Characterization of *Protomyces macrosporus* causing stem gall of coriander (*Coriandrum sativum*)

MUNISH LEHARWAN^{1*}, MEENU GUPTA², ANJALI CHAUHAN², ARTI SHUKLA³ and UTTAM KUMAR¹

Y S P University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh 173 230, India

Received: 14 November 2019; Accepted: 27 July 2020

ABSTRACT

Coriander (*Coriandrum sativum* L.) is an annual herbaceous plant and suffers from different fungal, bacterial and viral diseases of which stem gall caused by *Protomyces macrospores* is one among them. The disease appears in the form of tumour like swellings of leaf veins, leaf stalks, peduncles, stems and on fruits. The test pathogen was isolated from infected stem and seeds and pure culture of the fungus was obtained on potato dextrose agar enriched with yeast extract medium. The colour of colony was creamy white initially which later turned light brown. Morphological characters comprising chlamydospores were studied which were yellowish brown, spherical or globose to oval, single-celled, smooth and measured 40-80 µ in diameter. For molecular characterization, genomic DNA was isolated using standard procedures and a region of the nuclear rDNA gene containing the internal transcribed spacer regions was amplified from the genomic DNA. Phylogenetic tree showed that isolated strain A clustered close to *Protomyces inouyei* with 62% similarity only. Based on morphological characteristics, the isolated fungus was identified as *Protomyces macrosporus* but on the ITS sequences comparisons, strain A was similar to *Protomyces* sp. Pathogenicity tests showed that crushed powder of infected seed added to soil was better in disease development over mycelial suspension and the symptoms of the disease appeared in 42.5 days.

Keywords: Coriander, Morphological, Molecular, Protomyces, Stem gall

Coriander (Coriandrum sativum L.) is an annual herbaceous plant (2n=22) belonging to the family Apiaceae and generally grown in winter season as main crop in India (Singh and Verma 2015). It is commonly known as cilantro or dhania or Chinese parsley and has originated in Mediterranean region. In India, this seed crop is grown in about 662.3 thousand ha with an annual production of 609.3 thousand tonnes and productivity of 0.9 MT per ha (Spices Board 2017). The crop is widely grown and suffers from at least 20 fungal diseases (Mukherji and Bhasin 1986) and number of bacterial and viral diseases, of which stem gall of coriander caused by *Protomyces macrosporus* has been found to be predominant and destructive one and is of common occurrence wherever this crop is grown. The disease appears in the form of tumour like swellings of leaf veins, leaf stalks, peduncles, stems and also on fruits. Systemic infection provides greater distortion to the plant

Present address: ¹ICAR-The National Dairy Research Institute, Karnal; ²Dr Y S Parmar University of Horticulture and Forestry, Solan; ³KVK, Dr Y S Parmar University of Horticulture and Forestry, Solan. *Corresponding author: munishleharwan7@ gmail.com.

and plant parts. The inflorescence may show outgrowth on the surface and uniform invasion of the fruit making it abnormally large but partial invasion may lead to distortion (Rao 1972). The diseased seed are hypertrophied depending upon the stage of infection, ultimately lowering the crop yield and quality. Seed lose their value in respect to seed and consumption (Kumar *et al.* 2014). The genus *P. macrosporus* Unger has been reported to be obligate parasite within the *Apiaceae* family causing galls on stems, leaves, flowers and fruits by various researchers (Lakra *et al.* 2001 and Mishra *et al.* 2017). In the present study, the associated pathogen was isolated using modified media and its cultural, morphological, pathological and molecular characterization has been undertaken.

MATERIALS AND METHODS

Disease surveys: Various coriander growing localities of Solan district of Himachal Pradesh were surveyed during 2016–17 crop season to record the incidence and severity of stem gall of coriander. The infected aerial plant parts were collected in paper bags and brought to the laboratory for further studies. The percent disease incidence was calculated as per the standard formula (Lakra 2001). However, for recording the disease severity (%); a severity scale given by Lakra (1991) was followed (Table 1). The disease severity (%) was calculated according to the formula given by Mckinney (1923).

Table 1 Scale for recording severity of stem gall of coriander

Plant part	Score/Rating
Stem	30 points
Leaves	20 points
Pedicel	20 points
Fruits	30 points
Total	100 points

Isolation of causal organism: Infected seeds and stems with galls were used for the isolation of the test fungus under in vitro conditions. Small bits of 1 to 2 mm size were taken from the galls with the help of sterilized blade. These bits were surface sterilized with sodium hypochlorite (5%) for 2 to 5 min and then washed thrice with sterilized distilledwater and placed on sterilized filter paper to remove the excessive moisture and subsequently transferred to potato-dextrose agar (PDA) enriched with yeast extract (2%) media slants under aseptic conditions and incubated for 3-4 days at 20±1°C. The isolated fungus was purified by hyphal tip method and maintained on PDA medium at 4°C.

Cultural and morphological characterization: The isolated fungus was grown on PDA medium enriched with yeast extract and observations on colour and pattern of cultural colony were recorded. The morphological characters of the pathogen were studied on the host and sections from diseased portions from infected seeds and stems were examined under microscope in the laboratory. The size and shape of chlamydospores was also recorded. The identity was confirmed on the basis of morphological characters documented in standard authentic descriptions and taxonomic keys as given by Pavgi and Mukhopadhayay (1969).

The infected parts of coriander plant were collected and tested for Chlamydopsore germination. The method described by Haware and Pavgi (1976) where in diseased part of coriander bearing mature galls were collected and stored at 10°C. After 120-180 days, mature chlamydospores teased out of the diseased tissues were adhesized to glass slides by the alternate wetting and drying method described by Thirumalachar and Narasimhan (1953). The smears were steeped in acidulated water (0.5% H₂SO₄) for 20 min and rinsed in distilled water and then inverted over wet cotton towelling (Thirumalachar and Pavgi 1950). Germinating chlamydospores were fixed and stained using lactophenol to study the sequence in the process of germination and variations in the process.

Molecular characterization: Total genomic DNA was extracted from isolated *Protomyces* sp. by using standard method (Murray and Thompson 1980). Fungal tissues were harvested from 7 days old culture grown in potato dextrose broth enriched with yeast extract. Approximately 1 g of ground mycelium was taken into a micro centrifuge tube containing 1 ml of pre-warm (65°C) DNA extraction buffer (DNA extraction buffer: 100 mM Tris-HCl (pH 8.0), The 20 mM ETDA (pH 8.0), 1.4 M NaCl, 2% (w/v) CTAB,

0.2% (v/v) 2-mercaptoethanol. To analyse the isolated Protomyces sp. at sequence level, a region of the nuclear rDNA gene encoding for the regions (ITS) was amplified from the genomic DNA of Protomyces sp. by PCR using the primer ITS 1 (5'TCCGTAGGTGAACCTGCG3') and ITS 4 (5'CTCCGCTTATTGATATGCT3').

Polymerase Chain Reaction (PCR) was performed by following standard protocol. The PCR amplified product was eluted from the gel using GeneJET Gel Extraction Kit (Thermo Scientific) and Sanger sequenced (Xceleris Genomics, India). Sequenced data so obtained was analyzed with the help of Bioinformatics tools like NCBI-BLAST, Bio Edit, Clustal W and Mega 5.

Pathological characterization: Pathological characterization was done by following procedures.

Raising of plants: The healthy coriander seed of susceptible variety Rajendra Swati were sown in small sterilized pots (10 cm) which were filled with sterilized soil (570 g) in laboratory by placing at a depth of 2.5 cm in the soil. The standard crop raising practices were followed. After germination, 10 seedlings were maintained in each pot.

Preparation of inoculum

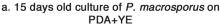
Chlamydospore inoculum and spore suspension: Diseased stem and seeds of coriander infected plants were collected from the field and were shade dried and powdered in a pestle mortar. This powdered material containing 7.5 × 10⁴ chlamydospores/g of crushed material was used as inoculum. Broth of potato-dextrose + yeast extract medium was inoculated with bits (6-8 mm) of culture of test fungus and was incubated at 20±1°C in BOD incubator. The spore suspension containing approximately 5.9×10⁴ spores/ml was used as inoculum.

Inoculation methods: The pathogenicity tests were conducted on coriander plants in pots by making the soil sick. Ten plants, maintained in each pot, were inoculated with the chlamydospores (2 g) or with spore suspension (5 ml) of the pathogen. After irrigation, these pots were kept in humidity chamber at 20+1°C temperature and 85% RH. The control pots were also maintained simultaneously without having inoculum. Observations on incubation period in days and disease progress in terms of disease severity after inoculation were recorded at weekly interval up to six weeks. Koch's postulates were established by re-isolating the fungus from diseased plants and were confirmed by comparing them with the original culture.

RESULTS AND DISCUSSION

Disease was observed in 12 out of 19 locations surveyed in Solan district, Himachal Pradesh and no disease was recorded at seven locations surveyed. The disease incidence was found highest at Pandah farm (56.33%) followed by Khaltoo farm (42.66%) and Naganji farm (23.57%) of the university. Similar trend was observed for disease severity which was also observed to be maximum at Pandah farm (53.24%) followed by Khaltoo farm (48.98%) and Naganji farm (36.76%) of the University. However, the disease was







b. Germinating chlamydospore of *P. macrosporus* (400X)

Fig 1 Cultural and morphological characters of Protomyces macrosporus.

either not found prevalent or low incidence and severity of the disease were recorded at farmer's fields surveyed in Solan district.

The non-prevalence or low incidence and severity of the disease at the farmer's fields surveyed could be due to the reason that the farmers grow the crop from the local collections of their own and do not procure the seed from the market and thus, it may be free from stem gall infection. The seed which was grown in the University farms was earlier purchased from the market and might be infected with the disease. Moreover, the inoculum from the soil might further aggravate the incidence as this pathogen is both seed as well as soil borne (Gupta 1954 and Mishra *et al.* 2017). Verma *et al.* (2014) reported its occurrence from different sites of Jabalpur, Madhya Pradesh. In Himachal Pradesh, Paul (1992) observed the disease intensity between 10.76–60%, 0.32–7.3% and 0.2–2.35% in the district of Kangra, Mandi and Kullu respectively.

Cultural characterization: The cultural characters of the test fungus were studied on PDA enriched with yeast-extract medium. The culture was slow growing and attained a diameter of 3-4 mm after 7 days of incubation at $20\pm1^{\circ}$ C. The colour of colony was creamy white initially (Fig 1a) which later turned light brown. The surface of the colony was pasty with slimy lobed in the appearance and surrounded by an uneven margins.

Many workers have reported this fungus to be obligate parasite within the *Apiaceae* family causing galls on stems, leaves, flowers and fruits (Lakra 2001, Khare *et al.* 2014 and Mishra *et al.* 2017), however, Pavgi and Mukhopadhyay (1969) isolated this fungus from the infected parts of the coriander plant on different cultural media such as malt agar, Richard's agar, Lilly and Barnett's semi-synthetic medium

and potato dextrose agar media and reported similar cultural characters. Pavgi and Haware (1970) purified the culture colonies of two *Protomycopsis* sp. by cultivating on potatodextrose agar enriched with 0.1% yeast-extract medium.

Morphological characterization: Infected stems and seeds kept from previous year were observed under microscope for shape and size of chlamydospores. The chlamydospores were yellowish brown, spherical or globose to oval, single celled, smooth and measured 40-80 μ in diameter. It had a thick covering whose diameter was 2-5 μm . In germination studies, the chlamydospores initiated germination after 24 hr of incubation at 24±1°C and swelled slightly by rupturing the outer layer and vacuoles appeared in the centre (Fig 1b). The extruded vesicle was globose to oval or cylindrical, thin-walled and elastic with hyaline to pale yellowish in colour. The germ tube of spore was hyaline, long and cylindrical.

Pavgi and Mukhopadhyay (1969) have also observed that the distinguishing morphological characteristics of *P. macrosporus* are the formation of chlamydospores or endospores. A mature chlamydospore was protected externally by hard, thick and unsculptured exospores enclosing a medium thick mesospore and membranous endospore. On the basis of cultural characteristics, viz. colour and type of colony and morphological characters, viz. shape, size and germination of chlamydospores, the fungus was identified as *Protomyces macrosporus*.

Molecular characterization: The genomic DNA was successfully isolated from the isolate and was found good to be used as a template for PCR amplification. PCR amplification using ITS1 and ITS4 primers revealed a unique band of 400 bp was obtained for *Protomyces* sp. Purified PCR products were sent to Xcleris Genomics, India under

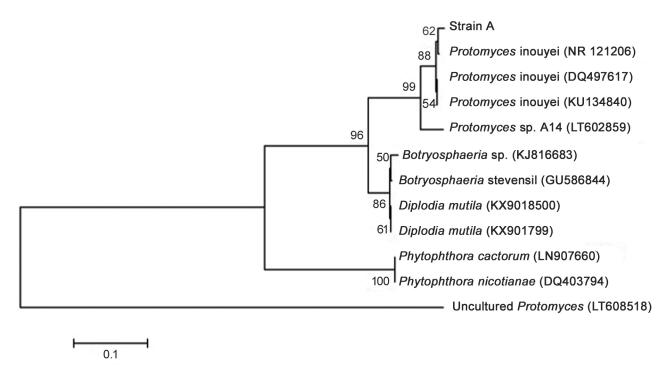


Fig 2 Phylogenetic tree based on the ITS sequences showing relationship between the isolated strain A and other sequences deposited in the NCBI Gene bank.

refrigerated conditions. A sequence of 400 bp corresponding to ITS region of *Protomyces* sp. was obtained. The sequence was BLAST analysed, which showed *Protomyces inouyei* as its closest match having 99% similarity with this strain. Weber *et al.* (2002) also reported the amplicon size of 573 bp for *Protomyces inouyei*.

Phylogenetic analysis of ITS region of isolated fungus was compared with the closest NCBI BLAST sequences, and a phylogenetic tree was constructed using maximum livelihood analysis (Fig 2). Phylogenetic tree showed that isolated strain A clustered close to Protomyces inouyei having accession no. NR121206. The other close associates of strain were Protomyces inouyei having accession no. DQ497617 and Protomyces sp. having accession no. LT602859. Based on morphological characteristics, the isolated fungus was identified as Protomyces macrospores but on the ITS sequences comparisons, it was found that strain A was similar to Protomyces sp. The genus Protomyces is very closely related to the genus Taphrina based on nSSU rRNA sequences (Nishida and Sugiyama 1994). Sjamsuridzal et al. (1997) found that Protomyces always appears as a monophyletic group.

Pathological characterization: Out of two inoculum sources, crushed powder of infected seed added to soil was better in disease development over spore suspension and the symptoms of the disease appeared in 42.5 days in comparison to spore suspension wherein symptoms developed in 51 days. The galls on stem arise mainly from the cortical and phloem cells by hypertrophy and hyperplasia. The galls formed on leaves were translucent and yellow-white. Galls formed on the mid-vein and lateral veins were minute and elongated

ones and showed puckering. These swellings later make the leaves rough and weak so that the leaves become crunched and bend down. Data also revealed that crushed powder of infected seed added to soil produced symptoms on stems in 40 days and on leaves in 45 days, whereas spore suspension resulted in symptom development on stem in 48 days and on leaves in 54 days which is the highest incubation period, while no symptom developed in control plants.

Pathogenicity tests of stem gall of coriander have been conducted by various workers (Pavgi and Mukhopadyay 1969, Chauhan et al. 1981, Valverde and Templeton 1984, Khan and Parveen 2016) and they have also revealed similar symptom development on stem and leaves. The longer incubation period on other plant part than stem may be ascribed to systemic infection of pathogen (Pavgi and Mukhopadhyay 1972). From the results, it can be concluded that associated fungus with stem gall of coriander was isolated in pure culture and identified as Protomyces macrosporus on the basis of morphological characteristics, i.e. the cultural characteristics of the pathogen, size and shape of chlamydospore of the fungus. The colour of colony was creamish white and chlamydospores were globose in shape and measured 40- 80 µm in size. The genera was further confirmed using molecular characterization and based on the ITS sequence comparisons, the isolated strain A clustered close to *Protomyces* sp. with 99% similarity with accession no. LT602859. Pathogenicity tests revealed that the symptoms on stems developed after 44 days of incubation, irrespective of type of inoculum and resulted in the formation of galls. Similarly, symptoms on leaves appeared after an incubation of 49.5 days, irrespective of type of inoculum used. The galls formed on leaves were translucent and yellow-white. Galls formed on the mid-vein and lateral veins were minute and elongated ones and showed puckering. These swellings later make the leaves rough and weak so that the leaves become crunched and bend down.

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