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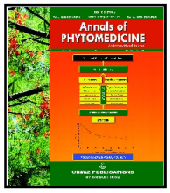

Original article

Morphological and molecular characterization of *Phytophthora* isolated from citrus orchards in Maharashtra

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Abstract

Citrus gummosis, caused by *Phytophthora* is one of the important disease of citrus, causing huge loss of yield every year in India. Diagnosis based on visible symptoms is generally unreliable and, thus advanced diagnostic tools, molecular characterization has revolutionized the detection test, making it feasible to test large number of samples. Soil samples from the rhizosphere of heavily infected, symptomatic citrus plants, showing damping off, foot rot, root rot, gummosis, *etc*., were collected from four different locations of Vidarbha region, Maharashtra during July-December in 2016-17 and *Phytophthora* was isolated from four different samples using leaf bait and soil suspension methods on selective PARPH-CMA medium. Four different morphological features were observed in the colonies when cultured on different media. The patterns of growth were stellate stiated, light rosette, cottony with slightly rossette and dense cottony structures on PARPH-CMA, CMA, V8 juice agar and PDA media, respectively. Molecular characterization was done with ITS primers. The PCR amplicons obtained from all the isolates were of expected size in all four isolates, because the specificity of primer combinations towards *Phytophthora.* No unspecific amplification was observed in any of the isolates, confirmed that above primer combinations, amplifies ITS region of *Phytophthora* only. The study conclusively revealed that *Phytophthora nicotianae* is the most prevalent species in citrus orchards of Vidarbha region, causing the widespread problem of citrus decline. To control the infection, proper hygiene and use of healthy planting material is necessary. To accomplish this, present investigation focus on the production of *Phytophthora* infection free, quality planting material, by screening both, scion source and rootstock through molecular methods before grafting.

Keywords: *Phytophthora*, citrus, ITS, morphology, Vidharbha

1. Introduction

Citrus is one of the major fruit crops of India, contributing estimated production of 11717 thousand tonnes and area cover of about 976 thousand acre. Various varieties (Rangapur lime, Kagazi lime, Malta, Sitagudi, *etc*.) of citrus grown in India from western to southern (Maharashtra, Punjab, Assam, Karnataka, *etc*.) region of India (Source: NBH, 2017). Citrus cultivated in variety of conditions in India, plagued many diseases. Among these, gummosis is major disease caused by *Phythophthora*, causing foot rot, root rot, citrus decline, damping off, brown rot of fruit, yellowing of leaves, *etc*. (Savita *et al*., 2012). The genus *Phytophthora* (meaning plant-destroyer in Greek), belongs to the class Oomycetes, currently placed in the kingdom Stramenopila, under the phylum Heterokonta, containing about 123 formally described species and 23 provisional species (http://www.phytophthoradb.org/ accessed April 21, 2015).

Phytophthora is the most damaging soil-borne pathogen in Vidarbha region of Maharashtra that attack citrus orchid, causing more than 20 per cent seedling mortality in central India. In Vidarbha region of Maharashtra, 20-50% Nagpur mandarin plants were found to be affected, resulting in severe decline due to *Phytophthora parasitica*, *P. citrophthora* along with *P. palmivora* (Savita *et al*., 2012). *Phytophthora* is major cause of short life span of the famous Nagpur mandarin orange (*Citrus reticulata* Blanco) crop in Vidarbha region of Maharashtra every year (Naqvi, 2002). The most important *Phytophthora* spp. affecting citrus worldwide are *Phytophthora nicotianae* (syn. *P. parasitica*), *P. palmivora*, and *P. citrophthora* (Bowman *et al*., 2007).

As *Phytophthora* is pseudo fungi, none of fungicide affects it. Only hygiene in field, used of healthy planting material, disease free soil, resistance root stock, *etc*., practices can be beneficial to prevent the pathogen. Detection of presence of pathogen at nursery stage has crucial value in prevention of gummosis, citrus decline, *etc*. Morphological features of sporangia, growth rate on selective medium, colony morphology parameters were observed in the cultures of *Phytophthora* to classify them (Das *et al*., 2016). However, these morphological methods are not reliable among the vast varieties of species of *Phytophthora.* DNA-based marker prove to be more reliable and rapid approach (Das *et al*., 2016). In present study, number of soil samples were collected from citrus orchids of different locations in Vidharbha region and *Phytophthora* isolated, characterised by morphological as well as molecular methods.

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2. Material and Methods

2.1 Field survey and sample collection

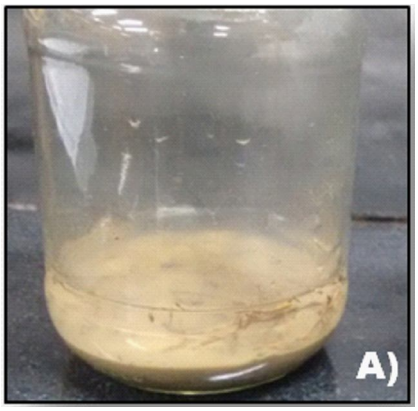
Soil samples from the root zone of heavily infected citrus plants, were collected (Table 1) from four different locations of Vidharbha regions (Citrus orchard, Dr. PDKV Akola main garden; Citrus orchard, Kaulkhed, Dist. Akola; Citrus orchard, Warud, Dist. Amravati ; AICRP Citrus field Dr. PDKV, Akola) during July-December in 2016-17 of Maharashtra state. Citrus plants were visually examined and soil samples were collected from symptomatic trees, showing damping off, foot rot, roots rot gummosis, *etc*., along with healthy leaf samples collected for leaf disc method of isolation.

2.2 Sample preparation

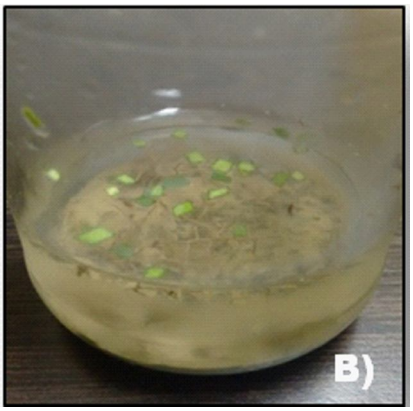
Two methods were adopted for isolation of pathogen. For leaf bait method, infected soil samples added in autoclaved distilled water along with 0.25% of agar and 2 mm of healthy leaf disc suspended in prepared soil suspension and kept at 23°C in dark for 72 h. One gram infected soil was suspended in 10 ml autoclaved distilled water along with 0.25% agar; serial dilutions were carried out (Plate 1).

Table 1: Characteristic symptoms of *Phytophthora* infested citrus

Sr.No.	Characteristic symptoms	Place of collection	Host	Isolate
1.	Gummosis, sap oozing out from the infected bark, root rot	AICRP on tropical fruit crops, Dr.PDKV, Akola	Nagpur mandarin	Phy1
2.	Gummosis, root rot, loss of leaves, decline	Main citrus nursery, Dr.PDKV, Akola	Nagpur mandarin	Phy2
3.	Gummosis, root rot, decline	Citrus orchard, Kaulkhed, Dist. Akola	Nagpur Mandarin	Phy3
4.	Gummosis, root rot, foot rot, decline, yellowing of veins	Citrus orchard, Temburkheda, Warud, Dist. Amravati	Nagpur Mandarin	Phy4

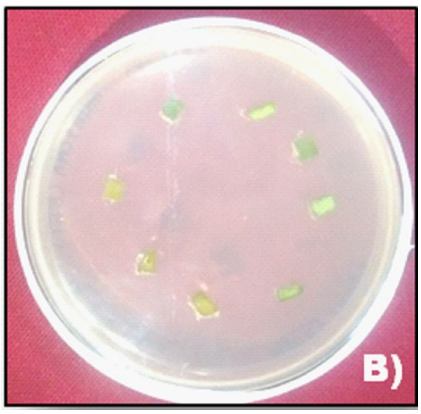


Soil suspension

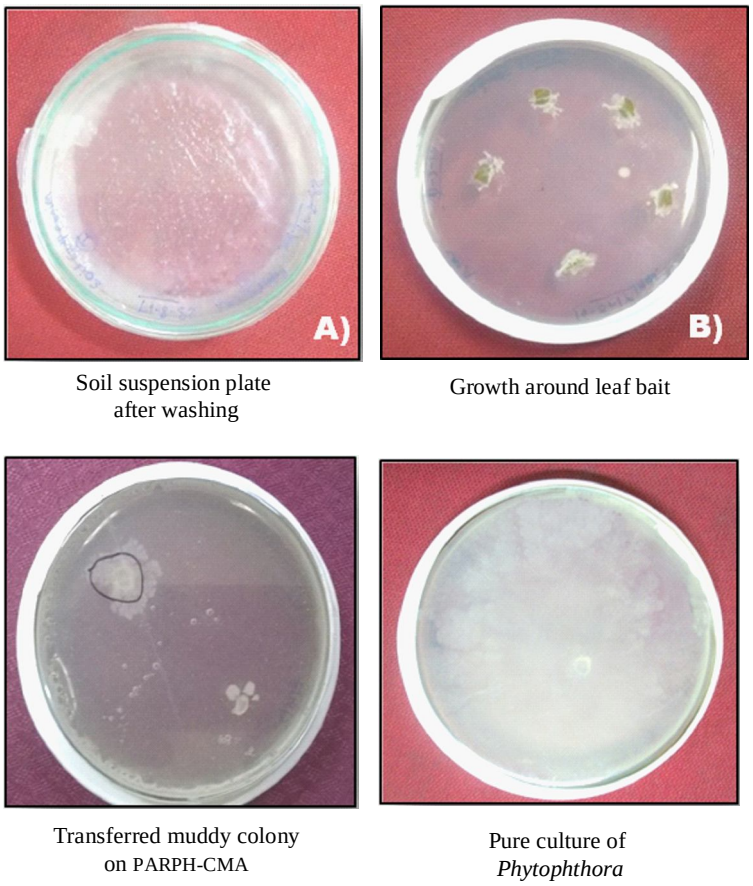


Leaf bait suspended in soil suspension

Suspension spread on PARPH-CMA



Leaf baits on PARPH-CMA



Soil suspension plate after washing

Growth around leaf bait

Transferred muddy colony on PARPH-CMA

Pure culture of *Phytophthora*

Plate1 : Isolation and purification of *Phytophthora* using (A) leaf, (B) soil suspension method.

2.3 Selective media

Selective PARPH media restrict the growth of other bacteria and fungus, allowing only *Phytophthora* to facillate growth, was prepared using pimaricin, ampicillin, rifampicin, penta-chloronitrobenzene and hymexazole antibiotics. PARPH used along with corn meal agar (CMA) (Jeffers, 2006) for isolation of pathogen.

2.4 Isolation, purification and maintenance of pure culture

For isolation from soil, prepared 1 ml soil suspension was spread on PARPH-CMA media plate under aseptic condition and incubated at 23°C in dark for 72 h. Plate was washed afterwards using autoclaved distilled water and sterile brush to remove the soil residue (Timmer *et al*., 1988). Muddy colonies were transfer to fresh PARPH-CMA and subsequently to CMA for purified culture. In leaf bait method, 2 mm of leaf bits of healthy citrus leaves kept in soil suspension in dark at 23°C for 72 h. Leaf baits were blot dried on sterile filter paper under laminar air flow and inoculated on PARPH-CMA by giving cut to media using sterile scalpel and inserting whole bait inside. Purified colonies from PARPH-CMA were then transferred to fresh PARPH-CMA and finally to CMA. All the culture were maintained at 23°C in dark and further transfer to fresh medium once in month.

2.5 Morphological characterization

To study the morphology, growth rate, colony pattern, shape of sporangium, *etc*., taken into account. Sporangium morphology was checked through agar-disc-in water technique (Al-Hedaithy and Tsao, 1985), 6 mm of plug from fresh culture of *Phytophthora* placed in empty sterile petriplate and the plate filled with sterile distilled water up to the point where plug is not fully immersed. The plate kept at 23°C under fluorescent light for 72 h and observed under the microscope to investigate sporangial morphology with respect to shape of sporangia. Colony morphology was recorded as pattern of growth of mycelium on different media, *i.e*., CMA, V8 juice agar, PARPH-CMA and PDA. To study growth rate of *Phytophthora*, the isolates were grown on four different media plates, *viz*., PARPH-CMA, CMA, V8 juice agar and PDA for study of growth rate with three replications. Plug of 6 mm diameter from advance margin of isolated pure culture of *Phytophthora* was inoculated on different media plate, incubated at 23°C and the observation of mycelial growth recorded on 5th day, 10th day and 15th day after inoculation.

2.6 DNA extraction and polymerase chain reaction

For isolating good quality of DNA, *Phytophthora* inoculated on PARPH-CMA for two weeks is used for DNA extraction. App. 4 gm of mycelium ground using sterile mortal pestle in 2 % of SDS for 5

min. Lysis buffer (EDTA 25 mM; Triton 1%; Tris-Cl 50 mM) added in above suspension and again agitated. After transferring the suspension in micro centrifuge tubes, equal volume of phenol : chloroform (1:1) added and centrifuged at 10,000 rpm for 10 min at 4°C. Supernant is carefully taken and 0.54 volume of supernatant, sodium acetate and 1/10th volume of supernant, isopropanol added in supernant prior to incubate at 2°C for 30 min. Sample centrifuged at 10,000 rpm for 10 min at 4°C and supernant discarded. Pellet washed twice using 70% ethanol and air dried (Personal communication). The pellet stored at – 20°C in T10E1 buffer. DNA quantification was carried out using spectrophotometer.

Polymerase chain reaction (PCR) carried out using ITS markers. (Table 2) (Cooke *et al.*, 2000). Each PCR reaction contains 10 × PCR buffer with 25 mM $MgCl_2$, dNTP mix 10 mM (ATP, GTP, TTP, CTP), each of primer and Taq DNA polymerase 5U/ µl and a DNA template 50 ng and the reaction mixture finally make up to volume 20 µl per reaction by nuclease free water. An initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec and extension step at 72°C for 10 min. Annealing conditions varies as per primer combination (Table 4). PCR amplification performed on eppendorf epigradient S master cycler. The amplified DNAs (PCR) were analysed on 2.0% agarose gel stained with ethidium bromide. The gel was observed under gel doc system (Bio-Rad).

Table 2: PCR reaction mixture consisted of following components

PCR Component	Quantity
Deionised nuclease free water	15.3 µl
10X PCR buffer	2.0 µl
2mM dNTPs	0.4 µl
Forward primer (10 pmol/µl)	0.5 µl
Reverse primer (10 pmol/µl)	0.5 µl
Taq DNA polymerase (5 U/µl)	0.3 µl
DNA sample template (5 ng)	1.0 µl
Total	20.0 µl

Table 3: List of primer used in detection study of *Phytophthora*

Sr.No.	Primer name	Primer sequence
1.	ITS 1	5' TCCGTAGGTGAACCTGCGG 3'
2.	ITS 2	5' GCTGCCGTTCTTCATCGATGC 3'
3.	ITS 4	5' TCCTCCGCTTATTGATATGC 3'
4.	ITS 5	5' GGAAGTAAAAGTCGTAACAAGG 3'
5.	ITS 6	5' GGAAGTAAAAGTCGTAACAAGG 3'

Table 4: Annealing conditions for primer (Lee and Taylor, 1992; Bonants *et al.*, 2000; Bowman *et al.*, 2007)

Sl.No.	Primer	Annealing temperature and duration
1	ITS 1-2	60°C for 30 sec
2	ITS 1-4	50°C for 30 sec
3	ITS 4-5	48°C for 1 min
4	ITS 4-6	50°C for 30 sec

3. Results

3.1 Isolation and maintenance of isolates

Phytophthora is isolated from four different samples, using leaf bait and soil suspension methods (Plate1). Culture maintained on PARPH-CMA plates for longer period at 23°C in Biotechnology Centre, Dr. P.D.K.V., Akola. Leaf bait method preferred than soil suspension because there are less chances of fungal and bacterial contamination.

3.2 Morphological characterization

3.2.1 Colony characterization

The characteristic, *viz.*, feature of *Phytophthora* was observed, *i.e.*, stellate stiated pattern, light rosette pattern, cottony with slightly rossette pattern and dense cottony mycelium on PARPH-CMA, corn meal agar, V8 juice agar and potato dextrose agar medium respectively; similar results were recorded by Mounde *et al.* (2012) and Das *et al.* (2016) (Plate 2). All the four isolates found to have showed similar colony pattern on same medium. The colonies show stellate stiated pattern mycelium to dense cottony mycelium on different growth medium indicates the growth of *Phytophthora* (Plate 2). From morphology, the growth is confirmed to be of *P. nicotianae* (Das *et al.*, 2016).

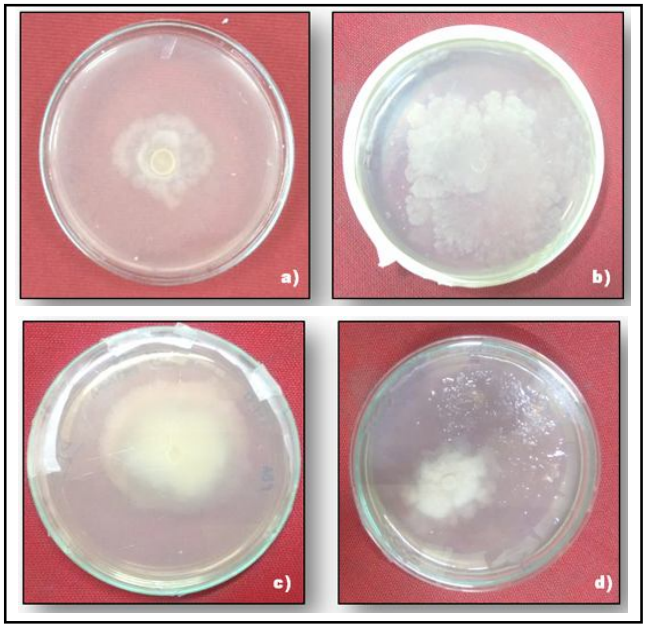


Plate 2: Colony morphology of *Phytophthora* spp. isolates on, (a) PARPH-CMA, (b) Corn meal agar, (c) V8 juice agar, (d) Potato dextrose agar, showing different colony pattern.

3.2.2 Sporangial morphology

Sporangial observation under compound microscope to determine shape caducity, papillation, found that sporangia were non-caducous, papillated, spheroid and smooth (Plate 3), indicates the growth of *P. nicotianae* as recorded by Bush *et al.* (2006) and Das *et al.* (2016) and also confirms the isolates collected from four different locations are of *P. nicotianae* in citrus field of Vidharbha region, causing gummosis were reported by Naqvi (2000); Das *et al.* (2011); Gade (2012); Das *et al.* (2016). Other species of *Phytophthora* were not

reported in the present study because of non-availability of morphological observations related to the other species.

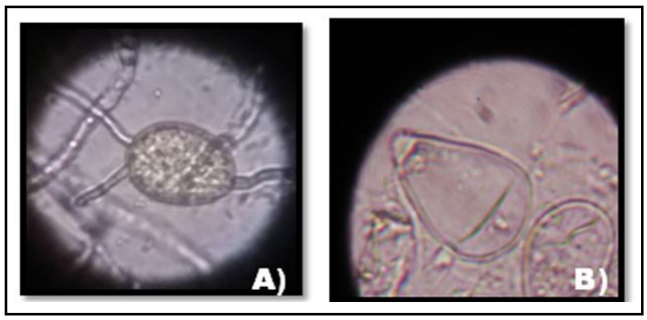


Plate 3: Sporangia character of *Phytophthora* spp. under 40x compound microscope. (A) Smooth sporangia, (B) Spheroid sporangia.

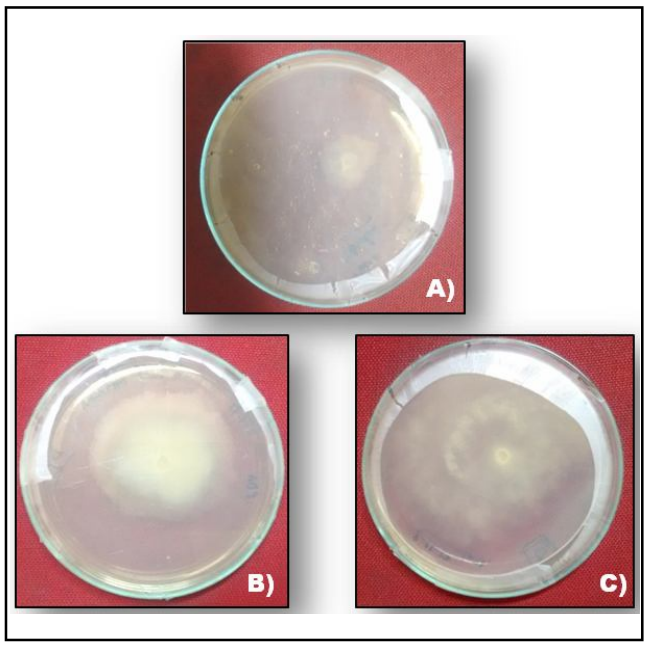


Plate 4: Growth of isolated *Phytophthora* spp. on V8 juice agar media after (A) 5th day, (B) 10th day of inoculation.

3.2.3 Colony growth rate

The statistical analysis shows that there is statistically significant difference in radial mycelial growth of *Phytophthora* spp. on different media. Among all the media plates, on 5th day after inoculation, growth on V8 juice agar (1.940 cm) found at par with potato dextrose agar (1.995 cm) and significantly superior with growth of mycelium on PARPH-CMA (1.000 cm) and corn meal agar media plates (1.403 cm). On 10th day after inoculation, growth on PDA (4.277 cm) found significantly superior to growth on V8 juice agar (2.999 cm), PARPH-CMA (1.733 cm) and CMA (2.178 cm) media plates. Whereas, on 15th day after inoculation, growth of *Phytophthora* isolate on V8 juice agar (5.033 cm) found significantly superior than PDA (4.309 cm), PARPH-CMA (2.301 cm) and CMA (2.325 cm). Observation of growth rate shows that *Phytophthora* isolate shows highest growth rate on V8 juice agar on 15th day after inoculation which found similar with results recorded by Mounde *et al*. (2012) and Das *et al*. (2016) on V8 juice agar (Plate 4).

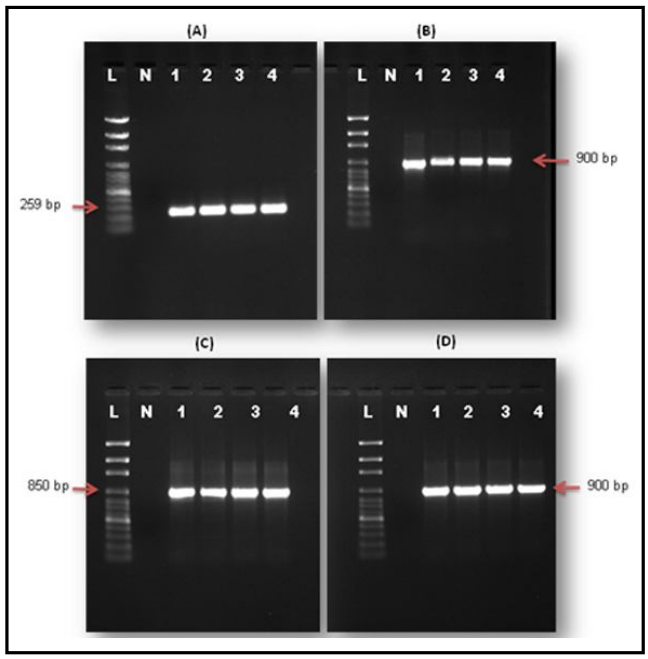


Plate 5: Amplification of ITS region in *Phytophthora* isolates from four different locations of L-100bp ladder, N-Negative control, (A) PCR product amplified with primer pair ITS1-ITS2, (B) PCR product amplified with primer pair ITS1-ITS4, (C) PCR product amplified with primer pair ITS4-ITS5, (D) PCR product amplified with primer pair ITS4-ITS6.

(1) Sample collected from AICRP on tropical fruits, Akola,

(2) Sample collected from main garden, Dr. PDKV, Akola,

(3) Sample collected from Citrus orchard Kaulkhed, Akola,

(4) Sample collected from citrus orchard, Tembhurkheda, Warud, Amravati.

3.3 PCR amplification of ITS region

The primers ITS 1-2, ITS 1-4, ITS 4-5 and ITS 4-6 (Table 2) (Lee and Taylor, 1992; Bonants *et al*., 2000, Bowman *et al*., 2007) were used for the study. The PCR amplicons obtained from all the isolates were 259 bp for ITS 1-2, 900 bp for ITS 1-4, 850 bp for ITS 4-5 and 900 bp for ITS 4-6 (Plate 5) because the specificity of primer combinations towards *Phytophthora*. ITS sequence form *P. nicotianae* ranged from 174 to 235 bp and the consensus length for the sequence alignment was 259 bp. No unspecific amplification was observed in any of the isolates, confirmed that above primer combinations, amplifies ITS region of *Phytophthora* only.

4. Discussion

In order to develop the molecular based detection method, *Phytophthora* spp. was isolated from citrus orchards of Vidarbha region of Maharashtra. The species was confirmed to be of *Phytophthora nicotianae* from the presented morphological, sporangial and molecular observation. Diversity in colony morphology was found on different media of the isolated *Phytophthora* spp. It was observed that colonies show Stellate stiated pattern mycelium to dense cottony mycelium on different growth medium indicates the growth of *Phytophthora*, earlier Mounde *et al.* (2012) and Das *et al.* (2016) reported the similar results. Although, isolation of the pathogen was carried out using

PARPH-CMA, but growth rate was found higher on V8 juice agar on 15th day of inoculation as compared to PDA, CMA and PARPH-CMA media plate, Mounde *et al.* (2012) and Das *et al.*(2016) got same growth rate on V8 juice agar. Sporangia of isolated *Phytophthora* were non-caducous, papillated, spheroid and smooth, which indicates the growth of *P. nicotianae* as recorded by Bush *et al.* (2006) and Das *et al.* (2016) and also confirms the isolates collected from four different locations are of *P. nicotianae* in citrus field of Vidharbha region, causing gummosis were reported by (Naqvi, 2000; Das *et al.*, 2011; Gade, 2012; Das *et al.*, 2016). Other species of *Phytophthora* were not reported in the present study because of non-availability of morphological observations related to the other species.

However, identification of *Phytophthora* spp. by traditional method like surveying and morphological produce results but both methods require labour, needs expertise, less reliable and expensive at large numbers of samples to screen. Hence, in this study we tried to revolutionize the method of identification of *Phytophthora* spp. with molecular identification with help of internal transcribed region (ITS) of ribosomal DNA. The ITS region of the species was demonstrated by Lee and Taylor (1992); Bonants *et al.* (2000); Bowman *et al.* (2007). ITS marker ITS1-2, ITS 1-4, ITS 4-5 and ITS 4-6 were used for detection of *Phytophthora* spp. to amplify isolated DNA by using polymerase chain reaction. Molecular tools like polymerase chain reaction based detection prove to be more efficient and less time consuming than morphological observation (Das *et al.*, 2011).

5. Conclusion

Morphological and molecular results obtained indicate that, *Phytophthora nicotianae* is prevalent in citrus orchards in Vidharbha region of Maharashtra. Molecular tools especially PCR amplification using ITS primers can be used for rapid and accurate detection of *Phytophthora* in citrus. As *Phytophthora* infected citrus nurseries acts as a hotspot for spread of disease, it's very essential to determine the infection at very early growth stage of grafted plant, it helps to prevent the spread of disease to large scale area, from nursery to farmers field. As pseudo nature of *Phytophthora*, prevention is most effective and only way to control the huge economical loss of citrus growing farmers and citrus industry from *Phytophthora* infestation. This would help us to produce certified quality planting material free of gummosis.

Conflict of interest

The authors declare that no conflict of interest exists in the course of conducting this research. All authors had final decision regarding the manuscript and the decision to submit the findings for publication.

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