First report of an atypical strain of Phytophthora inundata causing Kinnow mandarin decline in India

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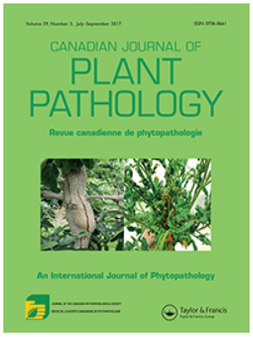

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Disease report/Rapport des maladies

First report of an atypical strain of *Phytophthora inundata* causing Kinnow mandarin decline in India

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Abstract: Kinnow mandarin is an important commercial fruit crop in India. A *Phytophthora* species was recovered from rhizosphere soil of declining Kinnow mandarin trees at Abohar area, Punjab state, India in January 2015. Morphological and physiological characteristics coupled with ITS-RFLP and sequence analysis of the ITS and *cox*1 gene regions revealed the isolate was an atypical strain of *P. inundata*. The isolate had ovoid to limoniform non-papillate sporangia and produced thick-walled, spherical oogonia with amphigynous antheridia. Thin-walled chlamydospores or hyphal swellings with radiating hyphae were formed readily in water in greater abundance than sporangia. This atypical strain of *P. inundata* was heterothallic (A1 mating type) and grew very slowly at 35°C. The optimum temperature for growth was 25°C and the isolate was metalaxyl-sensitive. Pathogenicity tests indicated that the strain of *P. inundata* has the potential to infect leaves and stems of Kinnow mandarin and was also pathogenic to rough lemon rootstock seedlings, causing root rot and decline. To our knowledge, this is the first report of *P. inundata* in India.

Keywords: *Citrus nobilis* Lour × *C. deliciosa* Tenora, ITS-RFLP, *Phytophthora inundata*, rough lemon

Résumé: La mandarine Kinnow est une importante culture fruitière commerciale en Inde. En janvier 2015, une espèce de *Phytophthora* a été récupérée du sol de la rhizosphère de mandariniers Kinnow dépérissants de la région d'Abohar, dans l'État du Pendjab, en Inde. Les caractéristiques morphologiques et physiologiques ainsi que le RFLP de l'ITS et l'analyse de la séquence des régions de l'ITS et du gène *cox*1 ont révélé que l'isolat était une souche atypique de *P. inundata*. L'isolat possédait des sporanges non papillés de forme ovoïde à forme de citron et produisait des oogones sphériques à paroi épaisse avec des anthéridies amphigynes. Des chlamydospores à paroi mince ou renflements d'hyphes, dont les hyphes étaient ramifiés, étaient formés facilement dans l'eau, et ce, en plus grande quantité que les sporanges. Cette souche atypique de *P. inundata* était hétérothallique (type sexuel A1) et croissait très lentement à 35°C. La température optimale de croissance était 25°C et l'isolat était sensible au métalaxil. Des tests de pathogénicité ont révélé que la souche de *P. inundata* pouvait infecter les feuilles et les tiges du mandarinier Kinnow et qu'il était également pathogène à l'égard des plantules porte-greffe du rough lemon, causant le pourridié des racines et le dépérissement. À notre connaissance, il s'agit de la première mention de *P. inundata* en Inde.

Mots clés: *Citrus nobilis* Lour × *C. deliciosa* Tenora, *Phytophthora inundata*, RFLP de l'ITS, rough lemon

Introduction

The oomycete genus *Phytophthora* includes soil- and water-borne plant pathogens that can cause significant damage in both agricultural and natural ecosystems. These pathogens pose major challenges to global biosecurity (Callaghan & Guest 2015). *Phytophthora* species affect a broad range of hosts worldwide, and with the expansion of global trade and human travel, there has been an escalation in the incidence of diseases they cause (Kroon et al. 2012). Several novel *Phytophthora* species have been described in the last decade. A *Phytophthora* species isolated from trees and shrubs in the early 1970s was designated as *Phytophthora* sp. 'O-group' (Brasier et al. 1993). This species was also isolated from a variety

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of riparian habitats in Europe. Through molecular characterization studies, it was found to be a member of ITS Clade 6 of the phylogeny-based classification of Cooke et al. (2000a) and a close relative of *P. humicola*. This pathogen was subsequently designated as a new species, *Phytophthora inundata* Brasier, Sánch. Hern. & S.A. Kirk (Brasier et al. 2003).

Phytophthora inundata is mainly associated with root and collar rots in ornamental and horticultural shrubs and trees. Ornamental plants such as *Aesculus* and *Salix* spp. and economically important plants including *Olea* and *Prunus* spp. are reported to be susceptible to *P. inundata* infection, usually during flooding or on very wet soils (Brasier et al. 2003). Besides this, *P. inundata* has been isolated from different regions and sources, including in California from alfalfa roots (Ho et al. 2006), infected roots and crown tissues of citrus trees in Chile (Vial et al. 2006), dead grasstree, *Xanthorrhoea preissii* in the south-west of Western Australia (Stukely et al. 2007), in Virginia, USA from *Nicotiana tabacum* (tobacco) stem pith and rhizosphere soil (Parkunan et al. 2010) and recently from necrotic root and crown tissues of olive trees in Turkey (Kurbetli et al. 2016).

Kinnow mandarin, a hybrid between King and Willowleaf mandarins (*Citrus nobilis* Lour × *C. deliciosa* Tenora) is one of the most important commercial fruit crops in India and its cultivation is highly profitable, especially in the north-western part of the country, consisting of Punjab state and the adjoining areas of Rajasthan state. In a field survey conducted in 2015, severe decline of Kinnow mandarin trees was observed in an orchard in the state of Punjab, India and a *Phytophthora* sp. was recovered from the rhizosphere soil of declining trees. The aim of this study was to identify the *Phytophthora* isolate employing morphological and molecular characterization data and to evaluate its pathogenicity on Kinnow mandarin.

Materials and methods

Isolation and morphological characteristics

Several declining Kinnow mandarin trees were observed in a 0.5 ha orchard in the village of Killanwali in Abohar, Punjab, India (30°8′45″N, 74°6′59″E) in January 2015. The grove was low-lying, water-logged and muddy. Affected trees showed low vigour and poor appearance with yellowing and dropping of leaves. A disease-ridden tree also exhibited die-back of twigs where dead shoots stood out prominently (Fig. 1). Fibrous roots and soil samples from the rhizosphere of declining Kinnow mandarin trees (budded on rough lemon (*Citrus jambhiri*) rootstock), were collected from the grove and immediately transported to the laboratory at the Central Citrus Research Institute (CCRI), Nagpur. For isolation of the pathogen, 20 cc of soil sample along with fibrous roots was suspended in 80 mL water supplemented with 0.25% of bacteriological agar. One ml of this suspension was spread on corn meal agar (CMA) – pimaricin-ampicillin-rifampicin-PCNB-hymexazol (PARPH) medium (Kannwischer & Mitchell 1978) in 90 mm Petri dishes and incubated in darkness for 48 h at 25°C. Dishes were washed with distilled water to remove the residual soil to observe the *Phytophthora* colonies. Agar discs of 5 mm diameter from the periphery of actively growing mycelia (of *Phytophthora*) were then placed on fresh PARPH medium to purify the isolate. After purification, one isolate (labelled as NRCPh-196) was grown at 25°C on CMA, V8 juice agar (V8A) and carrot agar (CA) media. A 5-day-old culture was used to obtain 5 mm diameter discs, cut from the leading edges of the growing culture for inoculation on CMA, V8A and CA and the dishes were incubated in the dark. Colony morphology was documented 5–7 days after transfer. The growth rate was recorded on all media by measuring the diameter (mm day^{-1}) of the culture daily after the 5th day till the 8th day. For cardinal growth temperature assessment, CMA plates were inoculated in triplicate and incubated at 5, 15, 20, 25, 30 and 35°C. Growth rate was recorded after 1 week. Tests were repeated twice for the range of 25–35°C.

Agar discs were cut from the growing edge of 5-day-old cultures grown on CA at 25°C in the dark, placed in Petri dishes, flooded with sterile distilled water and incubated at 25°C in the dark for 2–3 days to study the morphology of sporangia. Mating type of the isolate was determined by the single unknown isolate method

Fig. 1 (Colour online) Kinnow mandarin trees showing sparse canopy with die-back and decline symptoms at Killanwali village, Abohar, Punjab state, India.

(Kaosiri et al. 1980) with an A1 or A2 mating type tester isolate of *P. nicotianae* in dual cultures on CA. Sporangia and oogonia were observed at 40× magnification under a Nikon Eclipse Ni-U compound microscope.

Metalaxyl sensitivity

Metalaxyl sensitivity was determined by growing the isolate on CMA amended with metalaxyl (Krilaxyl Power containing 35% metalaxyl (Krishi Rasayan Export Pvt Ltd, New Delhi)) to the final concentration of 1, 5, 10 and 50 µg a.i. mL^{-1}. Five mm mycelial agar discs were placed on dishes of different concentrations and incubated in the dark at 25°C. The radius of the mycelial growth was recorded when the non-amended media dish (control) was completely covered by mycelial growth. An isolate was scored as sensitive (S) if colony growth on media amended with 5 µg mL^{-1} metalaxyl was less than 40% of the isolate growth on non-amended media (Silvar et al. 2006).

DNA extraction, PCR, sequence analysis and ITS-RFLP

Freshly colonized CMA plugs were subcultured in carrot broth and incubated at 25°C. After 5 days of incubation, mycelium was removed and blotted on sterile Whatman filter paper and DNA was extracted using the DNeasy Plant mini kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. The internal transcribed spacer (ITS) region (ITS1- 5.8S- ITS2) of the nuclear ribosomal DNA was amplified using the universal primers ITS6 (Cooke et al. 2000a) and ITS4 (White et al. 1990). Fragments of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene were also amplified using COXF4N and COXR4N primers (Kroon et al. 2004). Amplifications were performed in a Bio Rad T100 thermal cycler (Bio-rad Laboratories Inc, USA). The PCR products were sequenced using the same primers in both directions at Eurofins Genomics India Pvt Ltd (Bangalore, India) in an ABI Prism automated sequencer. Sequences of both directions were visualized with Finch TV v. 1.4.0. and aligned using Clustal W. Obtained sequences were compared with the sequences present in NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the BLASTn (Basic Local Alignment Search Tool) search algorithm to find highly similar sequences. Phylogenetic tree reconstruction was conducted in MEGA 5.1 (Tamura et al. 2011) by concatenation of the nuclear ITS region and the mitochondrial *cox*1 gene fragment with the maximum-likelihood method based on the Tamura–Nei model (Tamura & Nei 1993) with 1000 bootstrap replications. The GenBank accession numbers of *Phytophthora* species used for phylogenetic studies and accession numbers of the sequences derived in this study are shown in Table 1. *Pythium aphanidermatum* was included as an outgroup. The ITS–RFLP was performed by digesting ~900 bp amplified product using ITS4 and ITS6 primers with *Alu*I, *Msp*I, and *Rsa*I restriction enzymes (Fermentas, MD, USA), according to the manufacturer's instructions. The restricted products were electrophoresed in 3% agarose gel and documented.

Table 1. Accession numbers for *Phytophthora* spp. isolates obtained from the GenBank database used for phylogenetic analysis including the *P. inundata* isolate NRCPh-196 examined in this study.

Species	ITS[a]	COX-I[b]
P. boehmeriae	DQ297406	AY564165
P. infestans	AF266779	AY564150
P. mirabilis	AF266777	AY564153
P. nicotianae	AF266776	AY564196
P. cactorum	AF266772	AY564167
P. megakarya	AF266782	AY564193
P. palmivora	AF266780	AY564197
P. heveae	AF266770	AY564182
P. katsurae	AF266771	AY564190
P. citricola	AF266784	AY564170
P. ilicis	AJ131990	AY564186
P. inflata	AF266789	AY564187
P. citrophthora	AF266785	AY564171
P. colocasiae	AF266786	AY564173
P. taxon Walnut	AY659711	AY659731
P. gonapodyides	AF266793	AY564181
P. megasperma	AF266794	AY564194
P. humicola	AF266792	AY564184
P. inundata-SCRP644[c]	EF210200	EF210206
P. inundata-NRCPh-196[c]	KT633842	KU186666
P. fragariae	AF266762	AY564177
P. sojae	AF266769	AY564162
P. cinnamomi	AF266764	AY564169
P. cryptogea	AF266796	AY564174
P. drechsleri	AF266798	AY564175
P. syringae	AF266803	AY564203
P. lateralis	AF266804	AY564191
P. ramorum	AY54049	AY564208
P. insolita	AF271222	AY564188
Pythium aphanidermatum[d]	AF271227	AY564163

[a]ITS, internal transcribed spacers 1, 2 and 5.8S gene of rDNA (Cooke et al. 2000a).
[b]COX1, cytochrome c oxidase subunit I (Kroon et al. 2004).
[c]Isolate Code No. for *P. inundata* isolates.
[d]*Pythium aphanidermatum* was included as an outgroup.

Pathogenicity tests

Pathogenicity tests were conducted using detached leaf and excised stem tissues of Kinnow mandarin. For leaf inoculation, detached leaves were pin-pricked near the midrib and the inoculum of 5-mm-diameter CMA plugs

(face down) with actively growing mycelia were placed over the wounds. Control leaves were treated in the same manner but inoculated with sterile agar plugs with no mycelium. Similarly for excised stem inoculation, the surface of Kinnow mandarin stem was cut (2 cm) with a sterilized scalpel in such a way that only the vascular cambium was exposed and the green bark created a flap. The exposed cambium was inoculated with a CMA plug of the culture and covered with moist sterile cotton. Control stems were inoculated with plain CMA plugs without the culture. The leaves and stems were incubated at 25°C and monitored for lesion appearance on a regular basis. After 7–15 days, the diameter of the lesions was measured and photographed. Pieces of tissue from the leading edges of lesions were plated on CMA-PARPH for re-isolation of the pathogen.

Pathogenicity was also evaluated on rough lemon rootstock seedlings. Inoculum of the isolate was prepared in 50 mL carrot broth. After 4 days of growth at 25°C, the resulting mycelial mat was placed in a sterilized Petri dish containing 5 mL sterile distilled water, and incubated for another 4–5 days at 25°C under continuous white light to induce sporangial production. These cultures were then chilled at 4°C for 20 min and returned to room temperature for 1 h to induce zoospore release (Tjosvold et al. 2009). Ten 6-month-old healthy rough lemon plants were uprooted, and each was inoculated by immersion in 300 mL of 1.0×10^5 zoospores mL^{-1} of sterile distilled water for 24 h and repotted into 1.2 L pots with Soilrite Mix® growing medium. Five control rough lemon plants were inoculated by immersing roots in sterile distilled water alone. After treatment, pots were placed in shallow trays of water for 24 h to saturate the root zone, then removed from trays and incubated in a greenhouse at 25–30°C for disease development.

Results and discussion

The purified *Phytophthora* isolate, designated as NRCPh-196, formed slightly radiate to stellate pattern colonies with cottony mycelia on CA dishes, whereas on V8A, a petalloid pattern was observed with sparse aerial mycelium at 25°C after 5 days (Fig. 2*a*, *b*). Radial growth rate was 14.1 mm day^{-1} on CA and 11.1 mm day^{-1} on V8A at 25°C. Isolate NRCPh-196 showed growth at the minimum temperature of 5°C, with optimum growth at 25°C, and very slight colony growth at 35°C. Hyphal swellings (Fig. 2*c*) were observed in water culture. Thin walled chlamydospores (or globose hyphal swellings) with radiating hyphae were also noticed very frequently in water culture (Fig. 2*d*). Unlike earlier reports indicating no chlamydospore production (Brasier et al. 2003;

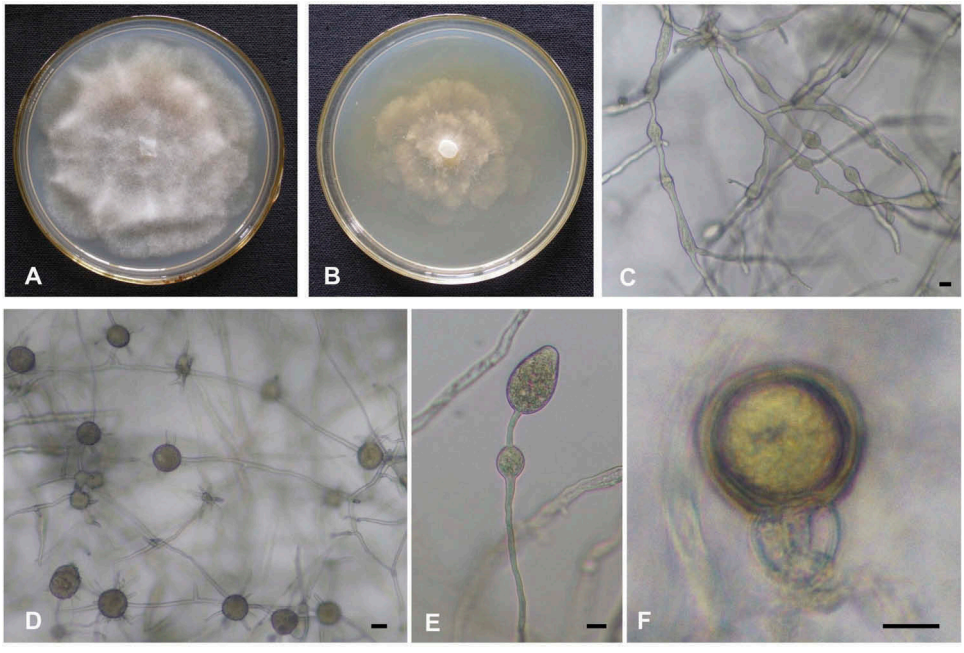

Fig. 2 Morphology of *Phytophthora inundata* isolate NRCPh 196. (**a**) Colony morphology on carrot agar, after 5 days at 25°C. (**b**) Colony morphology on V8 agar, after 5 days at 25°C. (**c**) Irregular catenulate hyphal swellings. (**d**) Globose hyphal swellings with radiating hyphae. (**e**) Limoniform sporangium with swelling on pedicel. (**f**) Oogonium with thick-walled oospore and amphigynous antheridia. Scale bars = 10 μm.

Safaiefarahani et al. 2013), the present isolate of *P. inundata* was observed to produce chlamydospores (average diameter was 20.8 μm). Sporangia (Fig. 2*e*) were non-papillate, non-caducous, ovoid to limoniform, and average (L × W) was 55.9 (range 39.1 – 71.8) × 32.8 (range 24.4–42.8) μm. The Length to Width ratio was 1.7.

The isolate was observed to be self-sterile and of A1 mating type, since it produced sexual spores when paired with A2 mating type tester of *P. nicotianae*. The isolate produced colourless, thick-walled, spherical oogonia with an average diameter of 29.6 μm (range 26.4–32.7 μm) on CA. Oospores (Fig. 2*f*) were aplerotic (average diametre 24.4 μm, range 22.9–27.4 μm), and the oospore wall was hyaline and averaged 5.2 μm in thickness. Isolate NRCPh-196 also displayed amphigynous antheridia averaging 14.5 (range 10.9–17.3) × 15.8 (range 14.1–17.9) μm (L × W). Based on these morphological characteristics, the isolate was tentatively identified as *Phytophthora inundata* Brasier, Sánch. Hern. & S.A. Kirk, particularly because of its heterothallic nature, which distinguishes it from the closest relative *P. humicola* which is self-fertile or homothallic (Jung et al. 2011). *Phytophthora inundata* has been reported to exhibit optimal growth at 30°C and a maximum upper temperature limit for growth of 35–37°C (Brasier et al. 2003). However, our isolate did not grow well at 35°C. Moreover, the sizes of oospores and sporangia appear to be smaller in the present isolate and the sporangia did not proliferate internally or externally, a trait typical of *P. inundata*. Hence, the isolate under present investigation can be described as an atypical strain of *P. inundata*. The isolate was deposited at the Microbial Culture Collection (MCC) (http://linux.nccs.res.in/mcc/general_deposit.html), Department of Biotechnology, Pune, India (accession no. MCC1237). The results of metalaxyl sensitivity tests revealed that the *P. inundata* isolate was sensitive to metalaxyl because the per cent growth rate on media amended with 5 μg mL^{-1} metalaxyl relative to the control was 22%, which is well below the 40% threshold.

Fig. 3 A maximum likelihood phylogenetic tree generated in MEGA 5.1. The tree was constructed using concatenated sequence data of the internal transcribed spacer (ITS) region and mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene fragment. The numbers on branches are bootstrap values (1000 replicates). The phylogenetic clades as designated by Cooke et al. (2000a) and Kroon et al. (2012) are indicated on the right. The *Phytophthora inundata* isolate examined in the present study is specified by a 'Purple dot'. *Pythium aphanidermatum* was included as an outgroup. Details of isolates are presented in Table 1.

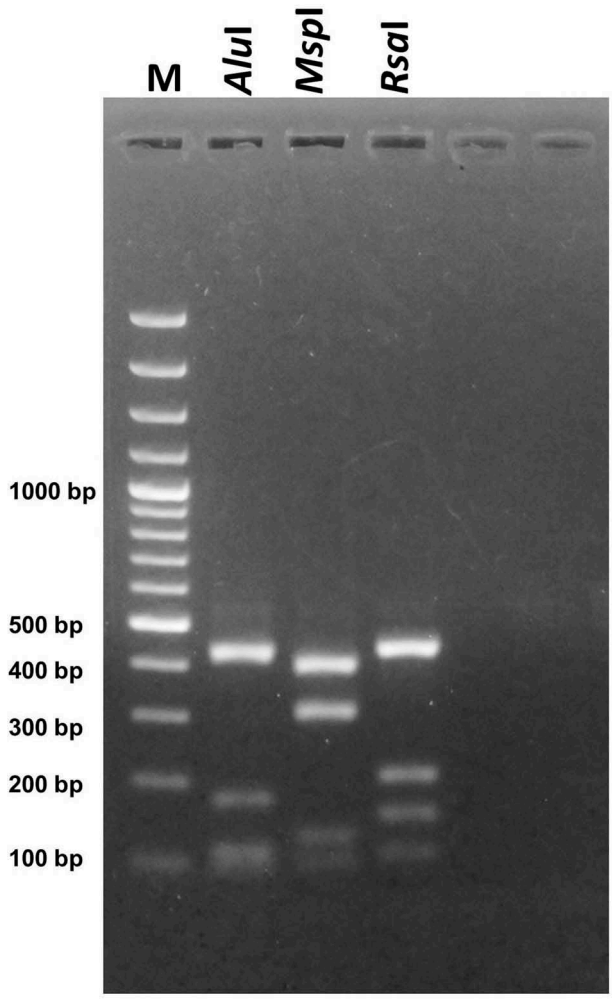

Fig. 4 (Colour online) Agarose gel showing restriction profiles of *Phytophthora inundata* isolate NRCPh-196 obtained after digestion of ITS6/ITS4 amplification products with restriction enzymes *Alu*I, *Msp*I and *Rsa*I. Lane M, 100 bp ladder.

Amplicon sequences from ITS and *cox*1 regions of isolate NRCPh-196 were compared with the existing GenBank accession of *P. inundata*, which showed maximum identity of 99% with KP420011 (Kurbetli et al. 2016) for the ITS region and 99% with EF210206 (Safaiefarahani et al. 2013) for the *cox*1 region. The nucleotide sequences of the ITS and *cox*1 regions of NRCPh-196 were submitted to GenBank with accession numbers KT633842 and KU186666, respectively. Our isolate also exhibited 99% similarity with some *P. humicola* isolates present in GenBank (e.g. Acc. No: AB367495 for ITS region). However, being heterothallic and with slightly growing colonies at 35ºC, our isolate of *P. inundata* was distinguished from *P. humicola*. *Phytophthora humicola* is a homothallic species and its maximum temperature for growth is 32ºC (Erwin & Ribeiro 1996; Gallegly & Hong 2008). Dual-loci (ITS and *cox*1)- based phylogeny (concatenated phylogenetic tree) revealed that isolate NRCPh-196 along with the *P. inundata* isolate SCRP644 segregated in one cluster when compared with closely related species of clade 6 and other *Phytophthora* species belonging to clades 1–10 (Fig. 3). *Phytophthora humicola* strain IMI302303 appeared as a sister group of *P. inundata*. On the basis of phylogenetic analysis, however, with 88% bootstrap value (Fig. 3), it is possible that isolate NRCPh-196 could be a new species of *Phytophthora* belonging to clade 6, closely related to *P. inundata*.

Restriction digestion of the NRCPh-196 ITS region (Fig. 4) with enzyme *Alu*I showed five bands of 427, 169, 112, 107 and 94 bp (last three seen as one broad band); while *Msp*I showed four bands of 402, 301, 125 and 98 bp, and *Rsa*I digestion showed four bands of 430, 210, 160 and 100 bp (Fig. 3). These results are in

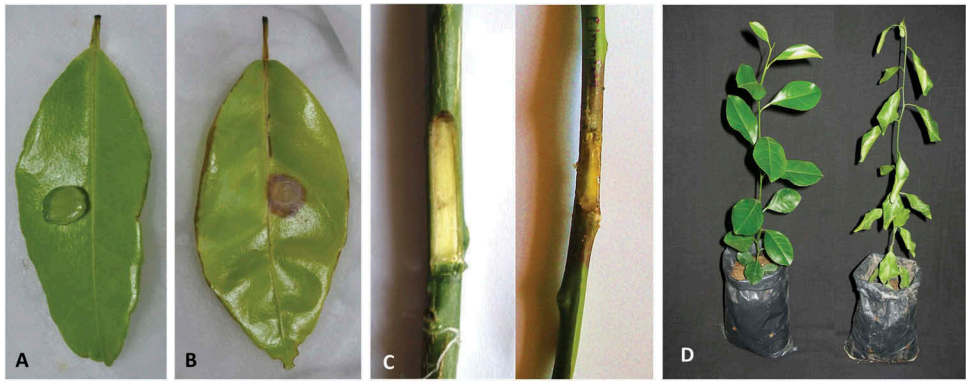

Fig. 5 (Colour online) Pathogenicity tests of *Phytophthora inundata* NRCPh-196 isolate. (**a**) Kinnow mandarin leaf control and (**b**) inoculated with *P. inundata*. (**c**) Development of brown lesion in Kinnow mandarin stem – Right, inoculated, Left, control. (**d**) Wilting and collapse of rough lemon seedling – Right, inoculated, Left, control after inoculation with *P. inundata*.

agreement with Cooke et al. (2000b) where these fragment sizes were obtained (with *Msp*I and *Alu*I restriction enzymes) in the case of *Phytophthora* sp. 'O' group. The ITS-RFLP profile of NRCPh-196 with *Rsa*I and *Msp*I clearly differentiated it from *P. humicola* and other *Phytophthora* species of clade 6, including *P. gonapodyides*, *P. megasperma* and *P. pinifolia* as reported previously (Durán et al. 2009).

Inoculated Kinnow mandarin leaves showed brownish-black necrotic lesion formation 7 days after inoculation (DAI) which indicated successful infection by *P. inundata* (Fig. 5*b*). Control leaves remained healthy (Fig. 5*a*). The pathogenicity trial using excised stems showed that the isolate was able to cause a progressive necrotic lesion from the site of inoculation in Kinnow mandarin stem (Fig. 5*c*), whereas the control inoculation produced no lesion. The length of lesion averaged 90 mm at 15 DAI. Inoculated rough lemon seedlings started showing foliar wilting at 5 DAI and at 10 DAI, seedlings showed complete wilting and decline symptoms (Fig. 5*d*). Roots were examined and brown necrosis and rotting symptoms were observed. Control rough lemon seedlings did not develop any disease symptoms (Fig. 5*d*). These results showed the susceptibility of rough lemon (commonly used rootstock for Kinnow mandarin in north-west India) to *P. inundata* infection. All the pathogenicity experiments were conducted twice with similar results. The pathogen (*P. inundata*) was re-isolated from all infected leaves, stems and roots on PARPH, indicating that successful colonization and infection occurred on the inoculated hosts.

The problem of waterlogging in south-west Punjab is broadly attributed to low-lying areas coupled with a lack of proper drainage, poor soil percolation because of impervious clay strata and constant seepage from the Rajasthan Feeder Canal and the Sirhind Feeder Canal, the main sources of irrigation water. *Phytophthora inundata*, as the name suggests, has a tendency to be found in areas of regular flooding (Brasier et al. 2003). It is of interest to note that the site from where *P. inundata* was recovered lies within 20 m of the water canal and is susceptible to regular flooding events. Hence preventing flooding or waterlogging, and establishing new plantations in well-drained soils, may help to circumvent the disease caused by *P. inundata*.

Members of the genus *Phytophthora* grouped with *P. inundata* in clade 6 are mainly reported as saprophytes or pathogens from riparian ecosystems and forests (Brasier et al. 2003). The pathogenicity of this aberrant strain of *P. inundata* to native flora in the Punjab state and elsewhere in India is unknown. In addition to citrus, it may pose a threat to other commercially cultivated species, especially under favourable soil and climatic conditions, based on its known host range. The extent of its distribution is also unclear. To the best of our knowledge, this is the first report of the occurrence of *P. inundata* in India and first record outside Europe, South America and Australia. Further investigations into its origin, infection mechanism and host range are warranted.

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