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Illustration of Key Morphological Characteristics of *Phytophthora cajani*-Pathogen of Phytophthora Blight of Pigeonpea

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ABSTRACT

Background: *Phytophthora cajani* causing the Phytophthora blight (PB) disease of pigeonpea. The disease will rampant during excessive rainfall coupled with hot and humid weather during the cropping season. The present study on micro and macro morphological characteristics can contribute to the identification and specification of biology of *Phytophthora* spp. There are no detailed studies concerning the characterization of the *P. cajani* are available with this backdrop the present investigation was taken.

Methods: *Phytophthora cajani* was isolated on V-8 PARP medium, whereas stimulation of zoospores and sporangia was done using the diluted tomato juice broth. Micro and macro morphological characteristics of *P. cajani* were studied using micrometry and Olympus CX41 phase-contrast microscope.

Result: The pathogen was homothallic with amphigynous antheridium and oogonium and able to produce oospore *in vitro*. Sporangium was nonpapillate, noncaducous, oviod-obpyriform shape. Further, the macro morphological characteristics like mycelial radial growth and colony type were studied. The colony characteristics were dull white, flat and rosette pattern. Other culture characteristics like optimum temperature and RH were mostly consistent with those reported former.

Key words: Morphological characterization, Physiological characterization, *Phytophthora cajani*, Sporangia, Zoospores.

INTRODUCTION

Pigeonpea (*Cajanus cajan* L.), one of the protein-rich food legumes of the semi-arid tropics grown throughout the tropical and subtropical regions of the world. The total acreage of the crop is 5.41 million ha with an annual production of 4.49 m tonnes and India alone contributes 72.5% of world cultivated area with 62.5% of world production (FAO, 2016). Indian subcontinent being the predominant country where, the crop is extensively grown in area lying between 14° and 28°N latitude, (Pramod *et al*. 2010). The crop occupies an area of 3.88 million ha with an annual production of 2.85 million tonnes in the country. The crop is reportedly a drought hardy with potential to tolerate vagaries of harsh environment. Despite being its hardy nature, it is being debilitated by an array of harmful microbial pathogens posing serious repercussions on yield and quality of the produce. Of the diseases reported be threatening the production, wilt and sterility mosaic diseases were important. However, the other important disease reported earlier is Phytophthora blight disease incited by *Phytophthora cajani.* The disease was first reported during 1966 by Williams *et al*. (1968). Presently, the disease has spread to most pigeonpea growing areas in Asia (Pal *et al*. 1970), Africa, America (Kannaiyan *et al*. 1984), Australia (Wearing and Birch, 1988), Dominican Republic, Kenya, Panama and Puerto Rico (Nene *et al*. 1996). A few years after the initial occurrence, disease came to halt (Kannaiyan *et al*. (1984). In the recent past, an alarming resurgence of PB in pigeonpea was observed irrespective of cropping system, soil types and cultivars especially when excessive rains fall within a short span of time and hot and humid weather persists during the crop season (Pande *et al*. 2011).

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The disease being persistently spreading nature calls for an urgent management practices to devised. However, the crucial need in devising management practice is the thorough understanding the biology of pathogen and also the exact identification up to species level. Traditional taxonomy of Phytophthora species was based on morphological characteristics as exemplified by the classic morphological key by Waterhouse (1963), which separated the genus into six groups and is still widely used today. Species identification was based primarily on morphological features of sporangia, antheridia, oogonia, oospore and chlamydospores along with other criteria like cardinal growth temperature, growth rate, colony morphology in culture

media and mating behaviour (Stamps *et al*. 1990). Various studies based on laboratory tests have pointed out the influence of culture medium (Duncan, 1988), temperature (Phillips and Weste, 1985) on variability in Phytophthora growth and formation of its reproductive structures. Information on mycelial growth, characteristics of oospores and sporangia, differences in size and shape of reproductive structures can contribute to identification and specification of biology of *Phytophthora* spp. in specific conditions (Bernadovicova and Juhasova, 2005).

There is no detailed studies concerning characterization of the *P. cajani* are available with this backdrop of this information, the present investigation was contemplated to elucidate the detailed morphology and biology of pathogen *Phytopthora cajani.*

MATERIALS AND METHODS

Fungal culture

Pigeonpea plants showing the typical symptoms of Phytophthora blight were collected from the pigeonpea fields of ICRISAT, Patancheru, Hyderabad. The isolation of pathogen was done according to tissue segment method (Rangaswamy, 1958). Stem bits consisting of 50 per cent infected and 50 per cent healthy were surface sterilized using 1 per cent sodium hypochlorite (NaOCl) for 60 seconds and then washed in sterile water thrice. The stem bits were blot dried and plated on petri plate containing V8 juice agar media (Himedia, Mumbai, India) amended with PARP antibiotics (pimarcin 400 µL; ampicillin 250 mg; rifampicin 1000 µL; and pentachloronitrobenzine 5 mlL^{-1} media). Plates were incubated at 30°C in the 12 h/12 h day-night photoperiod for 5 days. Putative Phytophthora colonies were selected and confirmed by cultural and morphological characteristics as described by (Erwin and Ribeiro, 1996). Morphology of the fungus was observed under Olympus CX41 phase contrast microscope with Q image micropublisher 5.0 RTV digital camera.

Morphological characteristics

Micro morphology

Micro morphological character of pathogen was studied by means of sporangium induction methodology as described above and images and micrometry observations were taken by using Olympus CX41 phase contrast microscope with Q image micropublisher 5.0 RTV digital camera.

Macro morphology

Colony pattern

Twelve solid culture were prepared according to the manufacturer instructions (HiMedia, India) and compositions (Dhingra and Sinclair, 1995). Disc of a 6 mm diameter fungus were taken from 5 day old pre-cultured petri plates on 20% tomato extract agar with the help of a cork borer and inoculated to petri plates and incubated at 30°C in the 12 h/12 h day-night photoperiod for 5 days. Colony type on different culture media was recorded on 7th day after incubation.

Radial growth

Study was conducted to determine best culture media for radial growth of the fungus using twelve solid culture media**.** The diameter of fungus was recorded in millimeters in two directions at right angles to each other. The mycelial growth and average colony diameter was recorded and calculated as described by Keith and Phillip (1971).

Physiological characterization

Temperature-mycelial growth relationships

Mycelail growth of pathogen at different temperatures *viz*. 5, 10, 15, 20, 25, 30, 35 or 40°C was ascertained using tomato extract agar medium. Five day old culture was inoculated in Petri plate and temperature was maintained constantly in incubators throughout the study. The diameter of fungus was recorded in millimeters in two directions at right angles to each other. The mycelial growth and average colony diameter was recorded and calculated as described by Keith and Phillip (1971). Three replicated plates were used for each temperature regime. The sporulation of the pathogen on different temperature was also observed at an interval of 5 days up to 30 days.

Relative humidity (RH)- mycelial growth relationships

The influence of RH on radial growth of pathogen was assessed in tomato extract agar medium at a RH of 50, 60, 70, 80, 85, 90, 95 and 100 per cent. The inoculation, incubation, replications, observation and other growth conditions were same as explained earlier.

Data analysis

The data were statistically analyzed (Gomez and Gomez, 1984) using the SAS 9.2 version developed by the SAS institute, NC, USA. Lab experiments were carried out using completely randomized design (CRD). Data was subjected to analysis of variance (ANOVA) at two significant levels ($P< 0.05$ and $P< 0.01$) and means were compared by Tukey's Honesty significant difference (HSD).

RESULTS AND DISCUSSION

Morphological characterization

Detailed recognition of features of mycelia and characteristics of asexual and sexual structures is very important for correct understanding of the whole infectious process and disease development. The features of the sporangium, like shape, type of a papilla, caducous or deciduous nature of sporangia and subsequent length of the subtending pedicel are important key features for identification of some species of Phytophthora (Erwin and Ribeiro, 1996). In the foregoing study, an effort was made document the various micro morphological characters of pathogen such as hyphae and hyphal swelling, sporangial morphology, gametangial morphology, chlamydospores and colony characters (Table 1 and Fig 1).

Table 1: Micro morphological characters.

Hyphae and hyphal swelling	
1. Mycelium	Hyaline, coenocytic and branched
2. Breadth of hyphae (µm)	4.07
3. Hyphal swelling	Intercalary and terminal
a. Length (µm)	17.9
b. Breadth (µm)	13
Sporangial morphology	
1. Sporangiophore	Simple sympodium
a. Length (µm)	83
b. Breadth (µm)	3.6
2. Sporangia shape	Oviod-obpyriform and non-pedicellate
3. Sporangia proliferation	Present
4. Sporangia	Non-papilate
a. Length (µm)	33.1
b. Breadth (µm)	21.2
c. L:B ratio	1.56
d. Sporangia exit pore breadth (µm)	7.3
5. Zoospore diameter (µm)	11.7
6. Caducity	No
Resting spore	
1. Chlamydospores	Absent
Gametangial morphology	
1. Mating type	Homothallism
2. Type of sexual reproduction	Amphigynous
3. Diameter of oogonium (µm)	23.1
4. Length of antheridium (µm)	6.6
Breadth of antheridium (µm)	9.3
5. Oogonium stalk length (µm)	7.8
6. Diameter of oospore (µm)	18.3

*Values are mean of ten replications.

Micro morphology

Hyphae and hyphal swelling

Mycelium was hyaline, branched, coenocytic filamentous measuring average breadth of 4.07 µm. Hyphal swelling was very common and it was both terminal and/or intercalary with lengths × breadth of 17.9 × 13 µm. The results are in corroboration with other member species of clade 7 group of Phytophthora *viz*., *P. vignae* (Gallegly and Hong, 2008) and *P. cinnamomi* (Robin *et al*. 2012) where hyphal swellings are abundant and positioned intercalary and rarely terminal.

Sporangial morphology

Size and shape of sporangia of *Phytophthora* vary due to culture media and environmental conditions (Waterhouse 1963). Size and shape differences among sporangia and oospores can contribute to easier identification of *Phytophthora* species from infected plant tissues (Hardham *et al*. 1994; Hardham, 1995). Kaosiri *et al*. 1978 reported that, caducity of sporangia is a useful taxonomic tool in identification of many *Phytophthora* species.

In the study sporangiaphore produced by *P. cajani* were simple or sympodial and average breadth is about 3.6 µm. These observations were in accordance with *P. cambivora* (Wicks and Lee, 1986), *P. melonis* (Ho *et al*. 1995), *P. cinnamomi* (Robin *et al*. 2012) and *P. sojae* (Faris *et al*. 1989) where they exhibit simple or sympodial branching and typically proliferate through the empty sporangium.

Sporangium was oviod-obpyriform with an LxB ratio of 1.56 µm; hence it was an oviod-obpyriform. Sporangia were of proliferating type which emerged externally from the base of previous sporangium and produced a new sporangium. Further sporangia were nonpapillate and noncaducous, where sporangia did not detach at maturity. Sporangial exit pore is very narrow with 7.3 µm breadth. Released zoospores were encysted with diameter of 11.7 µm.

Sporangial characteristics of *P. cajani* were in accordance with other species of clade-7 phylogenetic group of *Phytophthora* where *P. cambivora* (Wicks and Lee, 1986) and *P. alni* (Brasier *et al*. 2004) exhibits the oviod-obpyriform sporangium with L:B ratio of 1.6 µm and 1.32-1.62 µm respectively. *Phytophthora cinnamomi* (Robin *et al*. 2012), *P. alni* (Brasier *et al*. 2004; Cerny *et al*. 2008), *P. cambivora* (Vannini and Vettraino, 2011), *P. sojae* (Kaufmann and Gerdemann 1958), *P. fragariae* (Ho and Jong, 1988), *P. vignae* (Purss, 1957) where all the species were producing nonpapillate and noncaducous sporangia.

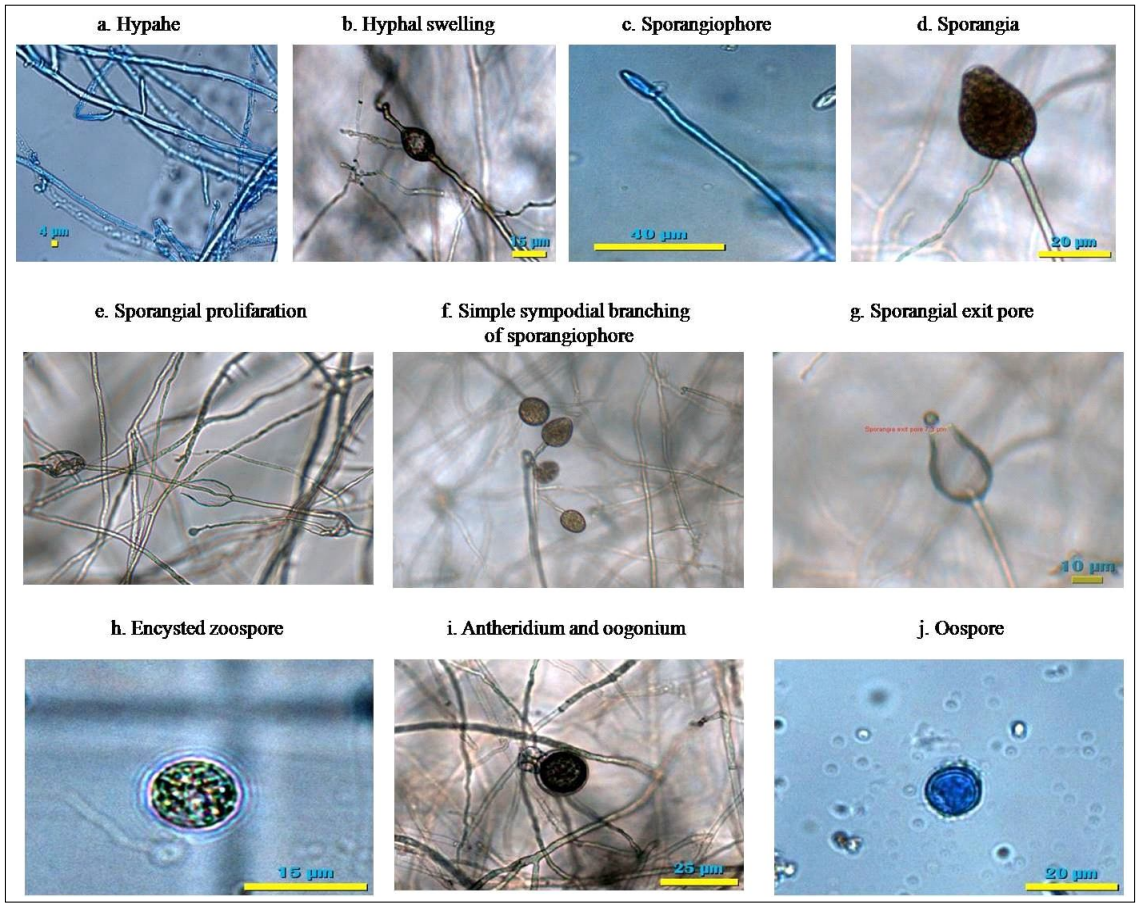

Fig 1: Morphological characterization of *Phytophthora cajani*.

Gametangial morphology

The mating behaviour of *Phytophthora* species is very important especially in relation to the survival of the species. *P. cajani* was homothallic and produced the male and female gametangia called oogonium and antheridium. Amphigynous type of sexual reproduction was observed where both male and female gametes orient perpendicular to themselves and produced a sexual spore called oospore. Average diameter of mature oogonia is about 23.1 µm whereas oogonium stalks length measures about 7.8 µm. Length and breadth of antheridium is of 6.6 × 9.3 µm. Average diameters of oospores is about 18.3 µm. *Phytophthora cajani* differs from some other of species of clade 7 group *viz*., *P. cambivora* (Heffer Link *et al*. *2002), P. melonis* (Ho, 1986)*, P. cinnamomi* (Robin *et al*. 2012) by being homothallic. Further *P. fragariae* (Ho and Jong, 1988) and *P. sojae* (Faris *et al*. 1989) were though homothallic but differs with *P. cajani* in antheridial configuration as they were either paragynous or amphigynous.

No chlamydospores were produced by test pathogen in any of the applied methods. Similar observation was observed in *P. inundata* (Brasier *et al*. 2003b), *P. alni* (Ho *et al*. 1984), *P. fragariae* (Milholland, 1994) and *P. sojae* (Kaufmann and Gerdemann 1958) where chlamydospores were not produced *in vitro*. In contradiction to *P. cajani* other phylogenetically related clade 7 group species *P. cinnamom* (Robin *et al*. 2012) and *P. vignae* (Purss, 1957) were able to produce chlamydospores *in vitro*.

Macro morphology

Colony characteristics and growth rates were useful as a first step in identification of *Phytophthora* species. Biological features and cultural characteristics of genus *Phytophthora* are very important for simpler identification (Cahill and Hardham, 1994).

Colony pattern

Colony patterns are dependent on both culture media and isolate and show great variability. *Phytophthora* spp. has a distinctive set of morphological traits that can be observed in culture on certain media (Erwin and Ribeiro, 1996). *P. cajani* colonies on tomato extract agar medium was characterised by dull white in colour, flat and rosette pattern type. The colony characteristics of *P. cajani* varied among the culture media from dull white to cottony white colour growth which was flat to aerial with smooth to irregular margins (Table 2). The results were in accordance with other researchers on different culture media on colony pattern of *Phytophthora* spp. (Erwin and Ribeiro, 1996). Variability of colony morphology on different culture media is common throughout the genus of *Phytophthora*. Thus, the usefulness

Table 2: Macro Morphological characters.

Temp. (°C)	Growth rate*	RH (%)	Growth rate*	Culture media	Growth rate*	Colony pattern
05	0.0^{f}	50	82.0^{b}	Oatmeal agar	52.17^{h}	Dull white and flat
10	0.0^{f}	55	82.0^{b}	Cornmeal agar	53.23^{h}	Irregular transparent and flat
15	46.4^{d}	60	83.1^{b}	V8 juice agar	89.03^{a}	Dull white and flat, rosette pattern
20	73.1^{c}	65	85.0^{b}	Pigeonpea seedmeal agar	66.13^{f}	Cottony white and aerial
25	87.4^{b}	75	90.0^{a}	Chickpea seed agar	64.60^{g}	Dull white and scanty flat
30	90.0^{a}	85	90.0^{a}	Potato dextrose agar	84.20^{b}	Dull white and rose petal type
35	23.5^{e}	95	90.0^{a}	Czapek-Dox agar	70.33^{e}	Dull white and flat
40	0.0^{f}	100	90.0^{a}	Carrot agar	40.67^{i}	Dull white and flat
				Tomato extract agar	90.00^{a}	Dull white, flat and rosette pattern
				Potato sucrose agar	82.07^{c}	Dull white and flat
				Potato glucose agar	75.63^{d}	Dull white, flat and rosette pattern
				Water agar	0.0^{h}	

*Growth rate on 7th day of incubation Mean of three replications.

Means followed by a common letter are not significantly different at 5 % level by Tukey's HSD.

of colony morphology as an identification aid beyond a supplementary purpose is questionable (Erwin and Ribeiro, 1996; Widmer, 2009).

Radial growth

The radial growth and sporulation of *P. cajani* was studied on different media (Table 2). After growing at 30°C for six days *P. cajani* showed the fastest growth on tomato extract agar medium (90 mm) and next best was V8 juice agar medium (89.0 mm) and least growth of 40.67 mm was observed on carrot agar medium. However sporulation was not observed in any of the medium tested. The results were in accordance with Ribeiro (1978), who reported that V8 juice agar as the best medium for the growth of many *Phytophthora* spp. Similarly, Dhingra and Sinclar (1995), stated tomato extract agar medium was ideal for growth and sporulation of *Phytophthora* spp. Lack of sporulation in *Phytophthora* spp. was probably due to a failure to meet some precise requirement mineral nutrition and temperature for this process to occur (Grant *et al*. 1984).

Physiological characterization

Temperature-mycelia growth relationships

Temperature is one of the pre-requisite for the growth and sporulation of the fungus which plays an important role in infection and disease development. The growth of *P. cajani* was tested on tomato extract agar medium at different temperatures of 5, 10, 15, 20, 25, 30, 35 and 40°C and the results are summarized in Table 2. The fastest growth was observed at 30°C (90.0 mm) followed by 25°C (87.4 mm). Pathogen did not show any growth when plates were incubated at temperature of 5, 10 and 40°C whereas 35°C showed the growth of 23.5 mm hence *P. cajani* can tolerate high temperature of 35°C. Sporulation was not observed in all the temperatures tested. The result fastest mycelia growth at 30°C concur with the findings of Pal and Grewal (1975); Kannaiyan *et al*. (1980) and Mishra *et al*. (2010) who reported maximum vegetative growth of *P. cajani* at 30°C.

Relative humidity (RH) - mycelia growth relationships

Relative humidity is another important epidemiological factor for influencing physiology of fungal growth and sporulation. *P. cajani* was growin at 30°C for six days at different RH. The result implied that, fungus prefers RH of 75 to 100% as evidenced by mycelial growth of 100 mm as against 82 mm in 50 and 55 % RH (Table 2). Sporulation of pathogen was absent at all the RH tested throughout the experiment. The results are in accordance with *Phytophthora capsici* (Granke, 2011) and *P. parasitica* (Prasad *et al*. 2017) where faster growth of the fungus was observed by increasing the RH.

CONCLUSION

The study established the information relating to micro, macro morphological and physiological trait of pathogen is significant to understanding the host, pathogen and environment interaction and ultimately to manage the disease.

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