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Morphological and Molecular Characterization of *Phytophthora colocasiae* Obtained from Fine Spatial Scale

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Abstract

Taro leaf blight caused by the oomycete pathogen *Phytophthora colocasiae* is the most devastating disease of taro with yield loss of 30-50%. The methods commonly used for *P. colocasiae* identification and isolation often include direct isolation from infected leaf tissue on semi-selective media or baiting from soil with leaf discs followed by isolation. Several studies have revealed the effect of culture media and physiological parameters on mycelial growth and sporangium production of *Phytophthora* sp. In view of this, the main objective of this research was to evaluate growth characteristics and sporulation of *P. colocasiae* obtained from fine spatial scale in different culture media. Morphological and molecular characterization was employed for assessing the genetic variability of those *P. colocasiae* isolates. All the isolates exhibited similar growth pattern and colony morphology within the individual media tested. Whereas, ITS characterization revealed detectable polymorphism among the *P. colocasiae* isolates. Maximum growth rate was recorded on onion agar followed by tomato agar, whereas soybean agar showed less linear growth after 7 days of inoculation. Evaluation of sporulation events revealed that carrot agar can induce sporulation earlier than other culture media tested. Physiological factors such as temperature, pH and incubation period greatly influenced the mycelial growth of the pathogen. Results of this study suggest that optimal isolation rates of *P. colocasiae* from infected leaf tissues are achieved by culturing fresh leaf tissues on Ampicillin supplemented PDA medium (100 mg l^{-1}).

Key words: *Phytophthora colocasiae*, growth characteristics, rDNA ITS characterization, pathogenicity test, culture characterization, sporulation, fine spatial scale

Introduction

Taro *(Colocasia esculenta* (L.) Schott) belongs to the monocotyledonous family *Araceae* is an excellent source of dietary energy, minerals, proteins and carbohydrates for millions of people in developing countries. The crop, first domesticated in South-East Asia, has continued to grow in humid tropical areas around the world. It has enormous medicinal properties to cure many diseases like tuberculosis, ulcers, pulmonary congestion and fungal infection. Moreover, taro corms are industrially important raw materials for the production of high fructose syrup and alcohols (Misra et al., 2008).Taro leaf blight caused by the oomycete pathogen *Phytophthora colocasiae* can reduce corm yield up to 50% in susceptible varieties (Jackson et al., 1980). Initial leaf blight symptoms appear as water soaked small circular spots on the leaf edges. As the disease progresses, these spots become enlarge and appears dark brown in color with yellow margins and finally within few weeks the entire leaf is devastated and resulted in severe foliar damage and decay of the plants (Bandyopadhyay et al., 2011). *P. colocasiae* is a serious oomycete pathogen which is highly evolving in nature and its hypervariability have been studied earlier by Nath et al. (2013).

Accurate identification and morphological characterization of the pathogen are necessary steps for the effective disease management. Morphological and colony characteristics are the important basic factors for identification of the pathogen and studying its variability. There are several reports stating the significant genetic diversity exhibited by *P. colocasiae* isolates having distant geographical origins (Lebot et al., 2003). Likewise, a study is necessary to assess the morphological and molecular level diversity of the isolates obtained from fine spatial scale.

The isolation and identification methods for *P. colocasiae* often comprises direct isolation from infected leaf tissue on semi-selective media or baiting from soil with leaf discs followed by isolation. *P. colocasiae* is comparatively short lived in infected taro leaf tissues, and the pathogen seems to possess a limited competitive saprobic ability. These aspects make the isolation and culturing of pathogen in artificial medium relatively unsuccessful. It is challenging to design experiments with artificial inoculation of its reproductive structures and fail to have a reliable diagnostic method for the accurate disease identification. Artificial inoculations using sporangia and mycelia are used for the disease initiation and infection development (Tsopmbeng et al., 2012). In different countries, several culture media have been used for the isolation and maintenance of *P. colocasiae* isolates (Misra et al., 2008; Tsopmbeng et al., 2012; Padmaja et al., 2015). It is necessary to supplement essential elements and compounds required for the normal growth and metabolism of the pathogen in the medium. Hence various artificial media were tried in the present study to hand pick the best medium suitable for the growth of *P. colocasiae*.

If infected leaf tissues are used for pathogen isolation, sample handling techniques which are suitable for fungal infected tissues may not be favorable for the conservation of oomycete viability. Moreover, many diagnostic laboratories are unacquainted with culture techniques for oomycetes and hasty bacterial growth from leaf specimens having secondary bacterial infection frequently precludes the *P. colocasiae* isolation (Tsao and Ocana, 1969). For the rapid and effective isolation of plant pathogenic oomycetes from infected plant tissues requires suitable antibiotic supplemented selective media and good laboratory techniques. Inhibition of both fungal and bacterial contaminants is very essential for the successful oomycete isolation. So the selective media used for this purpose often contains both antibacterial agents and drugs such as pimaricin that inhibits the growth of septate fungi (Hoy and Schneider, 1988). Hence evaluation of suitable culture media and physiological conditions for mycelial growth and sporangial production of *P. colocasiae* and assessing the morphological and molecular variability exhibited by the isolates at fine spatial scale have been done.

Materials and Methods

Sampling and pathogen isolation

Taro with typical leaf blight symptoms were sampled during May to July, 2017 from the fields of ICAR- Central Tuber Crops Research Institute (ICAR-CTCRI), Thiruvananthapuram and brought to laboratory in sterilized polythene bags. A single taro leaf having multiple leaf blight lesions were used for the isolation of the pathogen at fine spatial scale. Later the leaf was washed in running tap water to remove surface impurities. Small portion of diseased leaf (1-2 cm) along with some healthy tissue were excised with sterile scalpel and surface sterilized. The leaf bits were first sterilized in 70% ethanol for 1 minute, followed by 4% Sodium hypochlorite sterilization for 2 minutes, rinsed twice in distilled water and blotted dry using sterile Whatman filter papers. These leaf bits were then aseptically transferred to potato dextrose agar plates and kept for incubation at 25 ± 2^0C in an incubator for mycelial growth. Isolation of *P. colocasiae* from infected leaf tissues were evaluated on 3 different formulations of PDA amended with 100 mg tetracycline, 100 mg ampicillin and 10 mg rifampicin. Inoculated plates were incubated at 25ºC in the dark and examined within 2-3 days.

P. colocasiae colonies were initially identiûed based on the observation of sporangial and mycelial characters using standard mycological keys. This identiûcation was later conûrmed by PCR amplification using ITS and *P. colocasiae* -speciûc primers. DNA of *P. colocasiae* isolates were extracted using Genomic DNA purification kit (Fermentas, EU) according to manufacturer's protocol. The isolated DNA was used for amplification of rDNA-internal transcribed spacer (ITS) region with primer pair of ITS1 (52 -TCCGTAGGTGAACCTGCGG-32) and ITS4 (52 -TCCTCCGCTTATTGATATGC-32) (White et al., 1990) as described by Nath et al. (2015) and sequenced. The resulted high quality sequences were analyzed with NCBI BLASTn to confirm the authenticity of isolates. The sequences were aligned using the ClustalW software (Thompson et al. 1994) for analyzing the extent

of variations among the isolates. The genomic region RAS Ypt (monomeric GTP-binding proteins, essential in specific steps of vesicle transport and secretion) of *P. colocasiae* were ampliûed using primer pairs PCSP-RLF: GGTGTGGACTTT GTG AGTTTCAG and PCSP- RLR: AAGGGAGTTGGCACAACCATT as described by Nath et al.(2014). After 3 days of incubation, hyphae growing from the plated segments were evaluated under a light microscope (Nikon Eclipse 80i Nikon Corporation, Tokyo, Japan) for the presence of sporangia. Hyphal tips from the edge of growing mycelia were transferred onto water agar plates in order to obtain pure cultures.

Culture characterization

For the evaluation of suitable culture media and physiological conditions for mycelial growth and sporangial production of *P. colocasiae*, mycelial discs (1 cm) from water agar culture were transferred to 12 different culture media (Table 1) and incubated at 27 ^{0}C for one week. Following incubation, *P. colocasiae* morphology was characterized based on its colony texture of the mycelia. Three replicates were used for each isolate to confirm the characteristics at similar incubation conditions mentioned above. Data on radial growth rate of mycelia grown in different culture media were recorded on the 7th day of incubation in mm. Sporangial suspensions were prepared by scraping 7 days old *P. colocasiae* culture and dislodging sporangia in to 10 ml sterile distilled water with a drop of Tween 80. After removing mycelial fragments by double filtration through cheese

Table 1. Growth characteristics and sporulation of *P. colocasiae* in 12 different culture media

Culture medium	Culture characteristics	Colony diameter (mm)	*In vitro* sporulation (x10^6 sporangia/ml)
Carrot agar (CA)	Mycelia visible 24-48hr after introduction to the medium; thick cottony white aerial mycelia were produced.	84.08 ± 0.08^b	18^a
Carrot- potato agar (CPA)	Mycelia visible 24-48hr after introduction to the medium; plain white mycelia were produced. Growth rate is comparatively low with reference to carrot agar	74.41 ± 0.30^c	9^b
Czapek Dox agar (CDA)	Creeping whitish mycelia were visible after 2 days of inoculation	83.08 ± 0.08^b	8^b
Oat meal agar (OMA)	Creeping loosely matted whitish mycelia were visible after 2-3 days	74.25 ± 0.14^c	9^b
Onion agar (OA)	Mycelia visible 24-48hr after introduction to the medium; uniformly ûuffy cottony white aerial mycelia were produced.	91.83 ± 0.16^a	7^b
Potato dextrose agar (PDA)	Uniform whitish mycelia were visible after 2-3 days	50.33 ± 0.22^d	1^c
Soybean agar (SA)	Creeping whitish mycelia were visible after 5-6 days	22.08 ± 0.08^f	0^d
Taro leaf agar (TLA)	Creeping whitish mycelia were visible after 3-4 days	48.00 ± 0.14^e	0^d
Tomato agar (TA)	Mycelia visible 24-48hr after introduction to the medium; uniformly ûuffy cottony white aerial mycelia were produced.	86.08 ± 0.08^b	9^b
V8 Juice agar	Creeping whitish mycelia were visible after 2-3 days	82.91 ± 0.22^b	17^a
Yeast Peptone Glycerol agar (YPG)	Thick white mycelia with irregular boundaries	51.75 ± 0.14^d	0^d
Corn meal agar	Creeping loosely matted white mycelia visible after 2 days of inoculation	54.25 ± 0.14^d	7^b

Values are the mean ± SE of three replicates. Within columns, means followed by same letter do not differ significantly according to Duncan's multiple range test at $P \leq 0.05$.

cloth, a drop of the sporangial suspension was placed on a haemocytometer mounted on a light microscope (Nikon Eclipse 80i Nikon Corporation, Tokyo, Japan). The number of sporangia was counted at 40 X magnification and this experiment was repeated three times.

Colony morphology of *P. colocasiae* isolates collected at fine spatial scale was studied on 12 different media for characterizing the variation in colony morphology of each isolates. To study the effect of temperature on mycelial growth rate, *P. colocasiae* isolates were grown in triplicates at 7 different temperature conditions in a completely randomized design. *P. colocasiae*, mycelial discs (1 cm) from water agar culture were transferred to PDA medium and kept at 15, 20, 25, 28, 30, 35 and 40°C incubation. In order to study the effect of pH of the PDA medium on mycelial growth rate, *P. colocasiae* isolates were grown in triplicates at 7 different pH conditions in a completely randomized design. pH of the PDA was adjusted by 1N HCl and NaOH from 4 to 10 for inoculating *P. colocasiae*, mycelial discs (1 cm). After 7 days incubation at 27^0C, colony diameter of *P. colocasiae* cultures were measured as radial growth in mm. Each treatment was replicated three times.

Pathogenicity test

Pathological characterization of *P. colocasiae* isolates collected at fine spatial scale were performed by a floating leaf disc method of pathogenicity test using leaf blight susceptible variety 'Sree Kiran'. Five leaf disks (5x5 cm) were floated in 200-mm glass petri plates containing sterile distilled water. Mycelial disc of *P. colocasiae* cultures were excised and inoculated on each floating leaf disc. As control treatments leaf bits containing sterile agar plugs were used. The leaf discs were held at 15, 20, 25, 30 and 35°C and the disease symptoms were assessed for 4 days. The assay was repeated twice to confirm the virulence of the pathogen and re-isolation was made from all resulting lesions according to the Koch's postulate.

Statistical analysis

Analysis of variance was performed using SPSS statistical package to evaluate the values of mycelial growth and sporangia production. Mean comparison was performed by Duncan Multiple Range Test (DMRT) at P = 0.05 to evaluate the differences in the ability of culture media to promote mycelial growth using SPSS version 14.0.

Results and Discussion

Isolation of pathogen

A total of 5 *P. colocasiae* isolates were obtained from the leaf blight infected taro fields of ICAR- CTCRI at fine spatial scale. It was found that PDA medium supplemented with 100 mg/l Ampicillin supported *P. colocasiae* growth was very well (Fig 1.). Results of this study suggest that when appropriate sample handling and culture techniques are employed, the isolation of *P. colocasiae* from infected taro leaf tissues is not difûcult. Isolation of the pathogen is more successful, when fresh leaf tissues with earlier stage of infection were used. If the leaf tissue is dried or decayed, then the isolation became very difficult process. In such cases, infected leaf tissues were used as inoculum to produce fresh symptoms on healthy taro leaves and further re- isolation was performed. All the isolates were confirmed as *P. colocasiae* by PCR amplification using PCSP RL-F and PCSP RL-R primers

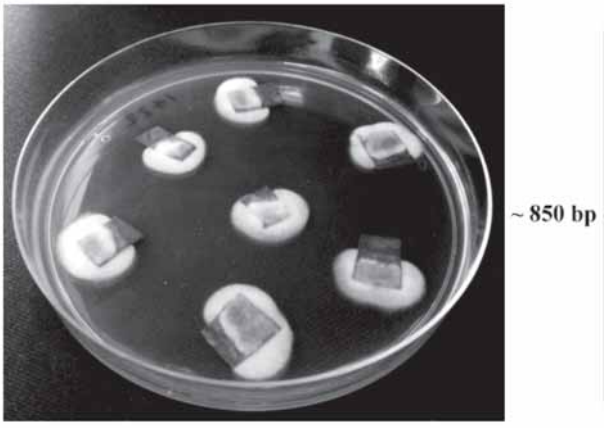

Fig. 1. *P. colocasiae* isolated from infected taro leaf tissues in 100 mg l^{-1} Ampicillin amended PDA medium.

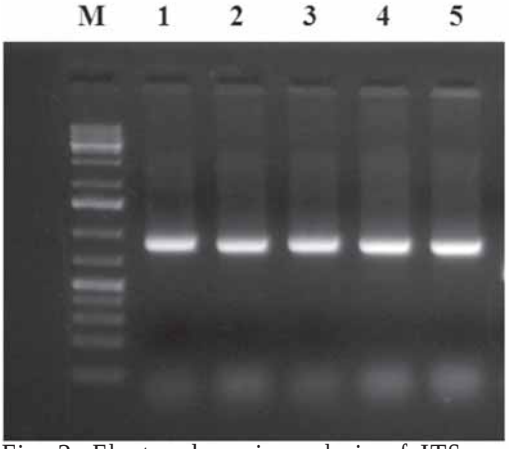

Fig. 2. Electrophoresis analysis of ITS amplification products; Lane 1-5 = *P. colocasiae*; Lane M = 1kb plus DNA ladder

and they produced an amplicon with size 206 bp. For all the *P. colocasiae* isolates PCR amplification using universal ITS primer yielded the expected 850 bp amplicon (Fig 2.)The ITS sequence analysis revealed 97–99 % nucleotide sequence similarity among the isolates of *P. colocasiae* available in the GenBank database. Alignment of sequences using Clustal W revealed considerable SNP variation in all the isolates obtained from fine spatial scale. The sequences were submitted to NCBI GenBank under the accession number KY432681, KY432682, KY432683, KY432684 and KY432685.

Evaluation of culture media

Culture characterization and sporangial morphology studies of *Phytophthora* are very important in accurate identification of the pathogen. Slower growth rate in culture media is exhibited by *P. colocasiae* in comparison with other species of *Phytophthora* namely *P. infestans P. colocasiae* isolates obtained from fine spatial scale were analyzed for any morphological variation on different culture media. All the isolates had produced a similar colony morphology and growth pattern within the tested individual media. There was no distinguishable morphological variation for any isolate. This suggests that with respect to colony morphology, all the isolates shared a common genetic base. But *P. colocasiae* isolates showed variable morphological characteristics, sporangial production and growth rates in different culture media (Fig. 3). *P. colocasiae* isolates grew in all the 12 tested culture media, but after 7 days incubation colony diameter was significantly larger on onion agar; but it recorded poor sporangial production (Table 1). DNA extraction from oomycete requires large quantities of mycelia. So it is possible to harvest large quantities of mycelia from *P. colocasiae* when it is grown in onion agar. While culturing in onion agar the pathogen failed in large scale sporangial production. For the conservation of oomycete viability, sporangial production is very essential. Optimum radial

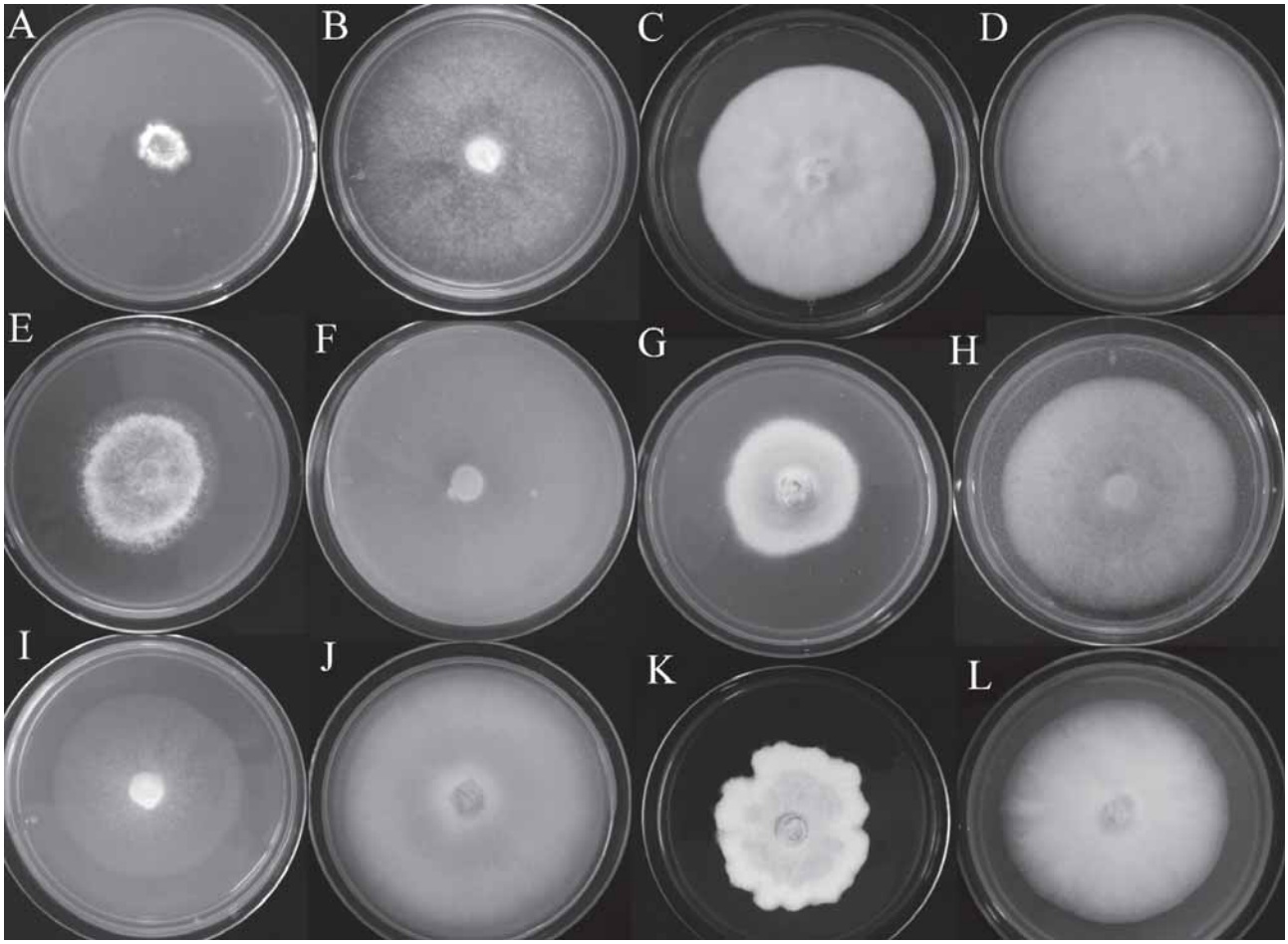

Fig. 3. Morphological variation exhibited by *P. colocasiae* in 12 different culture media. Culture media shown are A: SA; B: CDA; C: V8 juice agar; D: OA; E: TLA; F: OMA; G: PDA; H: CA; I: CMA; J: TA; K: YPG agar; L: CPA.

growth and sporangia production of *P. colocasiae* occurred on carrot agar followed by V8 juice agar, whereas soybean agar showed less linear growth and sporangial production. V 8 juice agar induced sporulation earlier than other culture media. The mycelia were found to be observed after 2-3 days of inoculation in the culture media; but in case of carrot agar, carrot potato agar and onion agar mycelia were visible after 24- 48 hours incubation. Cottony whitish mycelia were observed in carrot agar, onion agar and tomato agar. Creeping whitish mycelia were found in oat meal agar, cornmeal agar, V8 juice agar, taro leaf agar, potato dextrose agar, soybean agar and Czapek Dox agar (Table 1). It exhibits higher mycelial growth rate in onion agar, tomato agar, carrot agar, V8 juice agar and Czapek Dox agar. While on soy bean agar and taro leaf agar very low mycelial growth was observed (Fig. 3). The sporangia observed were semi- papillate, hyaline, ovoid to ellipsoid shaped structures and caduceus (Fig. 4). After 21 days of incubation microscopic culture examination revealed sporangial production of *P. colocasiae* in carrot agar, carrot potato agar, onion agar, tomato agar, cornmeal agar, oatmeal agar and V8 juice agar, but no sporangia were found in yeast peptone glycerol agar, soybean agar and taro leaf agar throughout the experiment.

Padmaja et al., (2015) reported that *P. colocasiae* can grow better in V6 and V8 juice agar. The Soybean agar medium had the least amount of mycelial growth likewise the reports of Medina and Platt (1999). This can be due to the fact that soybean oil is devoid of cholesterol, a compound reported to stimulate growth in several *Phytophthora* species including *P. infestans* (Hendrix and Lauder, 1966). The poor growth pattern of *P. colocasiae* on taro leaf agar could be due to the lack of certain minerals and higher concentration of carbohydrates. However, the role played by each nutrient in the growth of the pathogen *in vivo* could be investigated. According to Papavizas et al., (1980), formation of sporangia by a fungus is a vital criterion for the selection of an appropriate growth medium for its isolation and characterization. In such conditions, onion agar and tomato agar were found to be inappropriate for characterization and culture storage of *P. colocasiae* isolates. But for experiments which require large quantities of fungal mycelia, onion agar and tomato agar were found to be useful. Normally *P. colocasiae* grow best on media that contain thiamine, a suitable carbohydrate source, several organic additives, nitrogen sources and inorganic salts (Tsopmbeng et al., 2012).

Evaluation of physiological factors

The physiological factors such as temperature and pH greatly influence the mycelial growth and sporulation of *P. colocasiae* in PDA medium. When *P. colocasiae* grew on PDA and taro leaf discs over the temperature range 15–30°C, mycelial growth rate was maximum at 28°C after 7 -days incubation. Minimal growth rate was observed at 15°C, 35°C and 40°C. Tyson and Fullerton (2015) recorded 25-30 °C as the optimum temperature range for the growth and sporangial production of *Phytophthora* species. At lower temperature, sporangial production was found to be lower for *P. colocasiae*. At 25°C temperature the leaf blight lesions grew quickly and produced the greatest number of successful infections. This study has shown that *P. colocasiae* is strongly influenced by weather parameters like temperature. This explains the field experience, where symptom development was instant

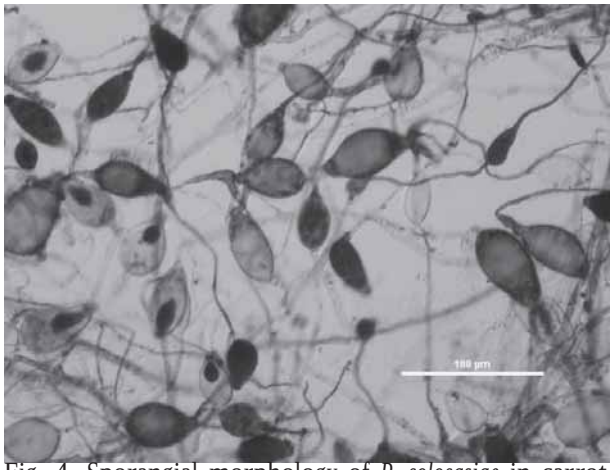

Fig. 4. Sporangial morphology of *P. colocasiae* in carrot agar

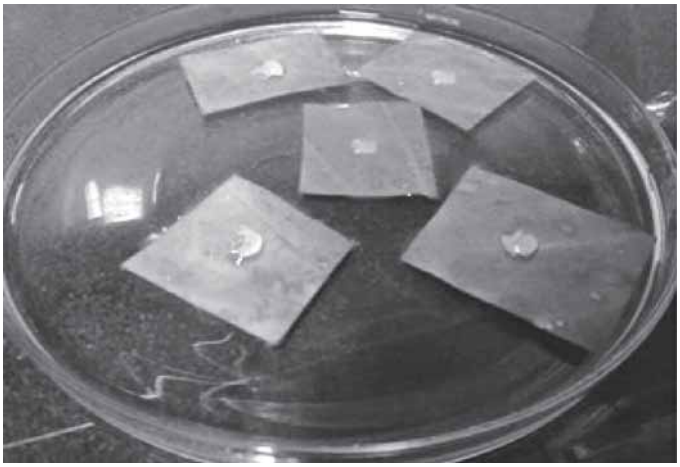

Fig. 5. Pathogenicity assay on taro leaf discs

when cool, wet conditions prevailed. If conditions were hot and dry, lesion development progress gradually, and in some cases lesion development failed to expand further. After 7- days incubation, maximum mycelial growth rate of *P. colocasiae* was found in PDA medium having pH 6. No mycelial growth was observed in PDA media with pH 3, 9 and 10. It is evident from the present study that *P. colocasiae* growth is well supported by acidic medium than alkaline medium. The pH of PDA medium ranges from 5- 7 was found to be optimum for better mycelial growth rate of *P. colocasiae*. But in case of *P. infestans*, its growth characteristics or growth rate at different pH range did not change much (Hussain and Hussain, 2016).

Pathogenicity tests

Typical leaf blight symptoms were produced by leaf discs up on 1-2 days of inoculation (Fig.5). The lesions developed were appeared to be yellow and water soaked in the beginning. As the disease progresses it becomes dark brown in colour. All the isolates were succeeded in producing an infection during pathogenicity test. Among the isolates collected at fine spatial scale, no significant difference in the lesion length was observed (P = 0.05). Previous study by Misra et al., (2011) have stated that isolates of *P. colocasiae* from Kerala to be less pathogenic, but in this study they were found to be aggressive and able to produce serious infection on taro leaf discs. Granke et al., (2011) observed that the long term storage of isolates may result in reduction of its virulence property. So this can be the possible reason behind the contradictory results of previous studies. The pathogen was successfully re- isolated from the lesions completing the Koch's postulate. The development of lesions on leaf discs was greatest at 25 and 28°C, but suppressed at 35°C.

Conclusion

The results of this study revealed that among all the media tested, maximum radial growth rate of *P. colocasiae* was recorded on onion agar (92mm). Whereas the *invitro* sporulation of the pathogen was maximum on carrot agar, followed by V8 juice agar. Based on the results, carrot agar and V8 juice agar were found to be appropriate culture media for the growth and sporulation of *P. colocasiae*. But for experiments that demand large quantities of mycelia, we can rely on onion agar. Physiological factors such as temperature and pH greatly influence the mycelial growth rate of this pathogen. ITS characterization revealed detectable polymorphism among isolates of *P. colocasiae* collected at fine spatial scale, but the morphological and pathological characterization failed to produce any detectable variation among them. These results will provide a basis for the future research on pathogen characterization and its management.

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