti-enzymatic and anti-obesity qualities, capsaicin, which is present in chili pepper leaves, can also boost the immune system and lower blood pressure (Alonso-Villegas *et al*., 2023). Antioxidants are also present in the chili plant which aid in the prevention of cancer, cataracts, heart disease, and macular degeneration (Kaur and Kapoor, 2001).

Worldwide, biotic and abiotic diseases have been shown to have an impact on chili yield, with 51 pathogens being identified as production-limiting

factors. Pests affect the chili crop during the growing season (Hussain and Abid, 2011). Aphids, thrips, leafhoppers, crickets, mites, root grubs, cutworms, flea Beatles, and other pests wreak havoc on the chili crop (Gulati and Kumari, 2013). The most frequent causes of yield loss in chili plants are fungal diseases such as damping-off, root rots, and wilting (Almaghasla *et al*., 2023). Chili crops' most widespread pathogen concern has been identified as *Fusarium*, which is the most devastating pathogen found in almost every area of the entire world (Parveen *et al*., 2020). It produces crescent-shaped spores of fungi that are found in a variety of soil conditions (Wang *et al*., 2007). Fusarium wilt of chili has recently resulted in crop losses of 15 to 20% in Pakistan's arid areas (Shafique *et al*., 2015). Wilting occurs at seedlings as an early infection, but it can occur at any stage of plant growth. Seed rot, seedling mortality, and complete plant death are all symptoms, as are curled and yellowing leaves and decreased development (Kraft *et al*., 2000). To prevent crop production losses, accurate pathogen identification and numerous controlled practices should be employed (McCartney *et al*., 2003). As a result, the current study sought to locate and identify the chili-specific organism responsible for wilt disease.

Materials and methods

Sample Collection

A survey was conducted in the Bahawalnagar district, specifically in the Tehsil Haroon Abad region with designations 139 6/r, 166 7/r, 138 6/r, and 425 6/r. The selection of these areas was based on the fact that these were chili growing areas in the stipulated time of survey. The objective was to gather specimens of infected chili plants to enhance comprehension of the pathogen responsible for chili wilt. While collecting samples, a variety of symptoms including leaf yellowing and water-soaked spores on leaves were seen. The chili plants showed signs of necrosis and wilting. Different leaf specimens from diseased plants were congregated in a completely randomized block design and placed in sterile plastic bags. Diseased samples were maintained in the lab's refrigerator at 4 °C until further use. The prevalence and severity of the disease among the affected plants in a few selected communities were measured.

Disease Rating Scale

A grading scale for the disease was formulated, taking into account the severity of the ailment. Disease incidence and severity were assessed and measured through visual observations. To determine the prevalence and severity of diseases, the following formulas were used

$$Disease\ severity = \frac{Area\ of\ plant\ part\ affected}{Total\ Area}\ x\ 100$$

$$Disease\ index = \frac{Number\ of\ diseased\ plants}{Total\ number\ of\ plants}\ x\ 100$$

Identification of Fungi based on Morphology

For precise pathogen identification, culture examination of microscopic and macroscopic data is always necessary. Using the MEA (malt extract agar) medium, which contains 2% MEA at a pH of 6.5, the pathogen was grown and identified. Using a surgical blade, the diseased samples' infected parts were carefully removed, and the surfaces were subsequently sterilized with sodium hypochlorite solution. Subsequently, three to four leaf segments, having undergone surface sterilization, were placed in a

sterile manner onto Petri plates containing agar with malt extract. Subsequently, the Petri plates were placed in an incubator set at a temperature range of 25–27 °C for a period of 4–7 days while the infected sample boundaries were frequently checked for the development of radiating mycelia. The developing colony was studied under a microscope to assess the morphological characteristics after 7 days of incubation. The colony's characteristics were studied with naked eye and with the aid of a microscope. From the front and rear, the colony culture was observed and the conidial kind, growth size, and colony size were assessed. The forms of growth of fungi were observed at magnifications 4X, 10X, 40X, and 100X.

Pathogenicity Trails

The detached leaf assay and pot experiments were used in this study for the evaluation of the pathogenicity of newly isolated fungal pathogen by looking at the symptoms that were induced in all four chili varieties after fungal infection. The leaves of the chili plants were taken out and put in the sterilized Petri plates lined with moistened filter paper for the detached leaf assay. The petioles of the disease-free leaves were positioned in a manner that brought them into contact with the surface of the Petri plates. The leaves were injected with 1 mL of a spore solution containing around $5x10^5$ spores/mL under aseptic conditions. Following this, the plates were transferred to an incubator at intervals, maintained at a temperature of 25–27 °C, with regular monitoring for the emergence of disease symptoms. To validate Koch's postulates regarding pathogenicity, the pathogen was subsequently re-isolated from the affected regions.

The seeds of all four varieties of chili were cultivated in disposable pots in a completely randomized block design as part of an experiment to prepare the nursery. For each variety, three seeds per pot were planted in four copies. Pots were watered as needed, and after 20 days, a chili seed nursery containing a variety of seeds was formed. Subsequently, each variety was pruned to retain only one plant per pot. Following that, in a sterile environment, each pot received 5 mL of a spore suspension containing 5×10^5 spores/mL. The spore suspension, however, was not given to the control plants; instead, they were just given water. The pots were covered with polythene bags for 24 hours after being sprayed with the suspension to preserve moisture and maintain an ideal temperature of 25-28 °C for disease growth. At regular intervals, the presence of symptoms was regularly observed.

Results

The present study was done to find out more about *Fusarium* wilt disease of chili. In November 2021, a survey was carried out, and the diseased leaves and stems from the corresponding fields were gathered. More than 55 to 60 percent of chili plants showed indications of leaf necrosis and wilting. Yellowing of plant leaves and the emergence of dark brown lesions near the soil line were disease indicators that led to the entire plant wilting (Fig. 1). To determine the incidence of the disease, the number of infected plants in a given field was counted. Village 166 7/R had the greatest disease incidence (65%), whereas village 138 6/R exhibited the second-highest disease incidence (60%) and village 425 6/R had the lowest disease incidence (45%) (Fig. 2). The survey analysis used for assessing the disease severity monitored infection rates per plant in each field. The village with the lowest disease severity (about 47%) was 166 7/R, while the village with the highest disease severity (around 57.67%) was 139 6/R. (Fig. 3).

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

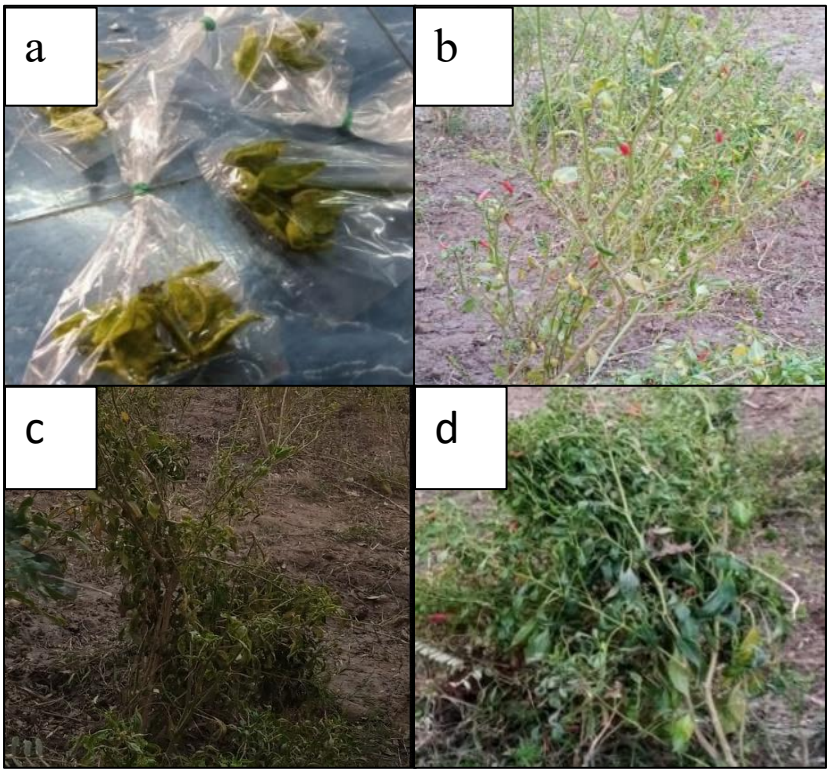

Figure 1: Infected plants in the field and samples collected in the field survey

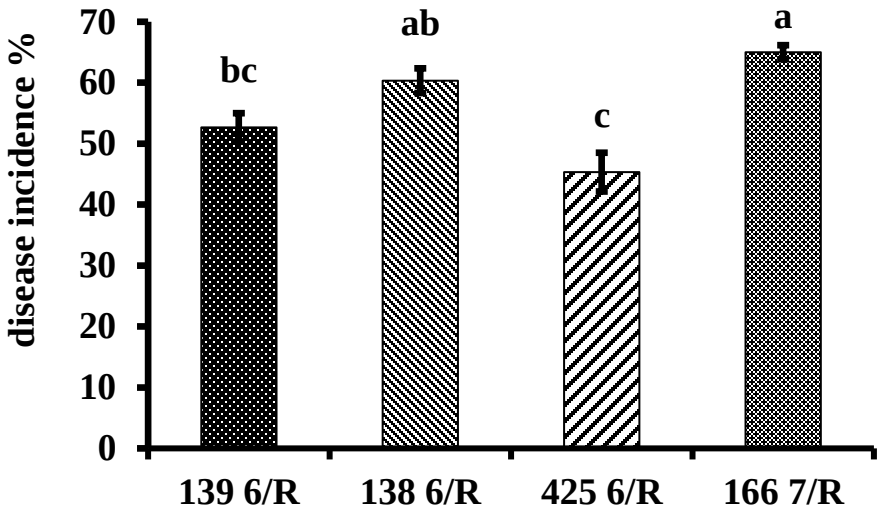

Figure 2: Analysis of disease incidence during field survey Vertical bars show standard errors of three replicates. Values with different letters show a significant difference by ANOVA ($p \leq 0.05$) as determined by DSAASTAT software, LSD test at $p = 0.05$.

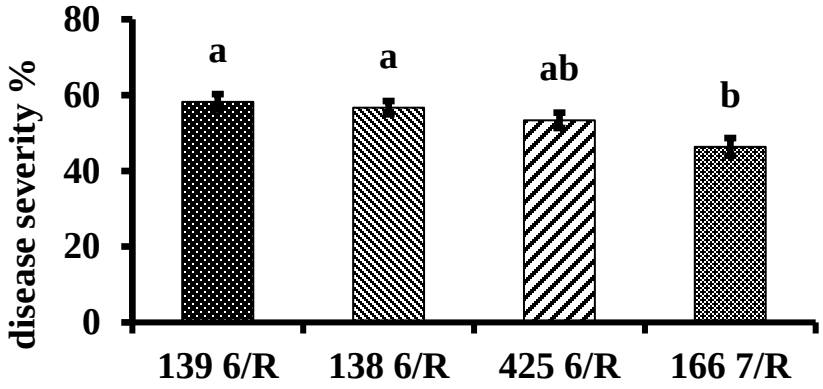

Figure 3: Analysis of disease severity during field survey Vertical bars show standard errors of three replicates. Values with different letters show a significant difference by ANOVA ($p \leq 0.05$) as determined by DSAASTAT software, LSD test at $p = 0.05$.

Identification of Pathogen on the Morphological Basis

First, a detailed examination of the morphology of the isolated pathogen from pure culture was carried out to identify the pathogen. Then, isolated pathogen-purified cultures were viewed under a microscope at various magnifications. Pure culture of the isolated fungi was grown on MEA medium plates for their morphological characterization. Colonies expanded swiftly, emerging into a fully developed plate in only seven days. The purified culture of the isolated pathogen was analyzed based on macroscopic characteristics, encompassing colony shape, color, fungal growth rate, as well as microscopic features of hyphae, spores, and conidia.

The correlations between these parameters in samples cultured on MEA were also taken into consideration. The pathogen exhibited a filamentous structure and produced mycelia, microconidia, and macroconidia that were elongated, slightly curved, and fusiform in shape. Mycelium initially appeared white before changing to a range of colours. The leading edge of the colony displayed a white hue with a pink tint, while the reverse side exhibited a range of tones from light brown to pale (Fig. 4). Based on morphology, the pathogen was determined to be *Fusarium oxysporum***.**

Pathogenicity Analysis

In-Vitro **Pathogenicity:** The application of the isolated pathogen and the examination on the test host plant substantiated Koch's postulates for pathogenicity, confirming the identity and impact of the pathogen**.** By artificially inoculating host plants with the identified pathogen, the pathogenic potential was verified by a detached leaf test in the lab. A disease rating system was used to assess the disease severity (Table 1).

To determine the pathogenicity of the pure fungal pathogen on several chili plants, a pathogenicity assessment was carried out using chili plants. Following the application of the *Fusarium oxysporum* suspension, the onset of leaf yellowing became apparent within 48 hours of inoculation. The symptoms manifested as dark spots, progressing to the systemic condition of necrosis. Disease progression on the leaves on Petri plates was shown to be quite sharp after 12 days, with 80% of the leaf area affected in variety Zenia (Fig. 5). Upon assessing the percentage

of the affected area, it was established that the Zenia variety exhibited the highest susceptibility. The analysis of the infected area revealed that Green-queen demonstrated the highest resistance to *Fusarium oxysporum*. (Fig. 6).

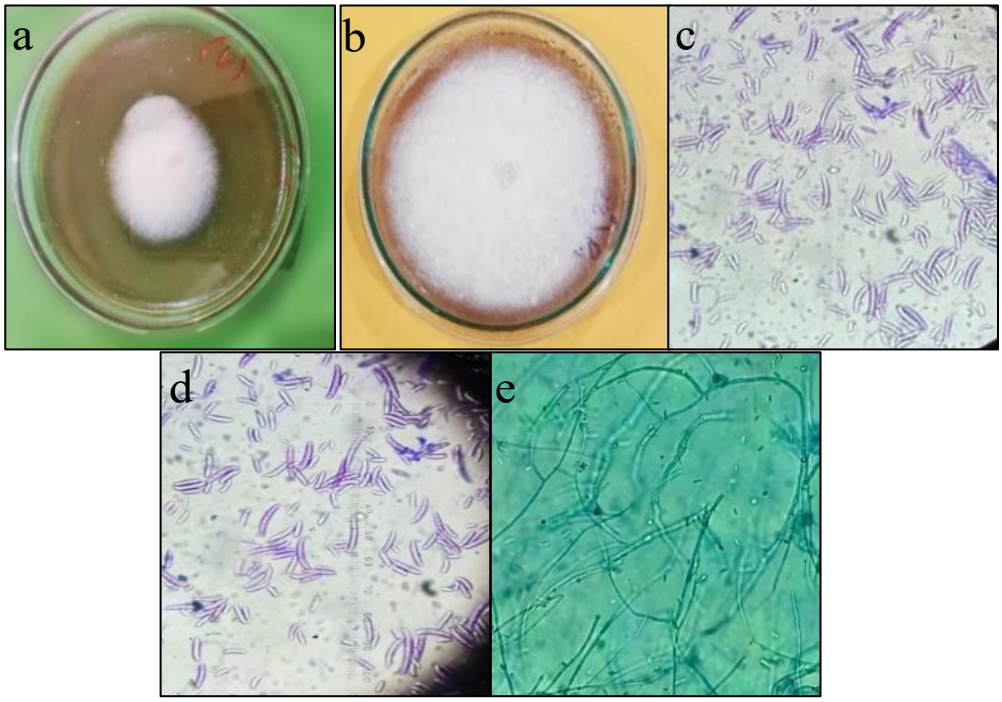

Figure 4: Cultural and Morphological characters of chili wilt pathogen *Fusarium oxysporum* (a): young colony from the front side; (b): mature colony from the front side; (c-d): microconidia and macroconidia under 40X magnification. (e): hyphae structure under 40X magnification.

Table 1: Disease Rating Scale

Infected Leaf Area (%)	Days after Inoculation	Status
0-20	1-2	Highly resistant
21-40	3-4	Resistant
41-60	5-6	Moderately susceptible
61-80	7-8	Susceptible
81-100	9-10	Highly susceptible

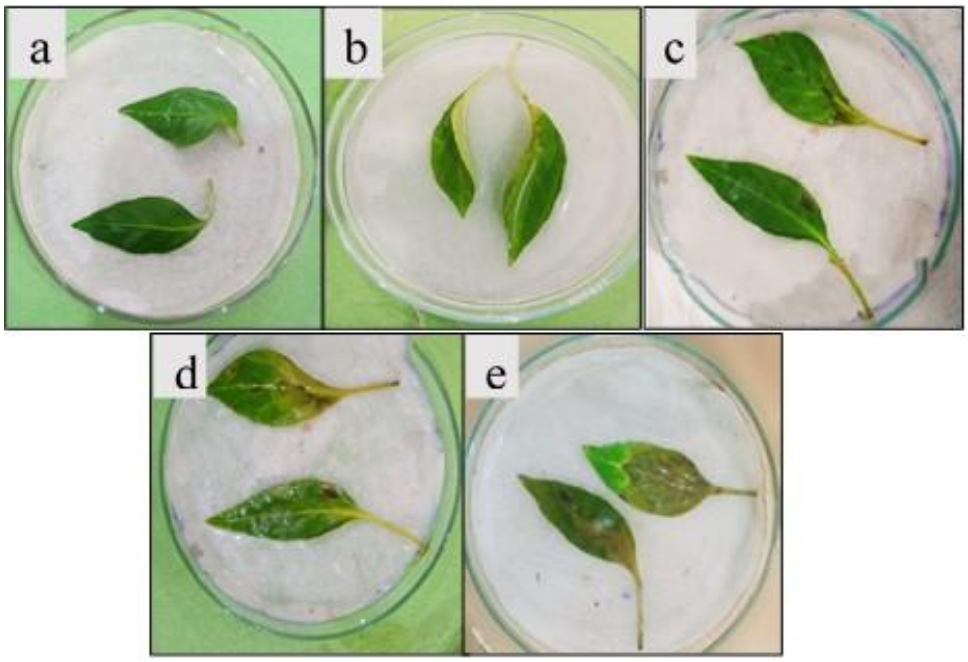

Figure 5: Detached leaf pathogenicity assay by *Fusarium oxysporum* on chili plants. a: Control Leaves; b-e: different stages of symptoms observed on leaves

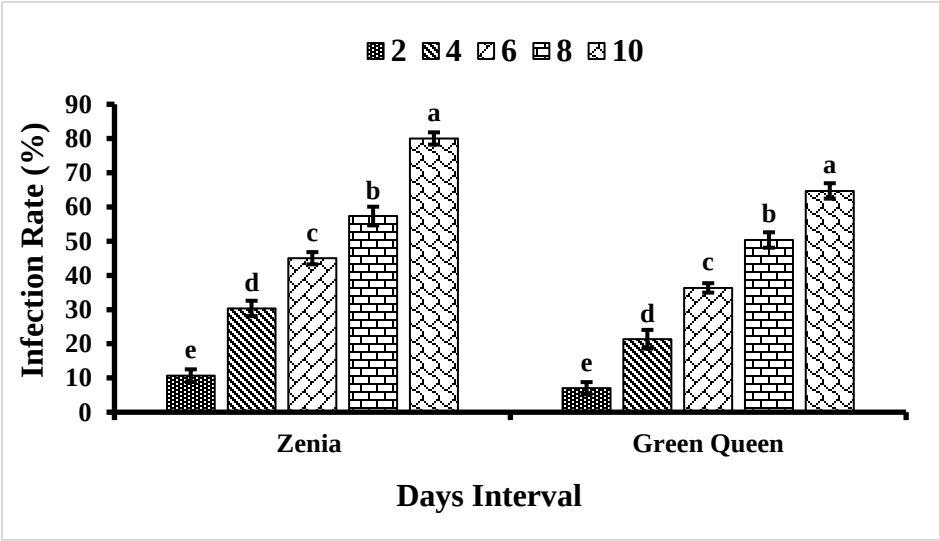

Figure 6: Disease progression curve for *Fusarium oxysporum* on represented chili plants based on the detached leaf method. Vertical bars show standard errors of three replicates. Values with different letters show a significant difference by ANOVA ($p \leq 0.05$) as determined by DSAASTAT software, LSD test at $p = 0.05$.

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

Pathogenicity Test in *in-vivo* Conditions: For the pathogenicity analysis in pot trials, seedlings were established by dispersing seeds in plastic pots. A subsequent pathogenicity test involved spraying 20-day-old chili plants of all selected varieties with a 5 mL spore suspension of the pathogen. Regular assessments were conducted on the plants to monitor the onset of disease symptoms in all types induced by *Fusarium oxysporum* (Fig. 7). The plant symptoms were discovered to be comparable to the disease samples previously collected. Yellowing of leaves was observed, succeeded by the development of dark brown lesions and complete necrosis, leading to the wilting of the entire plant. Analysis of data from pot experiments using *Fusarium oxysporum* as the tested pathogen revealed that *Fusarium oxysporum* induced the highest mortality across all chili cultivars, with the Zenia variety exhibiting the most severe infection at a 100% infection rate. Meanwhile, the Green Queen variety, identified as resistant, demonstrated approximately 68% disease severity against *Fusarium oxysporum* (Fig. 8).

0% Disease Severity	**20% Disease Severity**	**40% Disease Severity**	**60% Disease Severity**	**80% Disease Severity**
				
Healthy Plant	Yellowing started at the margin of the leaves	Plant shows Necrosis.	The plant shows reduced vigour and growth.	Wilting of the Whole Plant

Figure 7: Pictorial representation of disease rating scale of *Fusarium oxysporum* based on symptom development

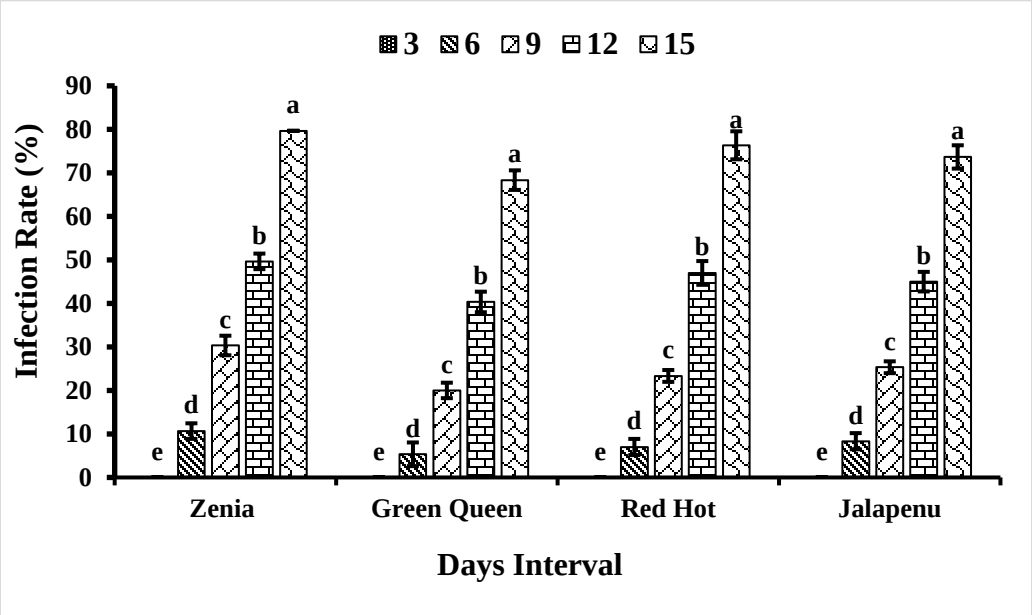

Figure 8: Disease severity of *Fusarium oxysporum* against four Chili varieties. Vertical bars show standard errors of three replicates. Values with different letters show a significant difference by ANOVA ($p \leq 0.05$) as determined by DSAASTAT software, LSD test at $p = 0.05$.

Discussion

Chili is one of the most economical crops which is widely cultivated on earth and commonly used as raw material for making chutneys and salads (Chen and Lin, 2019). Fungal infections are the most dangerous to chili output production (Tewksbury *et al*., 2008). Numerous fungal diseases affect chili plants and reduce yields, but the most common one that causes wilting is *fusarium* wilt, which is brought on by the *Fusarium oxysporum* (Bawa, 2016). It is vital to accurately identify the pathogen which is linked with wilting to manage *fusarium* wilt fungi in chili plants (Blaya *et al*., 2015). It is a valuable cash crop farmed for both domestic and international markets (Hill and Vigneri, 2014). In Punjab, Pakistan, this disease has been growing at an alarming rate and the growers facing significant losses in yield (Chauhan *et al*., 2012). As a result, the specific fungus causing wilting disease was emphasized in this investigation (Alabouvette *et al*., 2009).

Presently, an investigation into the prevalence, incidence, and severity of wilting disease is underway through a field study conducted in various villages of Tehsil Haroon Abad (139 6/r, 166 7/r, 138 6/r, and 425 6/r) in the Bahawalnagar district. A parallel study conducted by Shafique *et al*. (2022) focused on reporting the prevalence, incidence, and severity of leaf spot disease in spinach across four distinct regions during their survey. To effectively manage fungal diseases, the causative agent must first be accurately identified (McCartney *et al*., 2003). The most accurate traditional way for identifying diseases is by their morphology at the species level, however, mistakes occasionally do happen (Summerell *et al*., 2003). The isolated pathogen was identified in this study by evaluating its morphology, which included growth structure conidiophore and conidial size,

shape, and color among other things (Khodadadi *et al*., 2020).

The investigation aimed to assess the pathogenicity of the pathogen under study, employing both the detached leaf method and pot trials to observe the development of disease symptoms in chili plants. Upon visual inspection, it was evident that the disease symptoms induced by *Fusarium. oxysporum* were notably severe. Shafique *et al*. (2018) employed a detached leaf assay to validate the pathogenicity of *Alternaria arborescens* and *Phyllosticta ristolochiicola* in *Dracaena mariginata* and *Sonchus oleraceus* plants. In a similar vein, Mahmood (2010) utilized pot trials to assess the pathogenic potential of *Alternaria alternata* in tomato plants. The research findings indicated that among the tested varieties, the Zenia variety exhibited the highest susceptibility, while the Green Queen variety demonstrated the highest resistance to chili wilt. Additionally, *Fusarium oxysporum* was identified as a highly virulent pathogen responsible for causing chili wilt in both experimental trials. In their 2015 study, Shafique *et al*. investigated four strains of *Fusarium oxysporum f. sp. capsici* and examined their impact on ten chili varieties. The results indicated that, among the tested pathogens, strain B exhibited the highest level of pathogenicity across all the targeted crop varieties, the Sky Red variety demonstrated the highest susceptibility, whereas the Anchal variety exhibited the highest resistance against *Fusarium oxysporum* strain B.

CONCLUSION

According to the findings of the current study, it is concluded that *Fusarium oxysporum* is identified as the disease-causing pathogen of the chili and it causes great yield losses in the chili crop. This research will aid in the identification of the true biologic factor causing damage to crops, and suggests the development of a wide-ranging pathogen control approach that does not depend on fungicides and chemicals.

ACKNOWLEDGEMENTS

The fungal biotechnology research laboratory at the Department of Plant Pathology, Faculty of the Agricultural Sciences and the Plant Biotechnology laboratory at the School of the Biological Sciences University of Punjab assisted in this research.

CONFLICT OF INTEREST STATEMENT

The authors have no any financial or personal conflict of interest to declare.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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