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Phytophthora nicotianae Causing *Dendrobium* Blight in Yunnan Province, China

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ABSTRACT

Dendrobium is a famous Chinese herbal medicinal plant and cultivated on a large scale in Simao City, Yunnan Province, China. However, it has suffered from blight in recent years. The aim of present study was to determine the identity of the pathogen causing the disease. Isolates were obtained from diseased plant tissues on *Phytophthora* selective agar medium and the morphology was studied by growing them on clarified V-8 juice agar medium. The pathogen produced non-caduceus, terminal, papillate and mostly obpyriform sporangia, av. 46.2×34.9 μm with the mean length/breadth ratio as 1.34. Chlamydospores were spherical, thick-walled (av. 30.2 μm diam.). It was heterothallic producing spherical, smooth oogonia (av. 24.0 μm diam.) and short, cylindrical amphigynous antheridia. The cardinal growth temperatures were 9, 27 and 37°C. The Internal Transcribed Spacer 1 (ITS1), 5.8S Ribosomal RNA gene and the Internal Transcribed Spacer 2 (ITS2) and β-tubulin gene sequence of the pathogen were also studied. The results showed 98 and 100% similarity with two isolates of *P. nicotianae* in the Genbank, confirming the identification as *P. nicotianae* based on morphological characters. The pathogenicity of the isolates was confirmed by satisfying Koch's postulates. This is the first world record of *Phytophthora nicotianae* causing blight of *Dendrobium thyrsiflorum*, *D. chrysanthum*, *D. aurantiacum* and *D. chrysotoxum* in plantations.

Key words: Root and basal stem rot, ITS sequence, tubulin sequence, orchid disease, oomycetes, Chinese herbal medicine

INTRODUCTION

Dendrobium, a genus of the Family Orchidaceae, plays an important role in traditional Chinese medicine (CNP, 2010). It is considered as a valuable and commonly used medicine to nourish the stomach, promote production of body fluid and reduce fever (Jiangsu New Medical College, 1986). It has been proven to inhibit the proliferation of tumor cells, promote anti-aging, enhance immunity, dilate the blood vessels (Chen *et al*., 2008; Tsai *et al*., 2010), effectively treat cerebrovascular diseases and the chemical composition of the active ingredients have been determined (Bi *et al*., 2002; Liu and Zhao, 2003; Zhao and Zhao, 2003; Tang *et al*., 2004; Yang *et al*., 2004, 2006; Ying *et al*., 2009; Tsai *et al*., 2010). However, wild *Dendrobium* is on the

verge of extinction and field collection has been banned. The medical enterprises were encouraged by the Chinese government to increase *Dendrobium* production using tissue-culture technique to multiply the plant and cultivating the clones in the fields on a large scale (Sun *et al*., 2006). Most species of *Dendrobium* prefer growing in forests with mild, moist climate (Lai *et al*., 2006) which tends to favor disease development due to zoosporic pathogenic fungi.

Phytophthora, with more than 95 species, is a major genus of plant pathogens attacking a wide range of agriculturally and ornamentally important plants throughout the world (Erwin and Ribeiro, 1996). It has been classified traditionally as an *Oomycetous* or *Pythiaceous* fungus but with recent advances in chemical, ultrastructural and molecular studies, *Phytophthora* spp. are now considered as fungallike organisms or pseudofungi with characteristics more common with the heterokont, biflagellate algae and are placed in the Kingdom Chromista (Agrios, 1997) or Kingdom Straminopila (Alexopoulos *et al*., 1996), distinct from the true fungi of the Kingdom Fungi. Members of this genus often caused serious diseases. For instance, *P. infestans* caused late blight of potato, resulting in the Irish famine in the 19th century. *P. sojae* attacked soybean throughout the world in the form blight, costing the soybean industry millions of dollars each year. More recently, *P. ramorum* led to massive sudden death of live oak trees and large varieties of woody shrubs that inhabit the oak ecosystems (Appiah *et al*., 2004; Rizzo *et al*., 2005). Since, Erwin and Ribeiro (1996) published descriptions of all *Phytophthora* species up to that time, 36 new species and 2 hybrid species of *Phytophthora* (Ersek and Ribeiro, 2010) have now been added.

Orchids are often susceptible to *Phytophthora* species, causing diseases known as black rot, brown rot, crown rot, heart and leaf rot, stem and leaf rot, top and shoot rot, leaf spot as well as seedling damping-off. Several species of *Phytophthora* have been implicated. *P. palmivora* is the leading pathogen attacking orchids in Singapore (Rossetti, 1943), Ceylon (Thompson, 1959), Korea (Soon-Yeong and Seung-Weon, 1998), Taiwan (Yeh *et al*., 1998), Hawaii (Uchida and Aragaki, 1991; Uchida, 1994), Florida (Cating *et al*., 2009), Poland (Orlikowski and Szkuta, 2006), Indonesia (Purwantara *et al*., 2004) and Philippines (Portales, 2004). *P. parasitica* on orchids has been found in Buenos Aires (Rossetti, 1943), Taiwan (Chen and Hsieh, 1978; Ann, 1995), Hawaii (Uchida and Aragaki, 1991; Uchida, 1994) and Australia (Duff and Daly, 2002), whereas, *P. cactorum* attacks orchids in Florida (Burnett, 1974; Cating *et al*., 2009) and Hawaii (Uchida and Aragaki, 1991; Uchida, 1994). Other minor orchid Phytophthoras include *P. erythroseptica* var. *erythroseptica* in Australia (Hall, 1989), *P. multivesiculata* in Holland (Ilieva *et al*., 1998) and occasionally in Taiwan (Tsai *et al*., 2006), *P. cinnamomi* in Hawaii (Uchida and Aragaki, 1991; Uchida, 1994) as well as an unidentified *Phytophthora* species in Taiwan (Chern and Ann, 1996).

In this study, we described a severe disease of *Dendrobium* in plantations of Simao City, Yunnan Province and demonstrated that the causal agent was *P. nicotianae*.

MATERIALS AND METHODS

Isolation of the pathogen: *Phytophthora* isolates were obtained from diseased plants randomly collected from the *Dendrobium* plantations of Simao City, Yunnan Province, China. They were washed under running tap water for 3-5 min., surface-sterilized by immersion in 70% ethanol for 5-10 sec and dried on filter paper. The interior tissues of the advancing edge of the necrotic zone of phloem lesions in the middle section of the stem were plated on Corn Meal Agar (CMA), amended with PARP (25 μg mL^{-1} pimaricin, 100 μg mL^{-1} ampicilin, 25 μg mL^{-1} rifampicin and 25 μg mL^{-1} pentachloronitrobenzene) (Erwin and Ribeiro, 1996). The isolation plates were incubated at 26°C. As soon as *Phytophthora* had developed, they were transferred several times to new agar plates for purification. The isolates were stored on V8-juice Agar (V8A) or Lima Bean Agar (LBA) slants at 16°C in darkness.

Morphological characteristics: Pure isolates were grown on clarified V8A, LBA, CMA, Oatmeal Agar (OMA), Carrot Agar (CA), Potato-dextrose Agar (PDA) and Potato Sucrose Agar (PSA) for colony pattern description, whereas V8A was used for morphological studies. All media were prepared as described by Erwin and Ribeiro (1996). Mycelial growth rates for all four isolates were compared on V8A at temperatures from 9 to 38°C. Diameters of three colonies on 3 plates were measured along two perpendicular lines through the center of the colony, at 2 day intervals for 7 days and the average radial increment per day was calculated. To induce the production of sporangia, an agar disc (6 mm diam) from the advancing margin of a young colony growing on 10% V8A for 3 day at 26°C was transferred into 20 mL clarified V8 juice in a 6 mm diam Petri dish. After incubation under ambient temperature and light conditions for 2-3 days the disc was carefully rinsed twice per day with sterile distilled water and immersed in Petri's mineral salt solution in the same dish. Zoospore release was achieved by chilling the Petri dishes at 10°C for 30 min and then returning them to 26°C. Sexual structures were produced by pairing D1 or D4 with D2 or D3 on V8C or LBA plates at 26°C. For detailed examinations and measurements, mycelium with reproductive structures was mounted on glass slides in lactophenol. One hundred and twenty randomly selected sporangia, chlamydospores and sex organs were measured using an OLYMPUS VANOX microscope equipped with LY-WN-HP SUPER CCD video camera and complementary ScopePhoto 3.0.12.444 Software.

Pathogenicity test: Pathogenicity tests were conducted in the greenhouse, using 2 to 3 year old plants of *Dendrobium thyrsiflorum, D. chrysanthum, D. aurantiacum* and *D. chrysotoxum*. Six millimeters mycelium agar plugs from the growing edge of a 5 day old colony on V8A at 26°C were put at the mid-point surface of both needle-wounded and intact stems of 2 to 3 year old *Dendrobium* plants. They were misted with water and placed in sealed plastic bags for 48 h. Four plants were inoculated for each of the four isolates and as the control, *Dendrobium* plants of similar age were inoculated with sterile plugs. Plants were maintained, fully randomized in the greenhouse at approximately 26°C daytime and 18°C night temperatures and disease development was monitored daily for two weeks. In the field, inoculation experiments were also similarly conducted.

DNA Isolation, PCR reactions and sequencing: In order to determine the ITS and β tubulin gene sequences D1 isolate was used and the following procedure was followed. An agar block derived from a single zoospore culture, was grown for 3-5 days at 26°C in V8 broth. The mycelium was harvested, lyophilized and total DNA extracted immediately using a CTAB DNA extraction method (Gallegly and Hong, 2008). Primers of the Internal Transcribed Spacers 1 and 2 of the rRNA gene repeat for the isolate were amplified as described by Cooke *et al.* (2000). Amplification of the β-tubulin gene was done using the forward tubuF2 (5' ACGGCTCGAGGATGACCATG 3') and reverse TubuR1 (5' CCTGGTACTGCTGGTACTCAG 3') primers (Kroon *et al.*, 2004). The thermocycle sequence followed the procedure as described by Tao *et al.* (2010). The PCR product was cleaned using the Sangon PCR Purification Kit (Shanghai Sangon Biological Engineering Technology and Swevices Co., Ltd., Shanghai, China) and sent to the Sangon Bio. Corp. for sequencing.

RESULTS

Isolation of the pathogen: Since 2002, a severe disease has been detected in plantation fields of *D. thyrsiflorum, D. chrysanthum, D. aurantiacum* and *D. chrysotoxum* in Simao City of Yunnan

Fig. 1(a-d): *Dendrobium* symptoms of stem blight caused by *Phytophthora nicotianae* (a) *D. chrysanthum*, (b) *D. chrysotoxum*, (c) *D. thyrsiflorum* and (d) *D. aurantiacum*

Table 1: Growth rate of *Phytophthora* isolate D1 (*Dendrobium thyrsiflorum*)

Temperature (°C)	9	10	12	15	18	20	22	25	27	30	32	34	35	37	38
Growth rate (mm day^{-1})	0.00	0.36	2.61	4.25	4.97	6.55	7.55	8.13	9.04	8.70	7.20	7.83	7.17	0.67	0.00

Province, resulting in almost destruction of some plantations. Symptoms include root and basal stem rot leading to blight and defoliation with water-soaked and yellowish or brown lesions extending from the roots or wounded stems. The symptoms varied slightly among *Dendrobium* species and 1 to 3 year old plants showed typical symptoms of *Phytophthora* root and pseudostem rot (Fig. 1a-d).

Four *Phytophthora* isolates (D1, D2, D3 and D4) were recovered, respectively from diseased stem samples of *Dendrobium thyrsiflorum, D. chrysanthum, D. aurantiacum* and *D. chrysotoxum*.

Morphological description: Colonies showed a distinct rosette pattern on PDA and a cottony pattern on CA, V8A, CMA, OMA, LBA and PSA. The minimum and maximum temperatures for growth were 9 and 37°C, respectively. The optimum growth occurred at 27°C with the average daily growth rate being 9.04 mm (Table 1). The hyphae were nonseptate and the main hyphae were 2.62-6.94 (av. 5.05) μm wide (Table 2). Hyphal swellings were not observed. Sporangia (Fig. 2a-e) were noncaducous, terminal and occasionally intercalary, papillate often with various distorted shapes including pyriform, obpyriform, ellipsoid, ovoid and spherical, 23.07-70.31×17.09-57.91 μm (av. 46.20×34.89 μm) with the mean length/breadth ratio as 1.34 (0.95-1.95). Chlamydospores (Fig. 2f) were spherical, thick-walled, terminal or intercalary, 19.71-49.58 μm (av. 30.15 μm) in diameter. Oogonia and antheridia (Fig. 2g-h) were not produced in single cultures but formed abundantly by pairing the appropriate mating strains on the same agar plate after 15-30 days. Antheridia were amphigynous and spherical to short cylindrical;

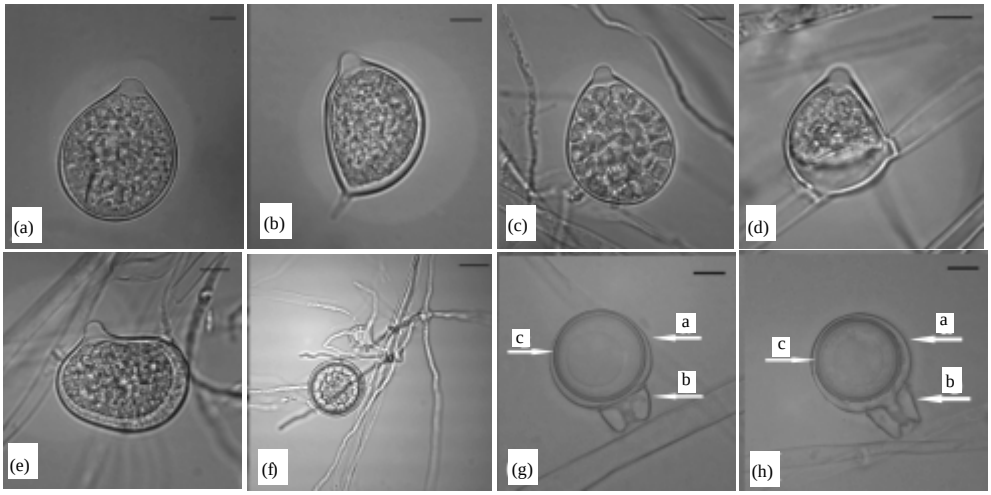

Fig. 2(a-h): Morphological characteristics of *Phytophthora nicotianae*. (a-e): zoosporangia, (f): chlamydospores, (g-h): a: Oogonia, b: Antheridia, c: Oospore, Bar = 10 μm

Table 2: Hyphal width of *Phytophthora* isolate D1

Hyphal width (μm)											
6.01	4.16	5.87	5.40	5.40	2.81	6.68	6.10	4.84	4.53	4.89	5.24
4.02	5.43	5.30	5.07	5.77	5.15	5.01	4.36	6.03	4.00	4.78	5.58
4.29	5.59	5.22	3.14	4.70	6.66	5.37	5.61	6.08	5.92	4.01	4.36
3.74	5.03	4.46	5.82	6.45	5.80	6.01	4.14	3.62	6.15	4.53	5.25
2.26	5.30	4.67	5.02	5.55	5.22	6.94	4.53	3.43	4.89	4.59	6.34
5.11	4.87	5.45	5.69	4.43	5.61	2.78	6.09	4.59	5.09	5.36	4.89
4.59	4.50	4.64	4.36	5.21	5.07	5.05	3.02	6.60	6.09	6.02	4.72
4.52	3.93	6.48	5.53	5.46	5.00	3.01	4.59	6.13	4.06	5.00	4.70
5.16	4.92	4.40	3.98	6.34	6.47	5.66	6.05	6.09	3.32	3.19	5.11
4.50	5.32	5.52	4.88	4.92	4.67	5.30	5.65	5.32	6.38	5.32	5.00

oogonia were smooth and spherical, 17.38-31.03 (av. 24.04) μm diam. All isolates of *Phytophthora* were identical in colony appearance on various agar plates, growth rates, growth/temperature relationships and characteristics of hyphae, sporangia, chlamydospores, oogonia and antheridia. Subsequently, they were identified as *Phytophthora nicotianae* (Gallegly and Hong, 2008).

Pathogenicity test: Isolates proved to be pathogenic to the inoculated wounded stems of all species of *Dendrobium,* resulting in characteristic symptoms found in nature and were consistently re-isolated from the lesions both in the laboratory and in the field. Lesions associated with the inoculations developed both above and below the inoculation sites but did not appear on the inoculated intact stems or un-inoculated controls. Thus Koch's postulates were satisfied, proving that the *Phytophthora* isolates caused stem rot of *Dendrobium*.

Diagnostic ITS and β-tubulin gene sequences: Internal transcribed spacer-1, 5.8S and large parts of the ITS2 region of the Isolate D1 yielded a single band (916 bp) and PCR amplification of the β-tubulin region yielded a single band (989 bp). The results of present phylogenetic analyses,

based on the alignment of nuclear DNA regions rDNA ITS region (GenBank Accession No.:GU931702) and β-tublin gene (GenBank Accession No.:GU931703) were homologous to the published Genbank database on *Phytophthora nicotianae* (Isolate: TARI 98147, Accession No.:GU111681; Isolate: PD_00391(P10318), Accession No.: EU080677) resulted in sequence identities of 98 and 100% for both regions, confirming that the pathogen was *P. nicotianae*.

DISCUSSION

P. nicotianae is one of the most widespread and destructive soil-borne plant pathogens, infecting 301 host species with a worldwide distribution (Erwin and Ribeiro, 1996). The nomenclature of *Phytophthora nicotianae* has been highly controversial due to the difficulty to differentiate it from *P. parasitica* (Tucker, 1931). Based on morphological and molecular studies of many isolates from all over the world, it is now widely accepted that these two taxa are conspecific with *P. nicotianae* having priority (Waterhouse, 1963; Ho and Jong, 1989; Gallegly and Hong, 2008), In this study, *P. parasitica* reported by various authors would be treated as synonymous with *P. nicotianae*.

Orchids have become important floriculture crops in many countries due to their beauty as cutting flowers and potted flowering plants. Common commercially cultivated include for example, *Cattleya, Cymbidium, Dendrobium, Epidendrum, Phalaenopsis* and *Vanda.*The total value of fresh cut orchids worldwide was US$56 million in 2004 (Laws, 2004). In mainland China, *Dendrobium* is of special interest besides its beauty because of its important medicinal value and has been cultivated on large scale in the field. In present study we have demonstrated that *Phytophthora nicotianae* caused root, stem root leading subsequently to the defoliation and blight of the entire *Dendrobium* plant. Three species of *Fusarioum*: *F. oxysporum*, *F. proliferatum* and *F. solani* were also found recently to cause root and stem rot of *Dendrobium* in nurseries in Malaysia but they differed from *P. nicotianae* in that the disease did not lead to host blight (Latiffah *et al*., 2009). Whereas, *P. nicotianae* worked alone in attacking *Dendrobium*, a complex of all three *Fusarium* species was responsible for the root and stem rot of this orchid. Based on pathogenicity tests, *F. oxysporum* could be the main cause agent of stem rot and *F. solani*, the main causal agent of root rot whereas *F. proliferatum* could cause both root and stem rots. Other fungi also caused dieseases in *Dendrobium* in various parts of the world. For instance, leaf spot was caused by *Alternaria, Cercospora* and *Phyllosticta,* floral fleck by *Bipolaria*, blossom blight by *Botrytis* and *Colletotrichum* and stem rot by *Pythium vexans* (Alfieri *et al*., 1984; Ito and Agragaki, 1977; Tao *et al*., 2010; Uchida, 1994). In addition, *Dendrobium* diseases could be caused by bacteria: *Erwinia* and *Pseudomonas* resulting in leaf spot, blight and soft rot of stems (Uchida, 1994) and viruses: *Cymbidium mosaic* and *Odontoglossum ringspot* leading to slower plant growth and lower yield of flowers (Khentry *et al*., 2006). *Phytophthora* spp., primarily *P. palmivora* (Ann, 1995; Thompson, 1959; Cating *et al*., 2009; Orlikowski and Szkuta, 2006), *P. parastica* (Ann, 1995; Uchida and Aragaki, 1991; Uchida, 1994) and *P. cactorum* (Burnett, 1974; Cating *et al*., 2009) posed the greatest threat to *Dendrobium* cultivation by causing major diseases like seedling damping-off, bud and flower rots, root rot, black rot and slow decline in growth often leading to the death of the entire plant. For diseases caused by *P. parastica* no specific species of *Dendrobium* was mentioned and the diseases took place in potted *Dendrobium* in glasshouses or nurseries. Li *et al*. (2008) reported for the first time, *P. nicotianae* causing *Dendrobium candidum* blight in cultivated fields in Zhejiang Province of China. In this report, we have proved that *P. nicotianae* is the pathogen causing blight of *D. thyrsifolorum, D. chrysanthum, D. aurantiacum* and *D. chrysotoxum* in plantations in Yunnan Province.

CONCLUSION

The pathogen identification is important for the management of the plants in plantations. Since wounds are usually required for infection great care should be taken to avoid any cultural practice that might cause wounds on the plants. *P. nicotianae* needs high humidity for the production of sporangia and zoospores which are the most important infective units. Thus, irrigation should be by dripping (Jayapiratha *et al*., 2010) instead of sprinkling to avoid the spread of zoospores. To provide further protection of roots from infection, natual antagonistic solutions can be added to the irrigation water

For instance, animal compost extract was found to be antagonistic to *P. cinnamomi* (Aryantha and Guest, 2006) and *Pythium ultimum,* a zoosporic Pythiaceous fungi related to *Phytophthora* (Kerkeni *et al*., 2007); Methanol extract of propolis, a sticky, gummy and resinous substance around honey hives in Turkey proved to inhibit or even completely stop the mycelial growth of *P. infestans*, *P. capsici* and *P. parasitica in vitro* (Yusuf *et al*., 2005). Garlic extract has been proven to suppress *Pythium ultimum* (Alhussaen *et al*., 2011). Similarly, dried preparation of microorganisms like *Trichoderma* spp. which are antagonistic to a wide variety of soil-borne plant pathogens (Daghman *et al*., 2010; Daami-Remadi *et al*., 2010; Jegathambigai *et al*., 2009) may be incorporated in the soil of *Dendrobium* plantations. However, the control of *Phytophthora* diseases is still based on the use of protectant fungicides like ridomil gold MZ68 (metalzyl+mancozeb) and cuvvax M (cymoxanil+mancozeb) (Boughalleb *et al*., 2006) and phosphonate (Aryantha and Guest, 2004). At present there are no resistant *Dendrobium* cultivars. Attempts should be made to search for any resistance gene among the wild strains and insert it into the susceptible cultivars using molecular techniques (Shanti *et al*., 2010). Alternatively, the tolerance of *Dendrobium* against *Phytophthora* might be enhanced by the incorporation of chitinase and glucanase genes (Sreeramanan *et al*., 2010).

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