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CM Bhaliya
Department of Plant Pathology, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

HJ Kapadiya
Department of Plant Pathology, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

JG Chavda
Department of Plant Pathology, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

DS Kelaiya
Department of Plant Pathology, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

RB Thanki
Department of Plant Pathology, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

Corresponding Author:
CM Bhaliya
Department of Plant Pathology, College of Agriculture, Junagadh Agricultural University, Junagadh, Gujarat, India

Inoculation techniques for assessing pathogenicity of *Fusarium* spp. and *Macrophomina phaseolina* on chickpea

CM Bhaliya, HJ Kapadiya, JG Chavda, DS Kelaiya and RB Thanki

Abstract
Chickpea (*Cicer arietinum* L.) is the second most important pulse crop grown in tropical, subtropical and temperate regions of the world. Among the different soil borne diseases, wilt and root rot complex caused by *Fusarium oxysporum* f. sp. *ciceris*, *F. solani* and *Macrophomina phaseolina* is the most important, devastating and challenging disease of chickpea. For the screening of any plants against any pathogens, a suitable inoculation method with appropriate inoculum density is essential. In present investigation, effect of different inoculation methods (soil inoculation and seed inoculation) on pathogenicity of *F. oxysporum* f. sp. *ciceris*, *F. solani* and *M. phaseolina* on chickpea were evaluated. All fungal pathogen isolate produce typical wilt and root rot complex disease symptoms in chickpea. The soil inoculation method with cent percent disease incidence was remain highly effective and the most suitable method to prove the pathogenicity of *F. oxysporum* f. sp. *ciceris*, *F. solani* and *M. phaseolina* in chickpea. In case of seed inoculation method, all the fungal pathogens exhibited drooping and yellowing of leaves followed by bark shredding and rooting of root in chickpea. *F. oxysporum* f. sp. *ciceris* was remain the most virulent pathogen among all the tested pathogen in seed inoculation method with 82.50 per cent disease incidence followed by *F. solani* (70.00%) and *M. phaseolina* (62.50%).

Keywords: *Chickpea*, *Fusarium*, *Inoculation*, *Macrophomina*, Root rot, Wilt

1. Introduction
Pulses popularly known as "*poor man's meat*" and "*rich man's vegetable*" play a vital role in resolving food and nutritional security challenges in India. Among the pulses, chickpea (*Cicer arietinum* L.) popularly known as bengal gram or chana is one of the important *rabi* pulse crop grown in temperate, sub-tropical and tropical climate throughout the world. Owing to high nutritional qualities of chickpea seed, it is consumed as dhal or a variety of snack foods, sweets and condiments. Green immature seeds of gram are used as vegetable while, husk and split beans are used as cattle feed.

The several biotic and abiotic stresses become the prime reason for its low productivity. Among the different biotic stresses, the damage caused by plant diseases is one of the major constraints. The chickpea crop is infected by 172 pathogens (67 fungi, 3 bacteria, 22 viruses, and 80 nematodes) in different parts of the world. Among them, wilt and root rot complex caused by *Fusarium oxysporum* f. sp. *ciceris*, *F. solani* and *Macrophomina phaseolina* is considered the most important, devastating and challenging disease that becomes major limiting factor in successful cultivation of chickpea crop in Saurashtra region of Gujarat state.

The characteristic symptoms of the wilt and root rot disease complex can develop at any stage of plant growth. Scattered drying of the plants occurs in the field, which is characterized shrinking of the collar region, drooping and chlorosis of petioles and leaves, internal discolouration of xylem vessels followed by rotting of tap roots with shredding of bark and lateral roots (Westerlund *et al*., 1974; Leslie and Summerell, 2006 and Nene *et al.*, 1991) [14, 7, 9]. The management of chickpea wilt and root rot complex is quite difficult to achieve, as pathogens are soil borne in nature and survive in the soil for many years even in the absence of host by producing resistant structures *i.e.* chlamydospores and sclerotia.

The proper inoculation techniques is the prime requirement to prove the pathogenicity and also to screen the germplasm for disease resistance. At present, number of inoculation techniques has been reported for soil borne pathogens such as root inoculation by dipping in conidial suspension, seed infestation method (Pande *et al.*, 2007) [11] and soil infestation method (Khan *et al*., 2004) [6].

The different methods of pathogen inoculation result in very different level of disease. The purpose of present study was to determine the best inoculation method for the pathogen *F. oxysporum* f.sp. *ciceris*, *F. solani* and *M. phaseolina* to establish optimum disease severity in chickpea plants.

2. Material and Method

2.1 Sample collection, isolation, purification and identification

The roving survey was conducted during *Rabi* 2020-21 in major chickpea growing districts of Saurashtra region for the collection of disease sample. The chickpea plants showing typical wilt and root rot complex symptoms were collected in paper bags separately, labelled properly and brought to the laboratory for isolation of the pathogen.

The standard tissue isolation procedure was followed for isolation of the pathogens (Tuite, 1969) [13]. The two species of *Fusarium* i.e. *Fusarium oxysporum* f.sp. *ciceris* and *F. solani* and *M. phaseolina* were isolated from infected sample which were purified by the hyphal tip method/single spore isolation technique and maintained on PDA slants for further investigations.

The identification of pathogens causing wilt and root rot complex of chickpea grown on PDA medium were identified based on their cultural and morphological characters. The genetic identification of isolated fungus was also carried out to identify the fungal pathogens at the species level.

2.2 Pathogenicity test

The pathogenicity was proved under net house conditions by artificial inoculation of *Fusarium oxysporum* f. sp. *ciceris*, *F. solani* and *M. phaseolina* separately by using susceptible cultivar JG 62 and following standard method of inoculation as mentioned below.

The black soil was blended with Farm Yard Manure (FYM) in a ratio of 3:1 by volume and steam sterilized for 2 hrs at 121^0 C and 1.2 kg/cm^2 pressure (15 psi) for three consecutive days before being placed into surface sterilized earthen pots for the investigation. The earthen pots of 30 cm diameter were washed thoroughly with tap water and disinfested with four per cent formaldehyde solution for two days. The surface sterilized earthen pot was filled with sterilized soil and FYM mixture @ 3 kg per pot (Bhaliya and Jadeja, 2013) [1].

For each methods of pathogenicity, five pots were prepared and one set of pot constituting five pots were filled with sterilized soil only. These pots were considered as uninoculated control. The pathogenicity test was performed by using representative isolates of each pathogen.

2.3 Inoculation techniques

2.3.1 Seed Inoculation

Ten apparently healthy surface-sterilized seeds were rolled separately on seven days old cultures of *F. oxysporum* f. sp. *ciceris*, *F. solani* and *M. phaseolina* thriving on PDA in Petri plates. These inoculated seeds were sown in earthen pots containing sterilized soil @ 10 seeds/pot. The uninoculated surface sterilized and healthy seeds were kept as a check. These pots were kept in the net house and watered as and when required. The observation for seed germination count was recorded after one week of sowing and the disease infection was recorded after 15 days of germination.

2.3.2 Soil Inoculation

The sick pot technique developed by Nene *et al.* (1981) [8] was followed for proving the pathogenicity. Autoclaved soil was filled in 30 cm earthen pots (3 kg/ pot) and inoculated separately with pathogens (*F. oxysporum* f.sp. *ciceris*, *F. solani* and *M. phaseolina*) multiplied on 100 g of 9:1 sand maize meal medium. The fungus grown on sand maize meal medium was added at 50 g/kg of soil. A check was also maintained without inoculum. After a week of colonization in soil, 10 seeds were sown in these pots.

These pots were kept in the net house and watered as and when required. The initial seedling emergence (germination percentage) was recorded and the disease incidence was recorded after 15 days of germination by using following formula (Parmar *et al.*, 2018) [12].

$$\text{Germination (\%)} = \frac{\text{Number of seed germinated}}{\text{Total number of seed sown}} \times 100$$

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plant}}{\text{Total number of plant observed}} \times 100$$

2.4 Re-isolation of the Test Fungus

The test fungus was re-isolated to confirm the Koch's postulates. The fungus was re-isolated from the infected plants showing typical symptoms by standard tissue isolation method and the identification of fungus was confirmed as per the original description. The culture obtained by re-isolation was transferred on PDA slants for comparison with original culture and further investigations.

2.5 Statistical analysis

The data obtained during the present investigations were subjected to statistical analysis by using analysis of variance technique. The standard methods of analysis of variance and completely randomized design were used in the experiments. The test of significance among the treatments was worked out by the 'F' test. The appropriate standard error of mean (S.Em.±) was computed in each case. For the treatment effects, which were found significant, the critical difference (CD) at 5 per cent level of probability was worked out to compare two treatment means. The effect of inoculation techniques of *F. oxysporum* f.sp. *ciceris*, *F. solani* and *M. phaseolina* were analyzed for each technique by using ANOVA followed by the Duncan Multiple Range Test (p<0.05).

3. Results and Discussion

The pathogenicity test of *F. oxysporum* f. sp. *ciceris*, *F. solani* and *M. phaseolina* was carried out by using *cv*. JG 62 by standard methods *i.e.* seed inoculation (Plate 1) and soil inoculation (Plate 2). The results of the study revealed that the diseases were successfully produced in both the tested methods (Table 1).

The disease incidence varied greatly in all treatments. The pathogen inoculation by all methods caused profound disease incidence (ranging from 62.50–100%) in chickpea plants inoculated with *F. oxysporum* f.sp. *ciceris*, *F. solani* and *M. phaseolina* as compared to the un-inoculated plants (control) which exhibited zero per cent mortality.

However, cent per cent disease incidence was recorded in soil inoculation method of all the tested pathogens followed by seed inoculation method which showed disease incidence in

the range of 62.50 to 82.50 per cent. The pathogen *F. oxysporum* f. sp. *ciceris* was remain the most virulent pathogen among all the tested pathogen in seed inoculation method with 82.50 per cent disease incidence followed by *F. solani* (70.00%) and *M. phaseolina* (62.50%).

Table 1: Effect of different inoculation methods on the seed germination and disease incidence of chickpea plants inoculated with wilt-root rot complex pathogens

Pathogens	Inoculation methods	Germination (%)	Disease Incidence (%)
F. oxysporum f. sp. *ciceris*	Soil inoculation	100.00 [a]	100.00[a]
	Seed inoculation	80.00 [b]	82.50[b]
	Control	100.00 [a]	0.00[c]
	S.Em.±	0.91	0.68
	C.D. at 5%	2.81	2.16
	C.V. %	2.19	2.51
F. solani	Soil inoculation	100.00 [a]	100.00[a]
	Seed inoculation	80.00 [b]	70.00[b]
	Control	100.00 [a]	0.00[c]
	S.Em.±	1.52	0.84
	C.D. at 5%	4.67	2.58
	C.V. %	3.63	3.30
M. phaseolina	Soil inoculation	100.00 [a]	100.00[a]
	Seed inoculation	80.00 [b]	62.50[b]
	Control	100.00 [a]	0.00[c]
	S.Em.±	1.11	0.61
	C.D. at 5%	3.42	1.87
	C.V. %	2.66	2.50

The least seed germination (80.00%) was recorded in the seed inoculation method. While soil inoculation method showed cent per cent seed germination in all the pathogens treatment.

In the case of *F. oxysporum* f. sp. *ciceris*, the visible typical wilt symptoms initially appeared after 25 days of inoculation. Symptoms manifested as drooping and yellowing of leaves, which later on withered and dried up. Finally, the whole plant gradually dried up. The plants of uninoculated control pots did not show any wilting symptoms and remained healthy in appearance.

The plants inoculated with *F. solani* exhibited typical symptoms after 20 days of inoculation. Affected plants turned yellow. The root portion became black, finer roots got shredded. While uninoculated control pots did not show any rotting symptoms and were healthy in appearance.

Concerning *M. phaseolina* inoculated plants, symptoms were initially expressed as drying without chlorosis of plant. Drooping of petioles and leaflet were observed at the very top of the plant. The collar region above the soil surface showed brownish, shredding of outer sheath of root and the plant could be easily pulled out confirmed its pathogenic nature producing the typical root rot symptoms. Whereas, uninoculated plants did not show any symptoms and remained healthy.

The reisolation was carried out separately on PDA from the roots of freshly infected plants collected from the pot and the cultures obtained were compared with original culture of *F. oxysporum* f. sp. *ciceris, F. solani* and *M. phaseolina*. The reisolated cultures of all the pathogens were similar to the original in all the respect. Thus, the pathogenicity of these isolates was confirmed by soil inoculation and seed inoculation method by employing Koch's postulates.

The pathogenicity test of these three pathogens reported here is in line with the findings of Biswas and Gupta (1981) [2], Kapoor and Sugha (1992) [4], Demirci *et al.* (1999) [3], Pandav (2002) [10], Zope (2005) [15] and Katariya *et al.* (2012) [5]. They reported that the soil inoculation method was the most effective method for proving pathogenicity in chickpea followed by

Biswas and Gupta (1981) [2] and Demirci *et al.* (1999) [3] confirmed the pathogenicity of *M. phaseolina, F. solani, F. oxysporum* and *S. rolfsii* by using sterilized soil inoculation method in pot trials under controlled environmental conditions and reported the symptoms of wilt and root rot complex in chickpea.

Kapoor and Sugha (1992) [4] reported that chickpea seedlings *cv*. JG 62 wilted within 20 days in laboratory conditions. Katariya *et al.* (2012) [5] also reported that *R. bataticola* from dry root rot of chickpea was most pathogenic in sterilized and unsterilized sandy loam soil.

Zope (2005) [15] also confirmed a pathogenicity test of wilt and root rot complex associated fungi *viz., F. oxysporum, S. rolfsii, R. solani* and *R. bataticola* isolated from infected chickpea plants through pot culture method.

It appeared that disease development was greatly dependent and influenced by the methods of inoculation. Among different methods of inoculation, maximum root infection by inoculated pathogen was recorded in plants inoculated by soil inoculation method followed by seed inoculation method.

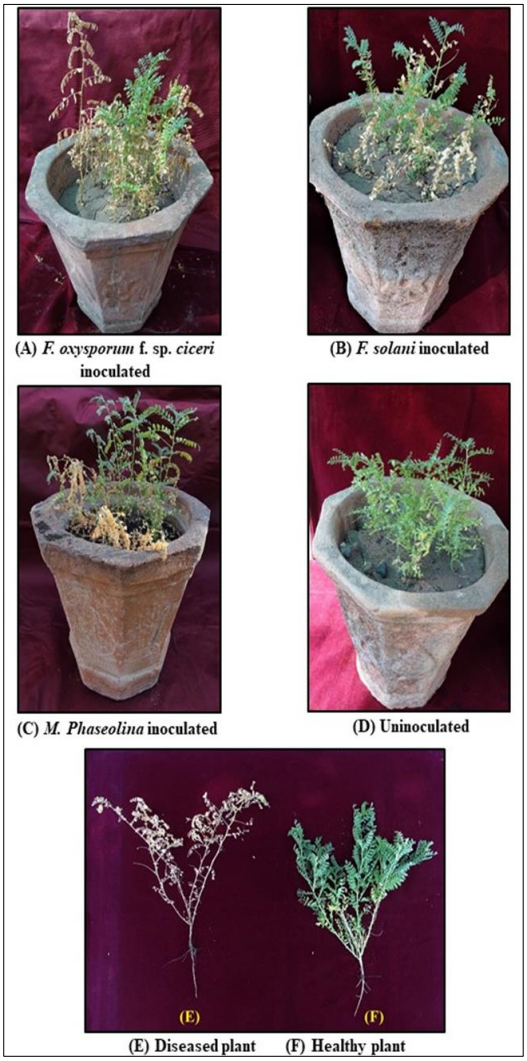

Plate 1: Pathology of wilt and root rot complex pathogens on chickpea by seed inoculation method

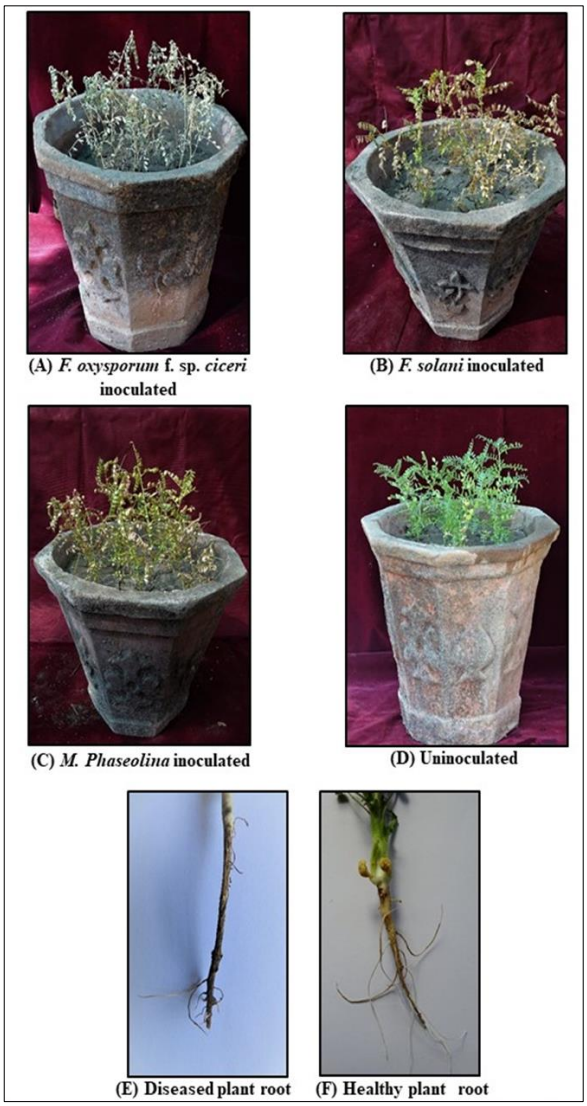

Plate 2: Pathogenicity of wilt and root rot complex pathogens on chickpea by soil inoculation method

4. Conclusions

The pathogenicity test confirmed the highly pathogenic nature of the test pathogen. The chickpea wilt-root rot complex pathogens *like as*, *F. oxysporum* f.sp. *ciceris*, *F. solani* and *M. phaseolina* causing considerable damage on artificially inoculated chickpea plants. The inoculation method of these pathogens influence the seed germination and pathogen infection of chickpea. Out of two different inoculation methods for pathogenicity test, the soil inoculation method was highly effective in establishing the *F. oxysporum* f.sp. *ciceris*, *F. solani* and *M. phaseolina* infection in chickpea plants. The disease was more pronounced in soil inoculation methods as compared seed inoculation methods.

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