



Virulence of *Pythium deliense* causing soft rot in Ginger, a new report from Kerala, India

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Abstract

Ginger (*Zingiber officinale* Rosc.) rhizome is widely used as a spice and medicine and, its large-scale production is being seriously affected by several fungal and bacterial diseases. Soft rot disease caused by various species of *Pythium* is the most destructive among them. Samples showing soft rot symptoms were collected from the ginger fields of Kozhikode and Wayanad districts of Kerala. Detailed study of the morphology, growth parameters and molecular characteristics of the fungal isolates confirmed the identity of *Pythium deliense*. The pathogenicity of the isolates was demonstrated by *in vitro* and *in vivo* methods. This is the first report of the pathogen, *P. deliense* causing soft rot in ginger from Kerala, India.

Keywords – isolation – pathogenicity - rhizome – spice – *Zingiber officinale*

Introduction

Ginger (*Zingiber officinale* Rosc. Zingiberaceae) is a perennial herb that is extensively used as a spice, herbal medicine and dietary adjunct that enhances the flavour and taste of foods. It has anti-inflammatory, antifungal, antiviral, antioxidant and anticancer properties (Naghsh 2015, Tohma et al. 2017, Ezzat et al. 2018, Hussein & Joo 2018, Yusuf et al. 2018, Kaushik et al. 2020, Kharisma et al. 2020) and hence the rhizome is used as an inevitable ingredient in Ayurvedic, Unani and Chinese herbal medicines to treat several diseases. Considering the importance of the crop, it has been cultivated in many tropical and subtropical areas of the world. The main producers are India, China, Nigeria and Nepal (FAOSTAT 2019).

Diseases are the most important production constraint of the ginger crop (Kumar & Hayward 2016). Apart from socio-economic problems, the main reasons for the decline in the productivity of ginger all over the world are biotic and abiotic stress issues. The main biotic threat faced by farmers is soft rot or rhizome rot disease caused by various species of *Pythium*. Dohroo (2005) described rhizome rot as the most destructive disease of ginger worldwide and estimated loss in a range of 50–90%. He listed eleven species of *Pythium* as causal organisms of soft rot of ginger from different parts of the world and among them, *Pythium aphanidermatum* and *P. myriotylum* are the species reported as the most commonly associated pathogen with the disease.

It was reported that the continuous climatic shifts with temperature rise and the soil moisture diminution adversely affected the growth of many crops and many weak pathogens may come to light as aggressive pathogens (Subila & Bhai 2020b). Considering the worldwide scenario, there are only a few reports available about *Pythium deliense* as a pathogen, compared to other pathogenic *Pythium* species. Meurs (1934) isolated *Pythium* from the tobacco plant and correctly identified and described it as *P. deliense*. In India, reports were available to cause rotting in tomato

(Singh & Srivastava 1953), mung bean (Ragunathan 1968), fish bean (Pandotra et al. 1971) and also in the rhizosphere and roots of black pepper (Subila & Bhai 2020b). It was also reported from some other countries like Nicaragua (Drechsler 1957) from tobacco, Pakistan (Lodhi et al. 2004) from *Piper betel*, Thailand (Intaparn et al. 2019) from *Catharanthus roseus*, etc. But from Kerala, it was reported from Eucalyptus (Sharma et al. 1984) and black pepper (Subila & Bhai 2020a).

The only report on the virulent effect of *P. deliense* in ginger soft rot was reported from India (Madhya Pradesh), by Haware & Joshi (1974) and Jooju (2005). To our best knowledge, there were no reports available about *P. deliense* as a soft rot causing pathogen in ginger from Kerala to date. Hence this work is aimed for a detailed study on the disease-causing ability of *P. deliense* in ginger.

Materials & methods

Diseased sample collection and pathogen isolation

From the ginger fields of Chakittappara and Vythiri areas of Kozhikode and Wayanad Districts, Kerala, three diseased ginger rhizome samples were collected (Fig. 1). The symptoms produced in infected ginger were noted in detail and documented. The samples were collected in polybags and brought into the plant pathology laboratory of The Zamorin's Guruvayurappan College, Kozhikode, Kerala for isolation of pathogen and identification. The rhizomes were washed thoroughly with tap water to remove the soil particles. Small cuts were made from the rot or lesion along with some healthy portions and the rhizome pieces were surface sterilized with 0.1% HgCl₂ for 1 min followed by washing in sterile distilled water three times. To remove adhered water completely from the surface of ginger pieces, they were kept on sterile blotting papers. It was then transferred onto pre-sterilized and solidified Potato Dextrose Agar (PDA) medium, in Petri plates. The plates with samples were incubated at room temperature (25±2°C) for two days. The fungal outgrowth, from actively growing edges of hyphal tips, were transferred onto PDA slants and maintained for further studies. The fungal cultures thus grown from the specimen were subcultured in PDA slants frequently to maintain the purity of the culture. All the cultures obtained are deposited at the ZGC herbarium, India. The facesoffungi number was registered as mentioned in Jayasiri et al. (2015).



Fig. 1 – Disease symptoms in the field.

Identification of the pathogen

Different media were tested to evaluate the sporulation of pathogen and also to identify the pathogen at the species level. Culture discs (5 mm size) were collected from the actively growing edges of culture and were inoculated at the centre of the 90 mm Petri plates containing media like potato dextrose agar (PDA) and Corn Meal Agar (CMA). These plates were then incubated for 96 h at room temperature. The growth rate of the isolates was measured at a 24 h interval and documented the colony morphology. The fungal hyphae taken onto glass slides under sterile conditions were stained with lactophenol cotton blue for microscopical observations. The microphotographs and measurements of fungal structures were taken using Magnus MX21iLED

(Microscope) Magcam-DC14 (Camera). They were compared with the details in the monograph of the genus *Pythium* (Plaats-Niterink 1981).

Production of Oospores

Oogonia and its structures are being used to characterize and identify various species of *Pythium*. For oogonia production in nutrient media, 5 mm mycelial plugs (disc with mycelia) were collected from the actively growing margins of 72 h old culture. The culture discs were kept in sterile distilled water along with sterile leaf blades of *Brachiaria ruziziensis* and incubated under humid conditions, to induce oogonia production. The oogonia production was observed under the microscope and documented all the characters as their size and structures.

Sporangial morphology

Sporangial characters are another important parameter widely used for *Pythium* species identification. The sporangial morphology was studied using the slide culture technique. Fungal culture disc (3 mm size) collected from an actively growing margin of 72 h old culture and placed on a sterile microscopic glass slide with an agar block. The microscopic glass slide with culture block was incubated in a humid chamber at $25\pm 1^{\circ}\text{C}$ for 3 days. The slide was fixed with lactophenol cotton blue for staining the culture and a coverslip was placed over the agar block and observed under a microscope for sporangia.

Molecular characterization

Though the preliminary identification of the isolates was done based on morphological characteristics, identity was confirmed using sequences of the internal transcribed spacer region of the ribosomal DNA (ITS rDNA) as described by White et al. (1990). About 100 mg of the tissue/mycelium was homogenized using liquid nitrogen and the powdered tissue was transferred to a microcentrifuge tube. DNA was extracted using a kit (NucleoSpin[®] Plant II Kit). The ITS rDNA region of the isolates was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR product was purified using ExoSAP-IT treatment. Sequencing was done using the BigDye Terminator v3.1 method. The sequences then aligned and were compared with the related *Pythium* sequences deposited at the National Center for Biotechnology Information (NCBI) using BLAST search.

Evaluation of pathogenicity of the isolates

The three *Pythium* isolates obtained were tested to prove Koch's Postulates (Pathogenicity) by *in vitro* and *in vivo* methods. To prove pathogenicity by *in vitro* method, healthy and mature seedlings of ginger were collected and washed with 70% ethyl alcohol followed by sterile distilled water. The seedlings were incubated in sterile plastic boxes containing wet filter paper and moistened cotton. For each isolate and also for the control, three seedlings were used. A small pin-prick was made at the base of each seedling using a sterile needle and an agar disc (*Pythium* culture) collected from the margin of actively growing fungal isolate was placed on the pricked area, in a way that the fungal mycelia are in contact with pin-prick. Seedlings with agar disc, without pathogen, were placed on pricked regions and maintained as control. The humidity inside the inoculation box was maintained by placing wet absorbent cotton and with frequent wetting. The box was incubated at room temperature for five days. The part of the rhizome of seedling which showed the infection was cut along with the healthy part and reisolated the pathogen by using PDA medium, as mentioned earlier.

For *in vivo* evaluation on pathogenicity, the ginger seedlings were raised in earthen pots filled with a potting mixture containing soil, sand and farmyard manure (2:1:1) and grown under greenhouse conditions. The isolates of *Pythium* were subcultured onto PD (Potato Dextrose) broth using mycelial plugs (5 mm) taken from the advancing margin of five days old pure culture of each isolate. Ten days old suspension culture of *Pythium* grown on PD broth was used for pathogenicity

tests. To prepare the inoculum for testing, the suspension culture, raised in a 250 ml conical flask was homogenized in a mixer grinder, made up to 1000 ml. The fungal suspension was inoculated to the soil, in pots with two months old ginger seedlings (50 ml/pot). The soil around the plant was mixed with culture suspension thoroughly for uniform distribution of pathogen in soil. The plants without inoculum served as control. For each isolate and control, three replications were maintained. After the incorporation of culture into the base of seedlings, the plants were irrigated regularly to maintain good moisture and humidity. Regular observations were made for the appearance and development of symptoms. The part of the rhizome which showed the infection was cut along with the healthy part and inoculated onto pre-sterilized PDA for reisolation of the pathogen as mentioned earlier.

Results

Collection, isolation and identification of the pathogen

Infected ginger samples showed marginal yellowing of the leaves, except for the midrib portion. Some pseudostems were drooped (Fig. 1). The rhizomes were partially rotten, showing signs of soft rot infections. After the 24 h incubation period as mentioned earlier, the plated ginger pieces began growing mycelium radially from the infected rhizome pieces.

Identification of *Pythium* isolates has been done based on morphological and molecular characteristics. The different isolates of *Pythium* grown on PDA and CMA exhibited different growth rates (av. 34.3 mm on PDA and 36.7 mm on CMA) but have floral cottony type colony morphology (Fig. 2a). The sizes of the main hyphae were 7–9 μm . Sporangia were observed as filamentous inflated or torulated structures with swollen side branches and mostly terminal (Fig. 2c). The encysted zoospores were 8–12 μm in diameter. The production of oogonia was observed in isolates after 48 h by the grass leaf method. They were smooth, terminal and globose, 16–24 μm (av. 19.7 μm) in diameter. These characters showed close similarity with *P. aphanidermatum* but the bending of oogonial stalks towards the antheridia was the unique character of *P. deliense* (Fig. 2b). Antheridia monoclinous, broad, apical and/or intercalary (6.2–8.3 μm length). Oospores were highly aplerotic, 15–19 μm (av. 16.3 μm) diameter and wall thickness were up to 2 μm . The identity of *Pythium* isolates was further confirmed molecularly. Amplicons of the expected size were sequenced and a BLAST search gives the closest similarity with *P. deliense* having 100% resemblance. The culture was registered and obtained the Facesoffungi number (FoF10628).

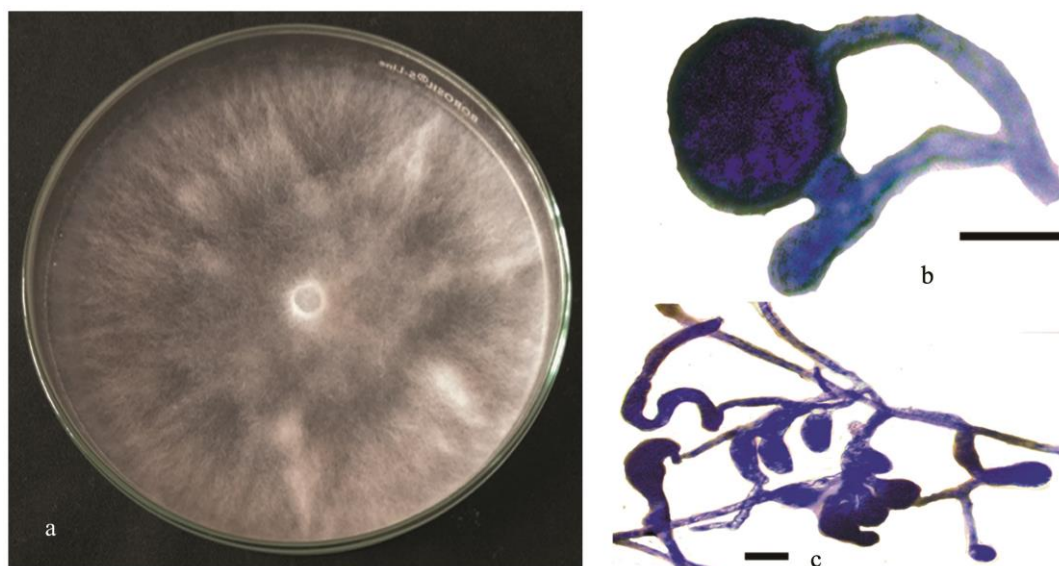


Fig. 2 – Morphology of the pathogen. a Colony morphology on PDA. b Oogonium and antheridium. c Sporangia. Scale bars: b-c = 10 μm .

Pathogenicity of the isolates

Pathogenicity of the different isolates of *P. deliense* was proved under both *in vitro* and *in vivo* conditions. Inoculation with *P. deliense* on ginger rhizomes developed symptoms after 48 h of incubation as brownish spots. The size of the lesion increased further after 96 h. The rhizome pieces along with a region of the dark coloured lesion were incised, cleaned, surface sterilized and inoculated onto PDA. White floral cottony mycelia were found to grow after 24 h of incubation showed the highest similarity and characteristics of the fungus *Pythium* which was fulfilling Koch's postulates (Fig. 3c-d).

On inoculating the plants with *P. deliense* under greenhouse conditions, symptoms such as foliar yellowing at the tip of the lower leaves started to appear within 5 days. It progressed along the margins of the leaves except for the midrib portion and after 15 days of inoculation, yellowing appeared on the upper leaves also. When the plants were uprooted, slight rotting of the rhizomes was observed. The pathogen was re-isolated and proved Koch's postulates (Fig. 3a-b).



Fig. 3 – Pathogenicity assay. a *in vivo* study controls. b *in vivo* study test. c *in vitro* study control. d *in vitro* study test.

Discussion

Rhizome rot caused by *Pythium* spp. is the most destructive disease of ginger resulting in heavy crop loss all over the world, wherever ginger is being grown (Dohroo 2005, Stirling et al. 2009, Chattopadhyaya 1997). Le et al. (2014) reported that about 15 *Pythium* species have been confirmed as pathogens that cause soft rot disease in ginger all over the world. In India, *Pythium* spp. causing soft rot disease in ginger was first reported long back, around 100 years ago (Butler 1907), from Surat (Gujrat) and it was identified as *P. gracile*. Thereafter, many other *Pythium* spp. affecting ginger has been recorded. Among them, there were only two reports (Haware & Joshi 1974, Jooju 2005), stating that *P. deliense* as soft rot causing pathogen in ginger. As mentioned earlier, there are many research findings, supported the pathogenicity of *P. deliense* as the soft rot pathogen in many crops, but soft rot incidence in ginger due to *P. deliense* was not reported from Kerala, hence, this is the first report. Moreover, the pathogen character shares its identity with the isolates reported by previous workers (Drechsler 1957, Lodhi et al. 2004).

As we understood, *P. deliense* is mainly a soil borne fungus and a water loving pathogen. The pathogen gets multiplied under high humid conditions during the rainy season and cause rotting on juvenile roots of ginger cause root rot. The rotting progress from roots and ultimately result in rotting on the rhizome. The infected rhizomes become brownish discolouration and infected tissues

become macerated. From the infected rhizome, the pseudostem comes off easily. As the already documented soft rot symptoms in ginger due to various species of *Pythium*, the symptoms due to *P. deliense* is also found the same with yellowing of leaves, progress from lower to upper leaves and ultimately the infected plants get toppled off. The rapid spread of infection in the fields under low drainage and with water stagnated conditions.

The morphological characteristics, pathogenicity studies and molecular characteristics confirmed the identity of *P. deliense*, a pathogenic species that causes soft rot in ginger in Kerala. The present study and identification of pathogen revealed the importance of *Pythium deliense* as a virulent pathogen, associated with soft rot disease in *Zingiber officinale* Rosc. in Kerala.

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