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Pathogenicity test of different *M. phaeolina* isolates

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Abstract
Dry root rot of clusterbean incited by *Macrophomina phaseolina* (Tassi) Goid is one of the most severe yield destabilizing factors causing serious yield losses every year. In recent years, *Macrophomina phaseolina* is becoming more prevalent in agricultural areas where climate change is leading to increased temperatures. An experiment was conducted for fungal isolation, purification, identification of the pathogen and the pathogenicity test for all the twelve isolates proven by using three inoculation techniques. The significant variation in disease symptoms was noticed at 25 days after sowing in isolate MP-BKN to 45 days after sowing in isolate MP-UDZ. It was also revealed that the maximum disease incidence (60.35%) was recorded in seed cum soil inoculation technique followed by soil inoculation techniques (52.45%).

Keywords: Pathogenicity, test, isolation, *Macrophomina phaseolina*

Introduction
Clusterbean [*Cyamopsis tetragonoloba* (L.)], also known as guar, is deep rooted annual legume crop of family *Leguminosae* (*Fabaceae*) known for its drought and high-temperature tolerance (Kumar and Rodge, 2012) [7]. Clusterbean is used for various purposes, *viz.*, vegetable, cattle feed, and fodder or green manuring. Clusterbean is rich in protein and fiber content; hence, offering several health benefits in vegetable form and its tender green pods are also a cheap source of nutrients. Further clusterbean seeds are using as high protein cattle feed (Rai and Dharmatti, 2013) [12]. Seeds of clusterbean contain 22–33 per cent gum (Choudhary *et al.*, 2014) [1] and consist of three parts: the testa (14-17%), the endosperm (35-42%), and their germ (43-47%). Recently guar gum is gaining much commercial importance for its unique galactomannan content. Guar gum, extracted from guar seeds, is a natural hydrocolloid, soluble in water and forms a thick solution. It is used as a natural polysaccharide, viscosity builder in the fracking process of petroleum extraction, paper, mining, textile, cosmetics, pharmaceuticals, and food industries as thickener and stabilizer (Punia *et al.*, 2009) [10]. Apart from its commercial use, guar gum is used for pain-relieving, to get rid of gastric ulcers, reduced hyperglycemia, hypertension, cholesterol, weight loss, and reduce obesity with the fulfillment of diet (Sharma and Gummagolmath, 2012) [14]. Globally, India is the preeminent clusterbean producing country and covering about 80 per cent of production. Due to the heavy demand for guar gum in the international market, it is also being introduced under progressive growing areas like Andhra Pradesh, Tamil Nadu, Karnataka, Maharashtra, and Chhattisgarh. Among all the clusterbean producing states in India, Rajasthan is the largest clusterbean producing state because it dominates the Indian production scenario by contributing 70 per cent of its total production, followed by Haryana Gujarat.

The production of the clusterbean crop has been stagnant because of its cultivation under rainfed areas, marginal and sub-marginal lands, low soil fertility, and biotic stresses. Among biotic stresses diseases, insects, nematodes, and parasitic weeds are account for significant crop losses. The significant diseases of clusterbean are *Alternaria* blight, Anthracnose, Dry Root rot, Bacterial blight, and Powdery mildew. Among these diseases, dry root rot incited by *Macrophomina phaseolina* (Tassi) Goid has become a major biotic threat in several regions of the country and causes considerable economic yield losses. The disease, dry root rot is most evident during the reproductive phases of plant growth, although the fungus can be isolated from plant roots throughout the season (Bruton *et al.*, 1987). This disease is most ubiquitous under a hot, dry climate during the post-flowering period of the crop. A temperature of 30 °C is considered favourable for disease development. The most characteristic symptom includes drying of taproot and several dark pinhead sclerotia developments underneath the bark (Leach and Garber, 1970) [8].

Materials and Methods

Collection, Isolation, purification and pathogenicity test of the pathogen

Collection of diseased samples

Dry root rot affected plants of clusterbean were collected from surveyed areas of Rajasthan *viz.*, Bikaner, Churu, Jaipur, Jhunjhunu and sikar. Some samples were also collected from other than surveyed areas: Alwar, Hanumangarh, Jaisalmer, Jodhpur, Nagaur, Sri ganganagr, and Udaipur.

Isolation and purification of the pathogen

The pathogen *M. phaseolina* was isolated from the diseased roots of clusterbean plants showing the typical dry root rot symptoms by tissue segment method on Potato Dextrose Agar (PDA) medium. A single hyphal tip obtained the axenic cultures of the different isolates of the pathogen with 1 per cent sodium hypochlorite solution for 1 minute followed by three washings items with sterilized distilled water. The surface sterilized pieces were transferred aseptically on Potato Dextrose Agar (PDA) slants in a Laminar Air Flow Cabinet and incubated at 28±2°C temperature in a B.O.D. incubator for seven days. A single hyphal tip isolation technique was adopted to obtain a pure culture of *Macrophomina phaseolina* (Rangaswami and Mahadevan, 2004). A single piece of hypha was demarcated under low power objective of microscope (10X) and cut with the help of dummy objective. An individual piece of hypha was transferred aseptically on PDA slants with the help of an inoculating needle. The isolated fungus were identified on the basis of morphological and colony characters *viz*., white to grey colony, turning black with age, septate hyphae and blackish colour spherical, irregular and oblong type sclerotia as *Macrophomina phaseolina*. These cultures were observed under a microscope, and the stock cultures prepared in slants were kept in a refrigerator for further studies and isolates were differentiated on the basis of place of their collection and codes were given (Table1).

Table 1: *Macrophomina phaseolina* isolates used in present study

S. No.	Code	Place of collection of isolates
1	Mp-AWR	Agricultural Research Station, Navgaon, Alwar
2	Mp-BKN	Farmer's field-Beechwal, Bikaner
3	Mp-CUR	Farmer's field- Rajgarh, Churu
4	Mp-DPA	RARI, Durgapura
5	Mp-HMH	Agricultural Research Sub Station,Hanumangarh
6	Mp-JJN	Farmer's field, Aabusar, Jhunjhunu
7	Mp-JU	Agricultural Research Station, Mandor, Jodhpur
8	Mp-JSM	Farmer's Field, Sonu, Jaisalmer
9	Mp-NGO	College of Agriculture, Nagore
10	Mp-SIKR	Farmer's field, Lakshmangarh, Sikar
11	Mp-SNGR	ARS, SriGanganagar
12	Mp-UDZ	Rajasthan College of Agriculture, Udaipur

Mass multiplication of *M. phaseolina* inoculum for inoculation

The isolates of the pathogen were multiplied on sorghum grains (Kataria and Grover 1976) [6]. The sorghum grains were soaked overnight in ordinary water. The excess water was drained out. About 150 grams of soaked sorghum grains and 30 ml of water were taken in each 250 ml conical flask, plugged with cotton and sterilized in an autoclave at 1.045 kg/cm^2 pressure for 30 minutes. The substrate in flasks was inoculated aseptically with 7 day old mycelial discs (5 mm) of the pathogen and incubated for 20 days at 28 ± 2°C.

Pathogenicity test

For proving pathogenicity, isolated and purified *Macrophomina phaseolina* cultures were multiplied on sterilized sorghum grains and proved pathogenicity by following Koch's postulated and through seed, soil and seed cum soil inoculation technique. Soil collected from the field was autoclaved at 1.045 kg/cm^2 pressure for 30 minutes for three consecutive days. The pots were also surface sterilized by 2 per cent formalin solution before filling the soil inoculums mixture. The inoculum multiplied on sorghum grains was added in the earthen pots (30 cm diameter) @ rate of 100 g/kg soil and suitably moistened and allowed for 72 hours to stabilize the inocula before sowing of clusterbean seeds. About ten clusterbean surface sterilized seeds with 1 per cent sodium hypochlorite solution were sown in each pot with six replications and maintained in cage house with need based irrigation. The pots without inoculum were served as control. Observations on disease symptoms were recorded periodically. Re-isolation of the pathogen was made from infested seedlings, identified under microscopic observation, and the culture thus obtained was compared with that of the original culture (Radha krishanan and Sen, 1985) [11] and the pathogenicity (Koch's postulates) was proved.

Seed inoculation technique

For this, seeds were rolled on 7 days old culture of fungus thriving on PDA contained in Petri plates. The inoculated seeds were sown in pots. The un-inoculated apparently healthy seeds served as check. These pots were kept in cage house and watered regularly as and when required.

Soil inoculation technique

Prior to sowing, pots (30 cm diameter) were sterilized with copper sulphate solution and filled with sterilized soil + FYM (Soil: FYM=3:1; sterilized at 1.045 kg/cm2 for one hour for three consecutive days). These pots were inoculated with inoculum, multiplied on sorghum grains @ 20 g/pot. 10 apparently healthy and surface sterilized clusterbean seeds (RGC-986) were sown in each pot with four replications. Surface sterilized seeds sown in un-inoculated sterilized soil, served as check. These pots were kept in cage house and watered regularly as and when required and maintained under identical conditions.

Seed cum soil inoculation technique

In this technique, both techniques referred above were carried out together, and inoculum of the pathogen was applied in soil and seed were also rolled with seven days old culture of fungus and surface sterilized seeds were sown in uninoculated sterilized soil which served as check.

Results and Discussion

Collection, isolation, purification

Disease infected plants of clusterbean were collected in *Kharif* 2019, from different clusterbean growing areas of Rajasthan *viz.*, Beechwal (Bikaner) Rajgarh (Churu). Aabusar (Jhunjhunu), Lakshmangarh (Sikar), Research Farm, Rajasthan Agricultural Research Institute (RARI), Durgapura (Jaipur), Navgaon (Alwar), Agricultural Research Sub Station (Hanumangarh), Agricultural Research Station, Mandor (Jodhpur), Sonu (Jaisalmer), College of Agriculture (Nagore), Agricultural Research Station, (Sri Ganganagar) and Rajasthan College of Agriculture (Udaipur) and the infected plant samples were carefully placed in paper bags, properly

tagged and brought to the laboratory. The main aim of sample collection was to explore the possibility of variability among the dry root rot pathogen, *M. phaseolina*. Later the fungus was isolated from root tissues of clusterbean bearing fungal sclerotia and showing characteristic dry root rot symptoms. The samples were cut into small pieces, and surface sterilized with 1% sodium hypochlorite for one minutes, then rinsed thrice in sterilized distilled water. The pieces were placed on sterilized Potato Dextrose Agar medium (PDA) in Petri dishes and incubated at 28±2ºC for seven days. Later, the fungus was purified with the help of the hyphal tip cut method.

Identification of the pathogen

The visible morphological and cultural characteristics served as primary tool for identification of the isolated fungus. Initially the mycelium was white in colour later which was converted to dark brown to black in colour. Production of aerial mycelium was also observed in some isolates. The culture was also sent to ITCC, Division of Plant Pathology, IARI, New Delhi for further confirmation or identification of fungus. The fungus was identified as *Macrophomina phaseolina* with I.D. No. 11,344.

Pathogenicity test of *Macrophomina phaseolina*

A pot experiment was conducted to prove the pathogenicity test by following Koch's postulates in different isolates of *Macrophomina phaseolina* collected from different clusterbean growing regions of Rajasthan. Results of the study depicted in Table 2 showed that all the twelve isolates of *M. phaseolina* showed variation in their pathogenic behavior and also had a significant variation on appearance of disease symptoms, which started to emerge at 25 days after sowing in isolate MP-BKN to 45 days after sowing in isolate MP-UDZ. The ascending order sequence of dry root rot symptoms appearance (days after sowing) of these isolates on clusterbean crop were as Mp-BKN(25) < Mp-CUR (27) < Mp-JSM (30) < Mp-DPA (32) < Mp-JU (34) < Mp-SIKR (36) < Mp-NGO (37) < Mp-JJN (39) < Mp-AWR (40) < Mp-SNGR < (41) < Mp-HMH (43) Mp-UDZ (45). Results tabulated in (Table 4.3a and Fig. 4.3a) revealed that all the twelve isolates showed significant variation in disease incidence (21.36-75.55%) under pot condition. The isolate Mp-BKN showing 75.55 disease incidence was found most virulent followed by Mp-CUR (71.72%), Mp-JSM (66.66%), Mp-DPA (62.50%), Mp-JU (53.03%), Mp-SIKR (46.51%), Mp-NGO (40.65%), Mp-JJN (38.46%), Mp-AWR (31.37%), Mp-SGNR (26.51%), Mp-HMH (23.14%) and Mp-UDZ (21.36%). The highest disease incidence (75.55%) was recorded in the Mp-BKN isolate, and the least virulent isolate was Mp-UDZ with (21.36%) dry root rot incidence. Later the pathogenicity test was conducted by using seed, soil and seed cum soil inoculation techniques. The fungus was found pathogenic and showed the typical disease symptoms on the root parts of the infected plants within 30-40 days after sowing. The first sign of the disease was the sudden wilting of seedlings of clusterbean. The infected leaves become yellowish and started to drop out within two- three week, and black coloured sclerotia developed on roots and within roots.

Finally, the profuse growth of fungus in the root system resulted in complete blockage of the vascular system of the root and consequences of this blockage. Roots were decayed from the ground surface. The results are presented in Table 3, revealed that the maximum disease incidence (60.35%) was recorded in seed cum soil inoculation technique followed by soil inoculation technique (52.45%). Likewise, minimum seed germination (40 days after sowing) (68.25%) was recorded in seed cum soil inoculation followed by soil inoculation (78.33%), and maximum seed germination was in uninoculated control (95.30%).

Discussion

Isolation, purification and identification

Collected samples of dry root rot infected plants from different locations were isolated to study the variability. The samples were grown on Potato dextrose agar (PDA) medium and purified using the "hyphal tip technique" under the aseptic condition in the laboratory.

For convenience, the isolates of *M. phaseolina* were named Mp-AWR Mp-BKN, Mp-CUR, Mp-DPA, Mp-HMH, Mp-JJN, Mp-JSM, Mp-JU, Mp-NGO, Mp-SIKR, Mp-SNGR, Mp-UDZ based on their place of collection. The pathogen formed whitish mycelial growth on Potato Dextrose Agar later, which turned brownish-black in colour and resulted in the development of black hard sclerotia in the periphery of the colony. Based on cultural and morphological characteristics *viz.*, colony colour, formation of round, irregular and oblong type sclerotial fungus was identified as *Macrophomina phaseolina* later identification of the fungus also confirmed by ITCC, Division of Plant Pathology, IARI, New Delhi with I.D. No. 11,344. Similarly, Su *et al*. (2001) reported that the fungus *Macrophomina phaseolina* (Tassi) Goid causes dry root rot, charcoal rot, dry weather wilt, seedling blight and ashy stem blight disease in over 500 plant species. Dhingra and Sinclair 1977; Lodha *et al.* 1986, Diourte *et al*. 1995) [2, 9, 3] reported that *M. phaseolina* is a pathogen of clusterbean and several pulse crops.

Pathogenicity

A pot experiment was conducted to prove the pathogenicity of the isolated fungus by following Koch's postulates and three methods of inoculation, *i.e*. seed, soil and seed cum soil inoculation techniques. Pathogenicity test was proven for all the *M. phaseolina* isolates using clusterbean variety RGC-986 and all the isolates were found virulent in sterilized soil. The significant variation in disease symptoms was also noticed at 25 days after sowing in isolate MP-BKN to 45 days after sowing in isolate MP-UDZ. The highest mortality and disease incidence (75.55%) were recorded in Mp-BKN isolate and developed symptoms after 25 days of seed sowing followed by Mp-CUR with (71.72%), Mp-JSM (66.66%) and Mp-DPA (62.50%). The least virulent isolate was Mp-UDZ, with 21.36 per cent dry root rot incidence and developed symptoms after 45 days of sowing. Among the tested three methods of inoculation, the seed cum soil inoculation method was proved highly effective with the highest disease incidence (60.35%) and lower seed germination (68.25%), followed by soil inoculation and seed inoculation technique with (52.45% and 78.33%), (40.25% and 85.33%) disease incidence and germination per cent, respectively. To prove the pathogenicity, Hinguera (1991) studied three different inoculation methods, *i.e*. toothpick inoculation and inoculation using rice seeds colonized by *M. phaseolina* in cowpea. The results concluded that soil-borne inoculum multiplied on rice seeds was more effective in disease development in cowpea seedlings. This finding confirms the present investigation. Similar results regarding the present study also obtained by Jaiman and Jain (2008) [5] who

conducted a pathogenicity test for *M. phaseolina* in clusterbean. They observed that seed inoculation with *M. phaseolina* resulted in 33 per cent pre and post emergence mortality and 59 per cent less seed germination and vigour index (856) in comparison to control that was 93 per cent.

Table 2: Pathogenicity of twelve *M. phaseolina* isolates causing dry root rot of Cluserbean (RGC-986)

S. No.	Isolates	Germination (%)	Numbers of Infected plants	Symptom appearance (DAS)	Disease incidence (%)
1	Mp-AWR	85.00	08	40	31.37
2	Mp-BKN	75.00	17	25	75.55
3	Mp-CUR	79.00	17	27	71.72
4	Mp-DPA	80.00	15	32	62.50
5	Mp-HMH	72.00	05	43	23.14
6	Mp-JJN	78.00	09	39	38.46
7	Mp-JSM	80.00	16	30	66.66
8	Mp-JU	88.00	14	34	53.03
9	Mp-NGO	82.00	10	37	40.65
10	Mp-SIKR	86.00	12	36	46.51
11	Mp-SNGR	88.00	07	41	26.51
12	Mp-UDZ	78.00	05	45	21.36
		S.Em±		1.69	
		CD at 5%		4.82	
		CV(%)		4.90	

Table 3: Pathogenicity test of *M. phaseolina* in clusterbean by different Inoculation techniques

Inoculation technique	Seed germination (%)*	Per cent disease incidence
Soil inoculation	78.33(62.26)	52.45
Seed inoculation	85.33(67.78)	40.25
Seed + Soil inoculation	68.25(55.70)	60.35
Uninoculated control	95.30(77.48)	-
S.Em±	1.15	
CD 5%	3.55	

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