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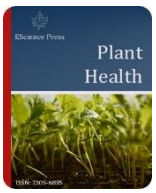

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Disease Surveillance and Pathogenic Variability of *Ascochyta Rabiei* causing Blight of Chickpea in Punjab Pakistan

[a]Muhammad Azhar Javed, [a]Yasir Mehmood*, [a]Amjad Shahzad Gondal, [a]Muhammad Sajid, [b]Muhammad Sheeraz, [c]Muhammad Irfan, [a]Syed Atif Hasan Naqvi, [a]Muhammad Abeer

[a] Department of Plant Pathology, Bahauddin Zakariya University Multan, Pakistan.
[b] Department of Agri. Business and Marketing, Bahauddin Zakariya University Multan, Pakistan.
[c] Department of Agronomy, Bahauddin Zakariya University Multan, Pakistan.

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ABSTRACT

Chickpea is an important legume crop and plays a significant role in global food security. Production is affected by many biotic and abiotic factors. The most significant biotic factor, *Ascochyta rabiei* is a fungal pathogen that causes Ascochyta blight in chickpeas, leading to substantial yield losses globally and is the primary cause in Pakistan. This pathogen causes heavy yield losses annually in the chickpea industry. The knowledge of the pathogenic variability of *A. rabiei* isolates can significantly impact the effectiveness of blight disease management strategies. A survey was conducted to collect blight samples to assess the pathogenic variability of A. rabiei isolates from different chickpea-growing regions of Punjab. The disease incidence and severity data were recorded using the quadrat square method in the fields. After isolation and purification, the pathogenicity test was conducted for conformation of the isolates. The virulence of ten isolates of *A. rabiei* was analyzed on six differential chickpea genotypes under artificial inoculation conditions in the glasshouse. The differential genotypes showed different levels of resistance and susceptibility to the isolates. Based on the reactions, the population of *A. rabiei* was grouped into different groups using a 1-9 rating scale. Only two isolates, BKR-01 and LAY-01, produced a high virulent effect against six germplasm. These isolates were collected from the Bhakkar and Layyah. The isolate MTN-1 were categorised as the least virulent. At the same time, the remaining isolates showed a moderate level of reaction.

Corresponding Author: Yasir Mahmood
Email: yasirmehmood@bzu.edu.pk

INTRODUCTION

Chickpea (*Cicerss arietinum L.)* belongs to the family *Fabaccea* and subfamily *Faboideae.* It is a self-pollinated leguminous crop and produces two seeds in each pod. It is annual a diploid crop with 12 chromosomes with genome of 740 Mb (Kayım *et al*., 2022). It is an important pulse crop in the world and is consumed in Asia and African countries (Merga and Haji, 2019). Chickpea is a significant source of great protein, carbohydrates and Minerals for animals and human food all through the chickpea developing regions all over the world (Adjou, *et al*., 2021). It provides protein to poor people and is also known as poor man's meat (Aslam, *et al*., 2021). Chickpea has an annual global production largely due to biotic and abiotic factors. One of the major limiting factors for chickpea production is Ascochyta blight.

Chickpea blight is cause by *Ascochyta rabiei* which is important foliar disease of chickpea worldwide that causes grain yield and quality losses of up to 100% (Duzdemir, *et al*., 2014). Humid, cool and cloudy climatic

are the most favorable condition for disease spread (Pande *et al*., 2011). The disease can cause 40-70% losses if optimum conditions last for 48 hours (Malik and Bashir, 1984). Under normal conditions, chickpea blight causes a 20–25% production loss annually (Jamil et al., 2010). Still, in Pakistan, epidemics of the disease have caused substantial output losses, prompting growers in Punjab's irrigated region to switch to other crops (Rubiales, *et al*., 2018). In Pakistan, the disease is severe in north-western region, major chickpea cultivated rain fed areas of Punjab Province, in which the districts include; Mianwali, Khushab, Muzafargarh, Rajan pur, Dera Ghazi Khan,Bhakkar, Chakwal, Faisalabad, Jhang and Layyah (Nawaz, *et al*., 2022). Pathogenic variability of *A. rabiei* has been reported by various researchers. Before this study, no systematic attempt was made to standardise differentials based on extensive scale screening of available genotypes or differentials used by other workers. Effectively managing Ascochyta blight proves challenging, and various control approaches exist ranging from cultural and chemical to genetic measures none guarantee a 100% success rate in controlling *A. rabiei* (Harveson, *et al*., 2013). Genetic resistance is the preferred tool for managing this disease, since it is an effective and inexpensive way of controlling biotic stresses. However, breeding for resistance to blight is difficult due to the frequent collapse of host plant resistance, possibly due to the variable nature of the pathogen (Sharma and Ghosh, 2016). Therefore, in order to identify stable and durable sources of resistance against the prevailing pathotypes of *A. rabiei* that occur in cultivated areas, it is very important to study pathogenic variability between *Ascochyta rabiei* isolates (Varshney *et al*., 2009). The current research aims to evaluate the aggressiveness of *Ascochyta rabiei* and resistance host both critical factors influencing chickpea blight incidence. Virulent isolates were collected from various blight-affected fields in Punjab. Understanding the pathogen's variability in population is vital in predicting blight incidence in chickpea crops. The knowledge about the population and available germplasm screening will provide valuable baseline information to develop disease management strategies.

MATERIALS AND METHODS

Survey and Field Assessment

A survey was carried out in six major regions of Punjab during the chickpea-growing season from December 2022 to March 2023 (Table 1). The primary objective of this survey was to measure the severity and incidence of chickpea blight disease. In each region, specific districts and locations were selected based on the presence of the disease. A combination of predefined distance criteria and the presence of the disease was utilised to select the fields for the survey. An efficient method was used to cover each sampling area, which included making stops at predetermined intervals along notable roads. The spacing between stops was adjusted based on the size of the sampling area and the availability of suitable chickpea fields.

Table 1. A survey of chickpea growing areas in Punjab was conducted during the 2022-23 cropping season.

District	Areas
Muzaffargarh	Chah Okab wala, Khakwani form
Multan	Ashiq wala, Musa Wala
Kot Adu	Chowk Munda, Jhaleren Shumali
Bhakkar	Azizabad(Chak 215 TDA), Mankera
Dera Ghazi khan	Choti Bala, Basti Notkani
Layyah	Chobara

Sample Selection

Samples of diseased plant specimens showing symptoms consistent with the disease were randomly selected from fields. This study assessed chickpea cultivation in six districts through a survey. The selection of survey fields was informed by various stakeholders, including farmers, local extension agents, and research institutions. Additionally, farmer cooperation, after being informed about the objectives of the study also played a role in field selection. The number of fields surveyed depended upon the abundance and occurrence of blight disease within each area. A systemic approach was employed where 10-20 plants were randomly selected while traversing diagonally through each field during data collection. The identification of blight disease in chickpea plants

relied on observing its visual symptoms. Plant samples were obtained from infected leaves, stems, flowers and pods to determine the disease's presence. Specifically, paper bags were utilised to collect, isolate and transport typical blighted specimens collected from distinct areas across a diagonal line within each selected field using a hierarchical sampling approach that encompassed ten plants at random (Shahjahan, *et al*., 2016). The gathered materials were subsequently subjected to further analysis in laboratory settings.

Pathogen Isolation

Samples of chickpea plants infected with Ascochyta blight were collected, along with their corresponding geographical coordinates, from the main chickpea growing districts of Punjab in both 2022 and 2023. Fifty samples were collected from ten fields across six Punjab districts (Table 2). To prepare these Ascochyta blight samples for analysis, all samples were initially surface sterilizated using the 1% sodium hypochlorite solution for 3-5 minutes. Subsequently, the samples were washed twice with sterilised distilled water. After this, they were dried on filter papers and directly positioned on the surface of acidified chickpea seed meal dextrose agar (40 g chickpea flour, 20 g dextrose, 20 g agar per litre of water). The culture plates were then placed in the incubator, set at a temperature of 20-22°C for 14 days, with alternating cycles of 12 hours of darkness and fluorescent light. Once the pycnidia were observed, they were collected using a sterilised needle and transferred to 1.5ml tubes filled with distilled water. Following this, they were vortexed and spread onto a 2% water agar medium. After two days, a single germinating spore was selected from the water agar and transferred to CSMA. Each isolate was subsequently cultured and multiplied on CSMA plates for further use.

Table 2. Different *Ascochyta rabiei* isolates along with geographical coordinates of the isolates.

Isolate	Source	Province	Latitude	Longitude
MTN-01	Ashiq wala	Punjab	30.362755°	71.358396°
MTN-02	Mosa wala	Punjab	30.351332°	71.324343°
MZG-01	Khakwani farm	Punjab	30.376455°	71.538396°
MZG-02	Chah okab wala	Punjab	30.332455°	71.838936°
BKR-01	Azizabad (Chak 215 TDA)	Punjab	31.355045°	71.274985°
BKR-02	Tehsil Mankera	Punjab	31.426021°	71.268303°
KTA-01	Chowk Munda	Punjab	30.479103°	71.232516°
KTA-02	Jhalaren Shumali	Punjab	30.299659°	71.363905°
DGK-01	Choti Bala	Punjab	29.810676°	70.24606°
LYA-01	Chobara	Punjab	32.406634°	74.788563°

Pathogenicity Test

A pathogenicity test was carried out on susceptible chickpea germplasm within controlled environmental conditions. Fifteen days old A.rabiei culture (10^5spore/ml) was used for inoculation. For the preparation of inoculum, fungal culture plate was filled with distilled water and macerated. Later, fungal culture material was passed through the muslin cloth and quantified with help of haemocytometer under the microscope and culture shifted into sprayer bottle for inoculation. The susceptible plants were inoculated, they were covered with a clear polyethylene sheet for a period of 48 hours, following which the sheet was taken off. To maintain high relative humidity, distilled water was sprayed on the plants and a soaked cotton plug was placed in the container until disease symptoms appeared. The assessment of disease symptoms was performed fifteen days post-inoculation, employing a rating scale ranging from 1 to 9 (Aslam *et al*., 2021). Re-isolation of the pathogen was done from the diseased plants to confirm the identity of the pathogen and establish the Koch's postulates.

Virulence Analysis

The virulence of ten *A. rabiei* isolates was assessed using a set of six chickpea germplasm that had been selected on the basis of resistant level analyzed from a prior screening experiment (Table 3). This evaluation was replicated three times and followed a completely randomized design. Six seeds were sown in each 10 cm diameter and 20 cm deep pot, filled with a sterile mixture of agro coir peat, clay soil, river sand, coarse sand, and quartz sand (1 kg/pot). Three replicates were

maintained for this setup. After germination, the plants were thinned down to three/pots. Subsequently, all the plants, except the control group, were inoculated as described in the previous section. The control plants were sprayed with distilled water only. The severity of the disease in each genotype was assessed 15 days after inoculation, utilizing a disease rating scale. The entire experiment adhered to a completely randomized design.

Table 3. Variety set used for virulence analysis of *Ascochyta rabiei* isolates.

Sr.	Variety	Reaction	References
1	Noor-2013	Highly susceptible	(Aslam, Shah *et al.* 2021).
2	Thall-2006	Susceptible	(Aslam, Shah *et al.* 2021).
3	Noor-2009	Moderately resistance	(Aslam, Shah *et al.* 2021).
4	TGK-1508	Susceptible	(Aslam, Shah *et al.* 2021).
5	Bittle-2016	Moderately resistance	(Aslam, Shah *et al.* 2021).
6	TG-1415	Susceptible	(Aslam, Shah *et al.* 2021).

Data Analysis

The disease severity was measured and converted to disease reactions using a standardized rating scale (Nene, 1981). Isolates with similar reactions on different genotypes were categorized as either resistant, moderately resistant, or susceptible. This classification helped determine the variability of *A. rabiei* based on the virulence behavior exhibited by these isolates.

RESULTS

The findings of a field survey carried out in Punjab to determine the occurrence of ascochyta blight disease in chickpea were presented. It is worth noting that despite having all-weather roads, access roads to these regions are inadequate due to their sandy soil composition and desert-like conditions suitable for cultivating only chickpea plants. The findings of the study indicated that there was a low occurrence of blight disease in the analyzed region of Punjab. Out of the twenty fields surveyed only ten exhibited symptoms of Ascochyta rabiei during the survey. The levels of ascochyta blight varied among districts but due to limited samples size in many areas. It was difficult to draw any definitive conclusion regarding these differences. Moreover, there was not enough data collected during this survey for statistical analysis purposes. During the 2022-2023 season, major chickpea growing regions experienced lower than averages rainfall conditions. The incidence of AB was noted solely in ten out of twenty fields, with an estimated incidence percentage ranging between 13 to 64.28%. Among all zones, Bhakkar exhibited the highest mean incidence rate with 64.28%. In Bhakkar and Muzaffargarh's analysis, AB's average frequency surpassed over half or more than 50 percent among the surveyed fields. Blight incidence was highest in Chak215 TDA and Ahmed form, with (64.28%) and (41%) respectively. According to recent research findings, Kot adu presented moderate disease incidence (36.84%), whereas Dera Ghazi Khan had a lower rate with only 15.5%. Additionally, Ashiq wala reflected an occurrence rate of 9.3%, accompanied by Musa wala at around 5%, Jhalaren Shumali reaching about 2.5%, and Okab wala presenting a minimal level at just over 35%. In particular, a high incidence roughly up to 40% affecting the Bhakkar district located within the Mankera region, showed evidence indicating that Blight disease has indeed spread considerably in this area as well. The current survey disclosed that in the area encompassing Chak- 294C Zone, ascochyta blight was widely distributed with a moderate degree of incidence. The disease severity of ascochyta blight was low in the fields and ranged from 10.9% to 36.8% (Table 4). The maximum disease severity was found in Bhakkar (36.8) and chak 210 TDA (17.9%). On the contrary, despite conducting a thorough survey of several regions, the severity of the disease was minimal in most areas of Multan. The disease severity in Muzaffargarh fields was up to 12.5%. The disease severity was observed in Ashiq wala, Musa wala and Dera haq with 14.5%, 10.9% and 11.98% respectively. The disease severity observed in Dera ghazi khan was up to 17.5%. The occurrence of disease in the Kot adu region was 15.7%, which was observed in Jhalaren Shumali. At the same time, disease severity in Layyah was 11.5% in the Chobara region. The pathogenicity test was conducted for the conformation of as Ascochyta rabiei causal organism of Ascochyta blight which produce typical symptom on the susceptible host. The disease rating scale showed 7-8

scale indicating the degree of virulence. The virulence of analysis of the isolates showed the varying degree of disease grade on the varieties set. Ten isolates reaction on six chickpea germplasm showed isolate variable reaction in term of symptoms development. Isolate BKR-02 and LAY-01 was proved to be highly virulent causing susceptible to highly susceptible reactions on all six chickpea germplasm. Meanwhile MTN-02 and BKR-01, proved to be virulent causing moderately susceptible to susceptible reaction on chickpea germplasm. Isolate (MTN-01) collected from Ashiq wala in Multan region was proved to be less virulent as compared to first two as it because MR to MS reaction on six genotypes (Table 5).

Figure 1. a) Ascochyta blight symptoms on pods b) Symptoms appeared on leaves in which plant death occurred and c) Symptoms appeared on stems.

Figure 2. Reactions of the differential genotypes of chickpea to different isolates of *Ascochyta rabiei*.

Table 4. Average disease incidence and severity of survey fields in Punjab (2022-23).

District	Area	No of field survey	Incidence	Severity
Multan	Ashiq Wala	3	29.6	14.5
	Musa wala	4	39	10.9
	Dera haq	2	13	11.98
Muzafargarh	Khakwani farm	2	40.90	12.5
	Chah okab wala	1	35	13
	Ahmed form	3	41	11.98
Bhakkar	Azizabad (Chak 215 TDA)	3	64.28	36.8
	Tehsil Mankera	4	45	13.8
	Chak 210 TDA	2	42.3	17.9
Kot Adu	Chowk Munda	2	36.84	13.6
	Jhalaren Shumali	3	26.31	15.7
	Rang Pur	2	33.3	12.5
Dera Ghazi khan	Choti Bala	3	26	17.5
	Basti Talpur	1	18.5	12.4
Layyah	Chobara	2	30	11.5

Table 5. Reaction of ten isolates against chickpea germplasm.

Sr.	District	Areas	Isolates	Reaction					
				Noor-2013	Thall-2006	Noor-2009	TGK-1508	Bittle-2016	TG-1415
1	Multan	Ashiq wala	MTN-01	MR	MS	MR	MS	MR	MR
2	Multan	Musa wala	MTN-02	MS	S	MS	S	S	HS
3	M.garh	Khakwani form	MZG-01	MR	MS	MR	MS	MR	MR
4	M.garh	Chah okab wala	MZG-02	MR	MS	R	S	MR	R
5	Bhakkar	Aziz abad Chak 215 TDA	BKR-01	MS	S	MS	S	S	HS
6	Bhakkar	Mankera	BKR-02	HS	S	HS	S	S	HS
7	Kot adu	Chowk Munda	KTA-01	MR	S	HS	HS	HS	MR
8	Kot adu	Jhaleren Shamali	KTA-02	S	S	S	S	HS	MR
9	DG.Khan	Choti Bala	DGK-01	HS	S	MS	HS	S	MS
10	Layyah	Chobara	LAY-01	HS	S	HS	S	S	HS

DISCUSSION

Understanding the pathogenic variability is important to cultivate the resistance varieties. In this current research the *A .rabiei* isolates obtained from various chickpea farming regions situated in Punjab were studied for their pathogenic diversity due to these areas being highly susceptible to ascochyta blight outbreak and being a hotspot for the disease mostly because of ideal environment conditions during cultivation periods (Khan *et al*., 1999). Field surveys were conducted in Punjab during the 2022-23 cropping season to assess the incidence and severity of the Ascochyta blight disease in chickpeas. This information was essential for developing integrated disease management strategies. The survey findings were consistent with previous reports, indicating that Ascochyta blight was a major problem in Punjab chickpea production areas. These results will be used to prioritize disease management efforts and develop new strategies for controlling Ascochyta blight. The survey was conducted in a certain area indicated that the incidence of disease ranged between13% to 64.28%, while the average severity varied from 10% to 17.7%. These findings were supported by another study, where three isolates were categorized into low, intermediate and highly virulent based on their pathogenicity test. There were varying degrees of susceptibility and resistance among the selected genotypes for virulence analysis which could account for these results. The isolates of *A. rabiei* exhibited varying reactions, leading to their

classification into distinct groups based on these differences. Each isolate was characterized by specific differential genotypes for identification purposes, with a total of isolates identified in his study (Chongo, *et al*., 2004a). The pathogen *A. rabiei* exhibited significant variability and had the ability to adapt into different pathotypes that specifically target certain variety of chickpea (Mahmood, *et al.*, 2019). The presence of the teleomorph stage contributes to the enhanced diversity observed in *A. rabiei* (Manjunatha *et al*., 2018). Understanding the pathogenic variability is important to cultivate the resistance varieties. In this current research the *A.rabiei* isolates obtained from various chickpea farming regions situated in Punjab were studied for their pathogenic diversity. Pathogenic variability was observed among the available ten isolates of when tested on a six germplasm with MTN-01 exhibited comparatively low virulence possibly due to the presence of weaker virulence genes. While isolates of LYA-01 and BKR-01 showed highly susceptible reactions on all six hosts. The isolates of *A. rabiei* exhibited varying reactions, leading to their classification into distinct groups based on these differences. The order of the *Ascochyta rabiei* was arranged according to virulence levels, from the least to the most virulent, which induced susceptibility in all genotypes tested, intermediate virulence was demonstrated by isolate KTA-01 and isolate KTA-02. The current findings are consistent with research conducted by previous scholars, who identified seven distinct pathotypes of *A. rabiei* based on reactions, however the samples size in our study was limited to only ten isolates. Prior studied have reported varying degrees of races (ranging from 2-12) worldwide for *A .rabiei* using differentials as testing procedures but comparison is challenging due to differences observed among cultivars and virulence levels within various isolate samples.

The determination of the pathotypes or races of *A. rabiei* can be difficult due to various factors such as the absence of standardized differentials and highly diverse populations. For instance, three pathotypes were identified in North Western Algeria (Benzohra, *et al*., 2011) and Pakistan using 11 chickpea lines, whereas four races/pathotypes were reported in Syria and Algeria while Turkey (Türkkan and Dolar, 2009) documented six races with seven chickpea cultivars being utilized for identification purposes (Farahani, *et al*., 2019). However, some studied like the one conducted by could not classify *A. rabiei* isolates into distinct categories because they lacked a standardized approach especially when working on strains that come from countries within West Iran's vicinity (Vafaei, *et al*., 2015). This study conducted utilized a sampling of ten isolates in Punjab where the disease is commonly observed due to favorable weather conditions. Previous research worldwide served as a basis for selecting the differentials, which were chosen based on variable reactions of common genotypes. These predominantly virulent and aggressive isolates pose a threat to chickpea production in Punjab. A similar observation was made by previous researchers who reported that there was an instance containing several aggressive isolates posing significant risks towards the Australian chickpea industry (Mehmood *et al*., 2017). This current research presents updated information about Pakistan's *Ascochyta rabiei* pathogenic variability and highlights its prevailing races, providing important insights regarding their existence (Ali, *et al*., 2009). In the near future, an analysis of virulence in *A. rabiei* isolates can be conducted using ten different chickpea genotypes. This information on pathogenic variation will help to choose proper control against prevailing isolates and being more cautious in areas where a virulent isolate is dominant. Additionally, this data is useful for plant breeders who seek to develop or improve resistance to specific pathotypes of *A. rabiei* within chickpea varieties. Without knowledge about pathogenic variability tracking changes that increase virulence over resistant cultivars proves challenging. Epidemiology and mechanisms of disease resistance must also undergo investigation for better disease management approaches against *A. rabiei* in chickpeas species.

CONCLUSION

Chickpea production in various regions of Punjab faces a significant threat from Ascochyta blight disease. In the 2022-23 growing season, an extensive survey was conducted to evaluate the incidence and severity of this disease in chickpea farming areas across Punjab. The survey revealed that Ascochyta blight disease had low incidence and severity levels. This study aimed to identify virulent isolates in the *A. rabiei* population. Which can be further used in chickpea resistant program.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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