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RESEARCH ARTICLE :

Variability studies among *Phytophthora nicotianae* (=*P. parasitica*) (Dastur) waterhouse; isolates collected from seedling blight affected castor (*Ricinus communis* L.) fields of Telangana state, India

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SUMMARY : The present investigation was conducted on seedling blight of castor caused by *Phytophthora nicotianae* (=*P. parasitica*) at Department of Plant Pathology, College of Agriculture, Professor JayashankarTelangana State Agriculture University, Rajendranagar, Hyderabad and Indian Institute of Oil Seed Research, Rajendranagar, Hyderabad. Nine pathogen isolates were collected from diseased castor seedlings, from different castor growing region of Telangana State, India and were designated as P_1 to P_9.Pathogenicity of all the nine isolates was proved by Koch's postulate of which isolate P_3 was significantly virulent compared to all other isolates. Among the three media *viz*., carrot agar medium, corn meal agar medium and potato carrot agar medium, carrot agar medium showed greyish, white colour and fluffy to slight fluffy mycelium with good colony growth rate and mycelium abundance. Studies on morphological characteristics among nine isolates showed two types of sporangia and they differed as pear shape, papillate and globose, semipapillate sporangia. Results of molecular polymorphism using RAPD markers indicated that there was no polymorphism among isolates. Based on cultural, morphological and molecular characters some variation among the isolates of *Phytophthoranicotianae* was observed.

KEY WORDS :
Phytopthora, Cultural, Morphological, Molecular

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BACKGROUND AND OBJECTIVES

Castor (*Ricinus communis* L.) is the most important non edible oilseed crop of arid and semi-arid regions of India. India is largest producer of castor seed in the world and contributes 55-60 per cent of total global production.In India castor is being grown in area of 12.33 lakh ha with castor seed

production of 19.64 lakh t with average productivity of 1592 kg ha^{-1} during 2012-13.Seedling blight is common disease of castor in India which was first reported from Pusa, Bihar in moderate to severe form with seedling mortality of 30 to 40%. The disease is favoured by ill drained and prolonged rainy season (Dastur, 1913). The symptom of disease appears as circular, dull green patch on both the surface of the cotyledon leaves which later spreads and causes rotting. The infection moves to stem withers and causes death of seedling. In mature plants, the infection initially appears on the young leaves and spreads to petiole and stem causing black discoloration and severe defoliation.In India the disease is distributed in states like Uttar Pradesh, Andhra Pradesh, Telangana, Karnataka, Bihar and Gujarat (Vaheeduddin, 1947 and Vasudeva, 1959).The aim of following study was to determine whether variability in cultural characteristics, sporangial morphology and it was affected by molecular variability or not.

RESOURCES AND METHODS

Isolation and identification of the pathogen :

Diseased leaves showing typical symptoms of castor seedling blight were collected from different castor growing areas of Telangana State (Ranga Reddy, Mahaboobnagar, Nalgonda districts). These leaves were put in sterilized polythene bags and brought to the laboratory for isolation and identification of the organism involved.

Isolation of the pathogen :

Castor leaves showing typical symptoms of the disease were selected and washed with sterile water. Small bits of diseased tissue along with some healthy tissue were cut with the help of a sterile scalpel and surface sterilized with 1% sodium hypochlorite solution for 1 minute. The surface sterilized leaf bits were transferred aseptically into sterilized Petri dishes containing solidified carrot agar and incubated at 18±2^{0}C in incubator for mycelial growth. After 3 days of incubation mycelial growth was absorbed along with diseased leaf bits. Hyphal tips from the advancing mycelia were transferred to the carrot agar medium slants.

Identification of pathogen :

The isolated pathogen was identified as *Phytophthora nicotianae* based on its mycelial and sporangial characters through standard mycological keys (Waterhouse, 1963 and Hemmes, 1993) and by CMI descriptions.

Morphological characters :

The morphology of mycelium, sporangium, oospore of *Phytophthora*were studied in seven day old culture of each isolate grown on carrot agar medium and stained with 0.1 per cent lacto phenol cotton blue and observed under compound microscope (40X). Observations on size and shape of sporangium, presence of papillae, and number of sporangium per ml were recorded

Length, width and size of sporangium :

Morphological characters such as length, width and the size of sporangium (LxB) were measured by using micrometer.

Cultural characters :

Observation on colony colour, growth rate, was recorded at 24 hrs interval and recorded at 7 days after incubation at 18±2^{0}C. Characters like sporulation and colour of spore were observed and recorded at 10 days after incubation. The *P. nicotianae* isolates were tested on four different media *viz.* carrot agar medium, potato carrot agar medium, potato dextrose agar medium and corn meal agar medium for growth characteristics.

Colony diameter and growth rate per day :

Twenty ml of carrot potato agar medium was poured in sterilized Petri plates and allowed to solidify. Mycelial disc of 5mm diameter were cut from the margin of the 7 day old culture of *Phytophthora nicotianae* placed in the center of the Petri plate under aseptic conditions. The plates were incubated for 8 days in an incubator at 18±2^{0}C and the diameter of the colony was measured and recorded. Three replications of each isolate were maintained for the study. Growth rate per day of each isolate was calculated by dividing the colony diameter with number of days kept for incubation.

Pathogenic variability :

The pathogenicity test was conducted in pots under glass house. The castor varieties DCH-519, 48-1, GCH-4, Kranti and Haritha which were susceptible to seedling blight disease was raised in pots and 20 day old culture grown on CA broth suspension containing zoospores was

inoculated to castor plant at 3-5 leaf stage with agar bit inoculation method and covered with polythene covers.

Molecular variability :

Molecular variability among *Phytophthora* isolates were checked by using RAPD primers and dendogram obtained was represented to indicate the variability among the isolates. DNA of pathogen was isolated by using CTAB method (Murray and Thompson, 1980). *Phytophthora* specific primers were used which were purchased from Bio Science Company, Hyderabad. The length of the primers was 10 bp with a scale of 50 nm. Ten RAPD primers were used at random *viz.* 1OPBA, 9OPBA, 12OPBA, 3OPBB, 7OPBB, 15OPBB, 2OPBC, 5OPBC, 9OPBC and 2OPBD. DNA Thermal Cycle was programmed for the following parameters: 94ºC for 5 min, followed by 35 cycles of 94ºC for 1 min, 36ºC for 1 min, 72ºC for 1 min and a cycle of 72ºC for 5 min. Amplification products were separated by electrophoresis at 110 V for 2 h on ethidium bromide-stained (0.5 ug.mm^{-1}), 3% agarose gels run in 0.5 x Tris-borate EDTA buffer and visualized under UV light.

OBSERVATIONS AND ANALYSIS

Morphological and colony characteristics of the fungus are the important basic factors for identification of a fungus and its variability. The morphological characteristics such as colour of the colony, branching of sporangiophore, shape, size of sporangium, presence and absence of papillae were studied among the isolates of *Phytophthora nicotianae*. The isolates were morphologically characterized by measuring the shape, size, length and width of sporangia and hyphae at a magnification of 40X.

Morphological characteristics :

Sporangial characters :

Sporangial characters like size of sporangium, shape, presence or absence of papillae, variation in length, width and their ratio were studied on carrot agar medium for all the isolates of *Phytophthora nicotianae* using Olympus microscope at a magnification of 40X and the data pertaining to sporangial characters are indicated in Table 1.

Length of sporangium :

The mean sporangial length of nine isolates of *Phytophthora nicotianae* ranged from 19.1 µm to 22.3 µm. The maximum length of sporangia was recorded in isolate P_7 (22.3 µm) followed by P_3 (21.6 µm), P_8 (21.3 µm), which were on par with each other. Isolate P_9 recorded sporangial length of (20.9 µm) which was on par with isolate P_8 (21.3 µm), P_4 (20.8 µm), P_3 (21.6 µm), P_1 (20.1 µm) P_2 (19.8 µm) and P_6 (19.6 µm), followed by isolate P_5 with minimum length (19.1 µm) of sporangium.

Width of sporangium :

The mean width of nine isolates of *Phytophthora nicotianae* ranged from 11.7 µm to 14.8 µm. The maximum width of sporangium was recorded in isolate P_7 (14.8 µm), followed by isolate P_8 (13.7 µm), isolate P_3 (13.5 µm), isolate P_6 (13.2 µm) and isolate P_2 (13.1 µm), which were on par with each other. Isolate P_1 recorded sporangial width of (12.8 µm), followed by isolate P_9 (12.6 µm) and isolate P_4 (11.9 µm). Isolate P_5 recorded minimum sporangial width of 11.7 µm.

Length and width ratio :

Maximum length and width ratio of *Phytophthora nicotianae* isolates was observed in isolate P_4 (1.75) followed by P_9 (1.66), P_5 (1.63), P_3 (1.60), P_1 (1.57), P_8 (1.55), P_2 and P_7 (1.51), while, P_6 recorded minimum L: W ratio of 1.48.

Size of the sporangium :

The isolates of *Phytophthora nicotianae* showed variation in size of the sporangium. The sporangial size varied from 330.04 µm^2 to 223.47 µm^2. Maximum size

Table 1 : Length, width and size of sporangia of different isolates of *Phytophthora nicotianae*

Isolate	Length (µm)	Width (µm)	Size (µm^2)	L:B ratio
P_1	20.1	12.8	257.28	1.57
P_2	19.8	13.1	259.38	1.51
P_3	21.6	13.5	291.6	1.60
P_4	20.8	11.9	247.52	1.75
P_5	19.1	11.7	223.47	1.63
P_6	19.6	13.2	258.72	1.48
P_7	22.3	14.8	330.04	1.51
P_8	21.3	13.7	291.81	1.55
P_9	20.9	12.6	263.34	1.66
C.D. (P=0.05)	1.73	0.92		
C.V. (%)	4.85	4.09		

(330.04 µm²)was observed in isolate P_7, followed by P_8 (291.81 µm²), P_3 (291.6 µm²), P_9 (263.34 µm²), P_2 (259.38 µm²), P_6 (258.72 µm²), P_1 (257.28 µm²) and P_4 (247.52 µm²) while minimum size of the sporangium was recorded in isolate P_5 (223.47 µm²).

Shape of the sporangium :

Four isolates of *Phytophthora nicotianae* P_2, P_3, P_8 and P_9 produced globose, semi papillate to nonpapillate whereas the sporangium of isolate P_1, P_4, P_5, P_6 and P_7 were pear shaped and papillate.

Sporangiophore :

The sporangiophore of isolates were simple sympodial to branched sympodial. The hyphal swellings were present in all the nine isolates of *Phytophthora nicotianae* and the hyphal width ranged from 0.7 µm to 1.2 µm. The data on sporangial length and width and colony colour of present investigation is in agreement with Gupta *et al.* (2012), Alvarz *et al.* (2007) and Mounde *et al.* (2012).

Cultural characters :

Colony characters of nine isolates of *Phytophthora nicotianae* designated as P_1 to P_9 grown on different culture media *viz.* carrot agar medium, corn meal agar medium and potato carrot agar medium were used for recording the radial growth of the mycelium at seven days after inoculation (DAI), growth rate (mm/day). The data is presented in Table 2 and 3.

Colour of the colony :

The colour of the colony varied from greyish to white . Out of the nine isolates, the colour of the two isolates (P_5 and P_7) was white, whereas the colony of other seven isolates was greyish in colour.

Radial growth of isolates on different media :

The growth of the mycelium was measured at 24 hours duration upto seven days after inoculation on different culture media and a variation in radial growth of the pathogen was observed among the isolates and the data are presented in Table 2.

Significant maximum growth was observed on carrot agar media in isolate P_7 (75.0 mm), followed by isolate P_3 (64.5 mm), isolate P_9 (58.0 mm) and isolate P_2 (52.5 mm), and isolate P_6 (50.0 mm). The isolate P_5 recorded a radial growth of 47.5 mm, followed by isolate P_4 and isolate P_1 which were on par with each other with a radial growth of 46.5 mm and 46.0 mm, respectively. Isolate P_8 recorded a minimum growth of 45.0 mm. Maximum radial growth of 43.5 mm was observed on corn meal agar medium in isolate P_7 while minimum growth was observed in isolate P_1 (24.5 mm). Isolate P_5 (42.5 mm) and P_2 (42.0 mm) were on par with each other. Isolate P_6 recorded a radial growth of 38.0 mm, followed by isolate P_8 (34.0 mm) and P_3 (31.0 mm). Isolate P_9 and P_4 showed similar growth of 30.0 mm and 29.5 mm, respectively.On potato carrot agar media significant maximum growth of 63.5 mm was observed in isolate P_7 followed by isolate P_2 and isolate P_6 with a radial growth of 54.5 mm. Isolate P_5 recorded a radial growth of 48.0 mm whereas isolate P_4 showed a growth of 30.0 mm followed by isolate P_9 (28.0 mm), isolate P_1 (25.0 mm) and isolate P_8 (24.0 mm). However the isolate P_3 recorded a minimum radial growth of 20.0 mm.

It was observed that among the three media tested, carrot agar medium supported maximum growth when compared to corn meal agar medium and potato carrot agar medium. Further among the nine isolates tested on three different media, isolate P_7 recorded significantly maximum growth.

Similar results were obtained by Singh and Dubey (2005) who studied the cultural characters of nine different isolates of *P. drechsleri* f. sp. *cajani* on corn meal agar medium. Based on mycelial growth, isolates were grouped into two categories *i.e.* fast and slow growing. Fast growing isolates had thicker mycelium and

Table 2 : Effect of media onradial growth of the *Phytophthora nicotianae* isolates

Isolates	Radial growth (mm) of *P. nicotianae* on different media		
	CA	CMA	PCA
P_1	46.0	24.5	25.0
P_2	52.5	42.0	54.5
P_3	64.5	31.0	20.0
P_4	46.5	29.5	30.0
P_5	47.5	42.5	48.0
P_6	50.0	38.0	54.5
P_7	75.0	43.5	63.5
P_8	45.0	34.0	24.0
P_9	58.0	30.0	28.0
C.D. (P=0.05)	4.20	2.29	3.31
C.V. (%)	4.50	3.78	4.96

higher L:B-ratio of sporangia. The results indicated the existence of morphological variability in the isolates of *P. drechsleri*f. sp. *cajani* collected. Padmaja (2013) also suggested that carrot agar medium is best among six different media used for abundant mycelium growth and sporangial production.

Rao *et al.* (1962) studied cultural characteristics of *Phytophthora parasitica* Dastur Var. *macrospora Ashby* which caused fruit rot disease of *Anonasquamosa*. The organism was grown on different types of both synthetic and non-synthetic solid media. They concluded that the *Phytophthora* isolate made best growth on oat meal, corn meal, lima-bean and rice meal agar with the production of abundant fluffy filamentous cottony-white aerial mycelium.

Colony growth rate :

All the nine isolates of *Phytophthora nicotianae* differed in growth rate (mm day^{-1}) of the colony and it ranged from 6.43 to 10.71 mm day^{-1} (Table 3). The isolate P_7 recorded mean maximum growth rate of 10.71 mm day^{-1}, followed by isolate P_3 (9.21 mm day^{-1}), isolate P_9 (8.29 mm day^{-1}). Isolates P_2 andisolate P_6 recorded a similar growth rate of 7.50 mm $day^{-1}$and $^{7.14}$ mm day^{-1}, respectively. It was also observed that the Isolate P_5, isolate P_4, and isolate P_1 recorded a radial growth of 6.79 mm day^{-1}, 6.64 mm day^{-1}, 6.57 mm day^{-1}), respectively. Minimum growth rate of 6.43 mm day^{-1} was recorded in isolate P_8.

Colony texture :

The isolates of *Phytophthora nicotianae* showed different types of colony texture on different media. Based on the texture and appearance of the colony, the isolates were categorized into two group's *i.e.* slightly fluffy and fluffy appearance of the colony on carrot agar medium. The isolates P_5 and P_7 produced fluffy colony whereas all other isolates produced slightly fluffy colony.

Mounde *et al.* (2012) characterized and identified *Phytophthora* species associated with citrus gummosis based on cultural traits as finely radiate, white rosette and slightly cottony colonies. The morphological characteristics of *P. nicotianae* mycelium were dense or loose rosette, with no pattern, spreading and arachnoid aerial.

Pathogenic variability of *Phytophthora nicotianae* isolates on different castor varities :

The pathogenicity test was conducted in pots in glasshouse as described under materials and methods (3.4.3). The castor varietiesDCS-107, 48-1, GCH-4, Kranti and Haritha were sown in pots and 10-day-old culture grown on CA containing zoospores was inoculated on leaves of 20 day old castor seedlings by agar bit inoculation method. The plants showed typical symptoms of blight after 7 days of inoculation. The data revealed that the isolate P_3 recorded maximum mean disease severity of 42.4 per cent followed by P_9 (36.0), P_8 (33.0) and P_7 (31.08) while minimum (29.6) was recorded in isolate P_2. Among the isolates the isolate P_3 was identified as most virulent followed by isolate P_9 which was on statistically at par with isolate P_8 followed by isolate P_7 which was at par with P_8 and P_2. However, it was observed that four isolates *viz.*, P_1, P_4, P_5 and P_6 failed to produce symptoms in any of the variety tested (Table 4). The highly virulent isolate (P_3) was used for

Table 3 : Cultural characteristics of different isolates of *Phytophthora nicotianae*

Name of the isolate	Colour of the colony	Radial growth of the colony (mm)	Growth rate/day(mm)	Texture of colony	Abundance of mycelium
P_1	Greyish	46.0	6.57	Slightly fluffy	Sparse
P_2	Greyish	52.5	7.50	Slightly fluffy	Sparse
P_3	Greyish	64.5	9.21	Slightly fluffy	Sparse
P_4	Greyish	46.5	6.64	Slightly fluffy	Sparse
P_5	White	47.5	6.79	Fluffy	Profuse
P_6	Greyish	50.0	7.14	Slightly fluffy	Sparse
P_7	White	75.0	10.71	Fluffy	Profuse
P_8	Greyish	45.0	6.43	Slightly fluffy	Sparse
P_9	Greyish	58.0	8.29	Slightly fluffy	Sparse
C.D.(P=0.05)			0.60		
C.V. (%)			4.50		

further studies.

Saadoun and Allagui (2008) grouped 15 different isolates of *Phytophthora nicotianae* into four pathogenic groups based upon pathogenic variability tested against six pepper (*Capsicum annuum* L.) varieties at the seedling stage. The first included the weakly virulent isolate Pnt317, which was able to attack only cvs. Beldi and Baker. Isolate Pnt341 constituted the second group which was more pathogenic than the first, being able to attack also cv. D'HIRAT. Isolates Pnt314, Pnt323 and Pnt326 formed the third group, characterized by a high pathogenic power on cvs. Beldi, Baker, D'hirat and Nabeul II. The fourth group, which comprised the widest isolate range was the most pathogenic since it attacked all the local varieties but not CM 334.

Molecular variability :

Screening of ten decamer oligonucleotide RAPD primers revealed that only three RAPD primers (12OPBA, 3OPBB and 2OPBC) could yield informative (polymorph), strong and reproducible DNA amplicons (bands) of *P. nicotianae* isolates by PCR. All the three RAPD primers generated 13 polymorphic bands. When fingerprints of these isolates were compared, some bands common to all isolates was observed, while others were unique to one or a few isolates. All of the RAPD bands produced by three primers in the five isolates of *Phytophthora nicotianae* were subjected to hierarchical cluster analysis based on the principle of UPGMA and a dendrogram was generated.

The similarity co-efficient ranged from 0.24 to 1.0, indicating that two isolates were 100% similar (Table 5). The highest similarity co-efficient (1.0) was between isolates P_1 and P_3. The UPGMA cluster analysis grouped the isolates into 2 categories showing less magnitude of genetic diversity among the isolates of *Phytophthora nicotianae*. Cluster I consisted of isolates namely P_1, P_2, P_3 and P_5, respectively. Cluster II consisted of only one isolate P_4. Both these clusters had the isolates from different locations and hosts. These isolates were not much dissimilar in phenotypic characters indicating that relationship exists between RAPD profile and the cultural and morphological characters.

Similarly, Ning and Zhang (2001) conducted Random amplified polymorphic DNA analysis of China isolates of *Phytophthora parasitica* var.*nicotianae* which were collected from different region of China. All the tested isolates differentiated into four cluster groups. Each cluster group contained different pathogenic group did not belong to the same cluster group. There was no significant different in RAPD pattern among highly virulence, intermediate virulence and weakly virulence groups in China. Therefore, the similarities and difference in banding patterns by RAPD could not be a useful molecular tool in identification and phylogenetic studies of the PGS of the same pathogen from the different

Table 4 : Pathogenic variability of *Phytophthora nicotianae* isolates on different castorvarieties

Phytophthora Isolates	Disease severity (%)					Mean
	Castor varieties					
	DCS-107	48-1	GCH-4	Kranti	Haritha	
P_2	30.0 (33.18)*	40.0 (39.21)*	8.0 (16.40)*	30.0 (33.17)*	40.0 (39.21)*	29.6 (32.93)*
P_3	58.0 (49.60)	46.0 (42.68)	18.0 (25.05)	35.0 (36.25)	55.0 (47.85)	42.4 (40.61)
P_7	40.4 (39.45)	35.0 (36.25)	10.0 (17.38)	25.0 (29.97)	45.0 (42.11)	31.08 (33.84)
P_8	50.0 (44.98)	35.0 (36.26)	10.0 (18.41)	30.0 (33.18)	40.0 (39.21)	33 (35.04)
P_9	55.0 (47.85)	40.0 (39.21)	10.0 (18.32)	30.0 (33.18)	45.0 (42.11)	36 (36.85)
C.D. (P=0.05)	3.28	3.18	3.40	3.33	2.83	3.30
C.V. (%)	4.14	4.29	9.64	5.45	3.65	5.00

* Figures in parenthesis are angular transformed values.

Table 5 : Genetic similarity co-efficient matrix for *Phytophthora nicotianae* isolates based on RAPD profile

	P_1	P_2	P_3	P_4	P_5
P_1	1.0000000				
P_2	0.5454545	1.0000000			
P_3	1.0000000	0.5454545	1.0000000		
P_4	0.3333333	0.1818182	0.3333333	1.0000000	
P_5	0.3636364	0.6000000	0.3636364	0.1000000	1.0000000

tobacco districts.

Conclusion :

Among the sporangial characters differences were observed between isolates of *Phythophthora nicotianae* in respect to size of sporangium, length, width and their ratio. Among the different media tested, maximum growth of the pathogen was recorded on Carrot Agar (75 mm), followed by Potato Carrot Agar medium (63.5 mm), Based upon result isolate P_3 considered as most virulent and used for further studies. Molecular variability studies among the pathogen isolates did not show much variation in the RAPD polymorphism analysis. In respect of cultural and morphological characters also they were mostly similar

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