First report of leaf spots caused by *Pestalotiopsis trachicarpicola* in *Gentiana rhodantha*

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Abstract

In August 2018, leaf spots were widely found with circular or irregular brown lesions on Gentiana rhodantha leaves in Liupanshui, Guizhou Province, China. Three strains of Pestalotiopsis spp were associated with leaf lesions by single-spore isolation. Based on asexual and sexual characteristics and multi-locus (ITS, TUB, tef-1) phylogenies data, strains were identified as Pestalotiopsis trachicarpicola . This is the first report of G. rhodantha leaf spot caused by P. trachicarpicola . The fungal culture tests showed that mycelia optimum growth temperature and pH were 20–25°C and pH 7–9, and for conidia germination were 25–30°C and pH 6–9, respectively.

Main Text

Gentian rhodantha Franch. ex Hemsl, a perennial herb belonging to the *Gentianaceae*, is mainly distributed at high altitudes (900–1800 m) in mountainous areas in southwest China. The aerial parts of this herb (including flower, leaf, and stem) are widely used in Tibetan and Miao traditional ethnomedicine for the treatment of cough, bronchitis, hepatitis, and dysentery (Xu et al. 2011).

In August 2018, leaf spots were widely observed in a survey of cultivated field of *G. rhodantha* in Liupanshui, Guizhou Province, China. The typical symptoms on leaves were small brown spots in center or edge of the leaves, which expand to circular or irregular spots with purple halo (Fig. 2a) and even completely wither. In this study, three *Pestalotiopsis*-like strains (LBB062904, LBB062905 and LBB062906) were isolated from leaf spots of *G. rhodantha* field by single-spore isolation (Chomnunti et al. 2011).

For molecular analysis, total genomic DNA of fresh pure cultures were extracted using the SDS-CTAB method (Suwannarach et al. 2010). The internal transcribed spacers (ITS) region of rDNA molecule was amplified using primer pairs ITS5 and ITS4, the β-tubulin gene *(TUB*) region with BT2A and BT2B primer pairs, and the translation elongation factor 1-alpha (*tef1*) gene using the EF1-728F/EF2 (Maharachchikumbura et al. 2012). PCR was performed with the 25 µl reaction system consisting of 19.75 µl of double distilled water, 2.5 µl of 10 × Taq buffer with $MgCl_2$, 0.5 µl of dNTP (10 mM each), 0.5 µl of each primer (10 µM), 0.25 µl Taq DNA polymerase (5 U/µl), and 1.0 µl of DNA template. PCR amplification protocols were performed as described by Maharachchikumbura et al. (2012). The DNA sequences obtained in this study have been deposited in the DDBJ/EMBL/GenBank database with accession numbers MT539135 to MT539137 and MT671942 to MT671947.

Phylogenetic tree was constructed by MEGA X (Kumar et al. 2018) for the combined data set of the ITS, *tef1* and *TUB* genes for 31 *Pestalotiopsis* strains (Table 1), *P. camelliae* CBS 443.62 was used as outgroup. Bootstrap value ≥ 50% (1000 replications) by the maximum likelihood (ML) methods were shown on the respective branch. The aligned results comprised 1413 characters including partial gaps (ITS: 1–509, *tef1*: 510–985 and *TUB*: 986–1413). Of these characters, 1275 were constant, 54 were variable and 106 were parsimony-informative. 31 *Pestalotiopsis* strains formed a strong clade (100% bootstrap support). Three strains obtained were clustered to *P. trachicarpicola* with an 82% bootstrap value (Fig. 1).

Strains sporulation were observed on synthetic nutrient-poor agar (SNA) amended with double-autoclaved pine needles placed on the agar surface, and incubated at 25°C for 15 days (Liu et al. 2017). Double-autoclaved *Taxus chinensis* needle was used to induce sexual forms, and the strains were inoculated in the center of SNA plates that contained needles and incubated at 20°C for 8 weeks.

The colonies on potato dextrose agar (PDA) were 7.30±0.51 cm (n=5) diameter at 25°C after 5 days, with undulate and redial edge, convex with plat surface, white to faint yellow on front, back pale honey-colored (Fig. 2b, c). Conidiomata pycnidial at pine needles in culture on SNA, globose, scattered, black, teardropform, 100–500 µm diameter (Fig. 2d–e); Conidiophores short, subcylindrical, hyaline, smooth, (Fig. 2f). Conidiogenous cells ampulliform, hyaline, 7.3–14.5×2.3–3.8 µm ($\bar{x}$±SD=8.23± 4.33×2.56±0.78 µm, n=15). Conidia fusoid, olivaceous to thin yellow, 4-septate, 20–26×5–9 µm ($\bar{x}$±SD=22.9±1.3×6.5±0.8

µm, n=30), thin-walled and verruculose wall (Fig. 2g–j); Basal cell obconic, thin-walled, hyaline, 3.5–6.4 µm long; Three median cells dolioform, versicolor, 13–17 µm long (x±SD=15.1±0.7 µm, n=30), verruculose, pale-yellow to brown, septa darker; apical cell conical, hyaline, smooth wall, 2.8–5.5 µm long, with 1–4 concurrent tubular apical appendages (mostly 3), filiform, unbranched, arising from the apical crest, 8–21 µm long; single basal appendage is straight, 3–7 µm long.

Ascomatas on *Taxus chinensis* needle were 130–320 µm diam, black, gregarious, immersed in leaves epidermis and raised slightly (Fig. 2k). Asci 8-spored, bitunicate, cylindrical, 67–94×6–10 µm (x±SD=84.8±7.6×9.4 ±1.3 µm, n=30) (Fig. 2 l–m), mature ascus without wall, indistinct J+ (amyloid ring) in apical apparatus (Fig. 2n). Ascospores 13.5–15.2×5.1–7.5 µm (x±SD=14.3±0.9 ×6.5±0.4 µm, n=30), uniseriate or interlaced, 2–4 cells (mostly 3) ascospore concolorous, light yellow to brown, oblong to fusiform, smooth or verrucose, brown septate and slightly constricted at the septa(Fig. 2o–r). Based on molecular analysis and unsexual and sexual characters, three strains were identified as *P. trachicarpicola* Y.M. Zhang & K.D. Hyde (Zhang et al. 2012), To the best of my knowledge, this is the first report of *P. trachicarpicola* causing leaf spot of *G. rhodantha*.

P. trachicarpicola have many asexual features similar to *P. neglecta*, *P. kenyana* and *P. oryzae,* which have thin and olivaceous walled conidia, concolourous medial cells and branches of apical appendages. But there are some significant differences between them (Table 2). The conidia of *P. trachicarpicola* (x=22.9×6.5 µm) are shorter than those of *P. kenyana* (x=25.5 µm) and *P. oryzae* (x=26.9 µm) and the apical appendages of *P. trachicarpicola* (8–21 µm) are shorter than *P. neglecta* and *P. oryzae* (Maharachchikumbura et al. 2014; Steyaert, 1953; Zhang et al. 2012). The asci and ascospores of *P. trachycarpicola* is morphologically mostly similar to *P. neglecta* and *P. accidenta*, but the asci of *P. trachycarpicola* are thinner than *P. neglecta*, and ends of the ascospores of *P. trachicarpicola* are more rounded and some are verrucose compare with *P. neglecta* and *P. accidenta* (Kobayashi et al.2001; Zhu et al, 1991).

In pathogenicity tests, detached leaves of *G. rhodantha* were surface-disinfected with 75% EtOH for 30 s and washed five times with sterilized water. Leaves were slightly wounded with a sterile needle and then inoculated a 2 mm diam PDA mycelial disk and inoculated sterilized PDA as controls and placed in Petri dishes at 25°C. There were 10 leaves for each treatment and performed three times. The inoculated leaves had observed symptoms consisted of circular, brown to black spots and highly similar to pathology characteristics of the specimen at 7th days after inoculation (Fig. 2s–t). None of the control leaves had symptoms. The same fungus was re-isolated from symptomatic lesion.

To determine the optimal temperature and pH on spore germination and mycelial growth of *P. trachicarpicola*, eight temperature gradients (5–40°C) and ten pH values (3.0–12.0) of PDA medium were designed as a randomized single factor experiment. All pates for pH tests were cultured at 25°C. Experiments were conducted 3 times in the dark. Germination rate were calculated we counted the number of total and germinated spore in 10 visions under low-power microscopic (10 × 10) at 12 h, and measured the diameters of colony after 7 days. The results showed that the mycelia grew at a temperature range of 10 to 35°C, with optimum growth at 20 to 25°C. The conidia sprout had a temperature range of 15–40°C, and the optimum temperature were 25–30°C (Fig. 3a). After treatment of medium with different pH values, mycelia could grow at pH 4 to 12, with optimum growth rate and conidia germination rate at 8 and 6–9, respectively (Fig. 3b).

Brown leaf spot and leaf blight caused by *Mycochaetophora gentianae* (Nekoduka et al. 2013) and *Septoria gentianae* (Verkley et al. 2013) were two serious disease on overground parts of *Gentiana* plants and widely occurred in fields, but they symptoms were significantly different with leaf spot in size, color and edge type in focuses. There were without any records of *Pestalotiopsis* diseases on wild and cultivated *G. rhodantha* yet. Therefore, we propose that leaf spot caused by *P. trachicarpicola* be added as novel disease on *G. rhodantha*.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Chomnunti P, Schoch CL, Aguirre-Hudson B, Ko-Ko TW, Hongsanan S, Jones EBG, Kodsueb R, Phookamsak R, Chukeatirote E, Bahkali AH, Hyde KD (2011) Capnodiaceae. Fungal Divers 51:103–134

Kobayashi T, Ishihara M, Ono Y (2001) A new species of *Pestalosphaeria*, the teleomorph of *Pestalotiopsis neglecta*. Mycoscience 42:211–216

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549

Liu F, Hou L, Raza M, Cai L (2017) *Pestalotiopsis* and allied genera from camellia, with description of 11 new species from China. Sci Rep 7:1–19

Maharachchikumbura SSN, Guo LD, Cai L, Chukeatirote E, Wu WP, Sun X, Crous PW, Bhat DJ, McKenzie EHC, Bahkali AH, Hyde KD (2012) A multi-locus backbone tree for *pestalotiopsis,* with a polyphasic characterization of 14 new species. Fungal Divers 56:95–129

Maharachchikumbura SSN, Hyde KD, Groenewald JZ, Xu J, Crous PW (2014) *Pestalotiopsis* revisited. Stud Mycol 79:121–186

Nekoduka S, Tanaka K, Sano T (2013) Overwintering of brown leaf spot fungus, *Mycochaetophora gentianae*, in infected gentian leaves as the primary inoculum source. J Gen Plant Pathol 79:175–177

Steyaert RL (1953) New and old species of *Pestalotiopsis*. Transactions of the British Mycological Society 36:81-89

Suwannarach N, Bussaban B, Hyde KD, Lumyong S (2010) *Muscodor cinnamomi*, a new endophytic species from *Cinnamomum bejolghota*. Mycotaxon 114:15–23

Verkley GJ, Quaedvlieg W, Shin HD, Crous PW (2013) A new approach to species delimitation in *Septoria*. Stud Mycol 75:213–305

Xu M, Zhang M, Wang D, Yang CR, Zhang YJ (2011) Phenolic compounds from the whole plants of *Gentiana rhodantha* (Gentianaceae). Chem Biodivers 8:1891–1900

Zhang YM, Maharachchikumbura S, Mckenzie E, Hyde KD (2012) A novel species of *Pestalotiopsis* causing leaf spots of *Trachycarpus fortune.* Cryptogamie Mycol 33:311–318

Zhu P, Ge Q, Xu T (1991) The perfect stage of *Pestalotiopsis* from China. Mycotaxon 40:129–140

Tables

Table 1 Strains used in this study with host, location, and GenBank accession number

Species	Strain/isolation No.	Host/Substrate	Location	GenBank accession		
				ITS	*tef1*	*TUB*
P. neglecta	TAP99M112	*Pieris japonica*	Japan	AB482211	AB453853	AB453882
	TAP20O063	*Cupressus macrocarpa*	Japan	AB482209	AB453889	AB453841
P. oryzae	CBS 171.26	Unknown	Italy	MH854881	KM199494	KM199397
	CBS 353.69	*Oryza*	Denmark	KM199299	KM199496	KM199398
	CBS 111522*	*Telopea*	Hawaii	KM199294	KM199493	KM199394
P. trachicarpicola	LBB062904	*Gentiana rhodantha*	China	MT539135	MT671942	MT671945
	LBB062905	*Gentiana rhodantha*	China	MT539136	MT671943	MT671946
	LBB062906	*Gentiana rhodantha*	China	MT539137	MT671944	MT671947
	OP068*	*Trachycarpus fortunei*	China	JQ845947	JQ845946	JQ845945
	CBS 111507	Unknown	Zimbabwe	MH553960	MH554378	MH554619
	CBS 297.76	Soil	Spain	MH554027	MH554462	MH554704
P. kenyana	CBS 442.67*	Coffea	Kenya	MH859026	MH870724	KM199395
	CBS 911.96	Agar	Unknown	KM199303	KM199503	KM199396
	LC6633	*Camellia sinensis*	China	KX895027	KX895246	KX895360
P. rhodomyrtus	LC4458*	*Camellia sinensis*	China	KX895010	KX895228	KX895342
	LC3413	*Camellia sinensis*	China	KX894981	KX895198	KX895313
P. australasiae	CBS 114126*	*Knightia* sp.	New Zealand	KM199297	KM199499	KM199409
	CBS 114141	*Protea* sp.	Australia,	KM199298	KM199501	KM199410
P. telopeae	CBS 114137	*Protea*	Australia	KM199301	KM199559	KM199469
	CBS 113606*	Telope	Australia	KM199295	KM199498	KM199402
P. brachiata	LC2988	*Camellia* sp.	China	KX894933	KX895150	KX895265
	LC8189*	*Camellia* sp.	China	KY464143	KY464153	KY464163
P. biciliata	CBS 790.68	Unknown	Netherlands	MH859228	KM199507	KM199400
	CBS 124463*	*Platanus× hispanica*	Slovakia	KM199308	KM199505	KM199399
P. disseminata	CBS 143904	*Persea americana*	New Zealand	MH554152	MH554587	MH554825
	CBS 118552*	*Eucalyptus botryoides*	New Zealand	MH553986	MH554410	MH554652
P. adusta	MFLUCC10-0146*	*Prunus cerasus*	USA	X399007	JX399071	JX399038
	ICMP6088	Unknown	Fiji	JX399006	JX399070	JX399037
P. knightiae	CBS 111963	*Knightia* sp.	New Zealand	KM199311	KM199495	KM199406
	CBS 114138*	*Knightia* sp.	New Zealand	KM199310	KM199497	KM199408
P. camelliae	CBS 443.62*	*camelliae*	China	MH858206	KM199512	KM199424

* means type strain, epitype strain or outgroup strain.

Table 2 Sexual and asexual forms of *P. trachicarpicola* and its related species

Species/strain	Anamorph						Teleomorph			
	Conidia size (µm)	Color of three medial cell	Apical appendage		Basal appendage		Asci size (µm)	Ascospore		
			Length (µm)	Quantity	Length (µm)	Quantity		Size (µm)	No. of Septate	Wall
P. trachicarpicola LBB062904	20–26×5–9 (22.9×6.5) [a]	Concolourous, olivaceous to light yellow	8–21	1–4 (mostly 3)	3–7	1	67–94×6–10 (84.8×9.4)	13.5–15×5–7.5 (14.3×6.5)	1–3 (mostly 2)	Smooth or verrucose
P. trachicarpicola OP068 [b]	19–24.9×5–6.3 (22×6.0)	Concolorous, olivaceous	9.4–17.8	2–3 (mostly 3)	2.8–11	1–2 (mostly 1)	65–76×5–14 (73.6 × 9.3)	12–16×5–8 (14.1×6.5)	2–3 (mostly 2)	Smooth or verrucose
P. kenyana [c]	23–28×7–9 (25.5×8)	Concolourous, olivaceous	3–20	2–3	1–4	1–2 (mostly 2)	–	–	–	–
P. neglecta [d] (*Ps. gubae* [e])	19–25×6–8 (21× 6.6)	Concolourous, brown	9–22	2–3 (mostly 3)	0–7	1	75–88×10–12	9.5–17.5×5–7	2–3 (mostly 2)	Smooth
P. oryzae [c]	24.5–29×6–8 (26.9×7)	Concolourous, olivaceous	9–27	2–3 (mostly 3)	3–6	1	–	–	–	–
P. baarnensis (*Ps. accidenta*) [f]	16.5–27×5.6–6.7	Concolourous, reddish brown	10–32.5	2–3 (mostly 2)	2.3–6.8	1	62.5–72.9× 8.3–8.9	13–17×4.8–6.8	1–3 (mostly 2)	Smooth

[a] The average values of length and width were listed in brackets.

[b] Zhang et al. 2012

[c] Maharachchikumbura et al. 2014

[d] Steyaert, 1953

[e] Kobayashi et al.2001

[f] Zhu et al. 1991

Figures

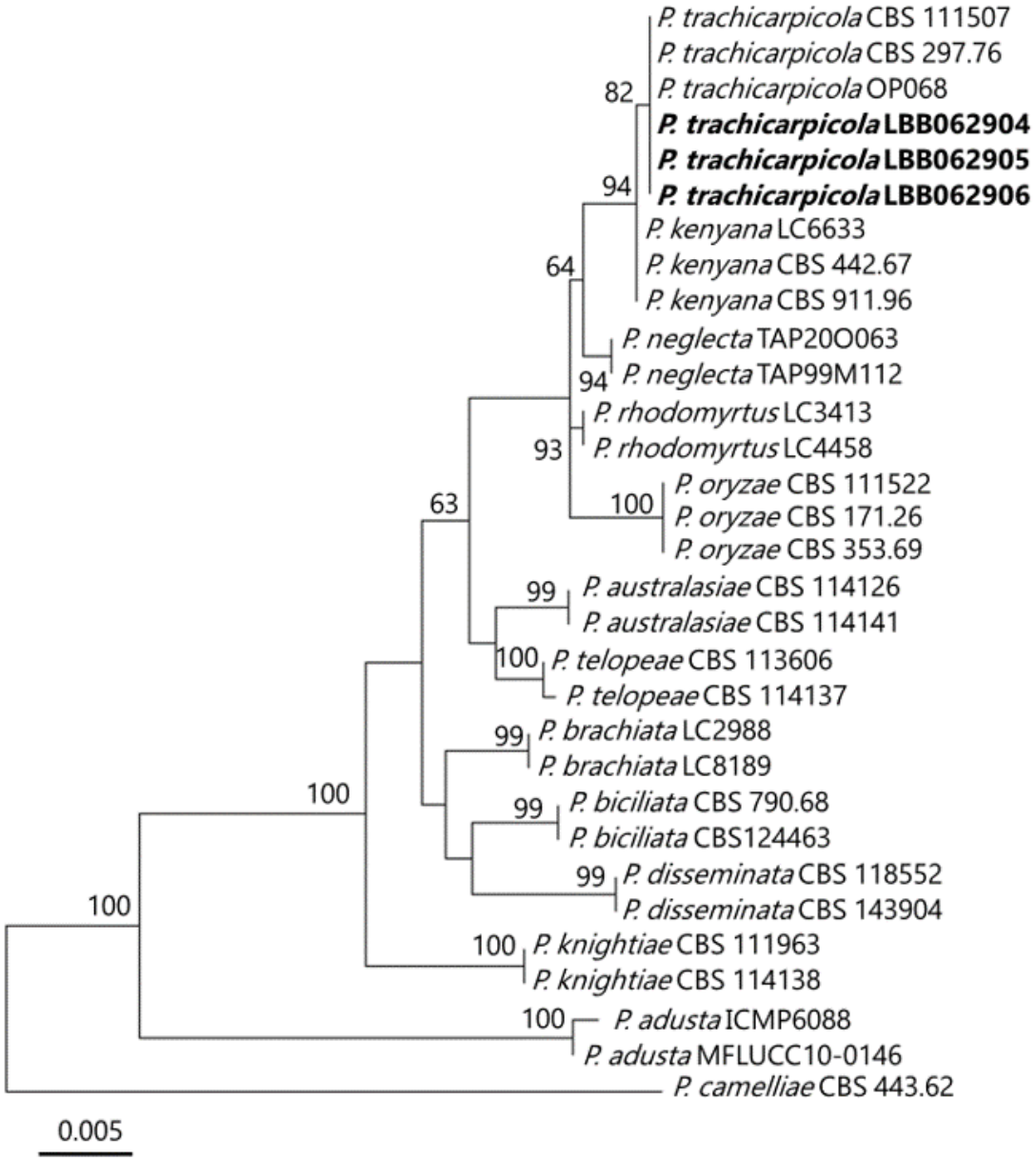

Figure 1

Topology showing the maximum likelihood tree, inferred from combined ITS, TUB and tef1 gene regions. Bootstrap values smaller than 50% are not shown. Strain Pestalotiopsis camelliae CBS 443.62 was used as the outgroup taxon. 3 strains from G. trachicarpicola in this study are thickened.

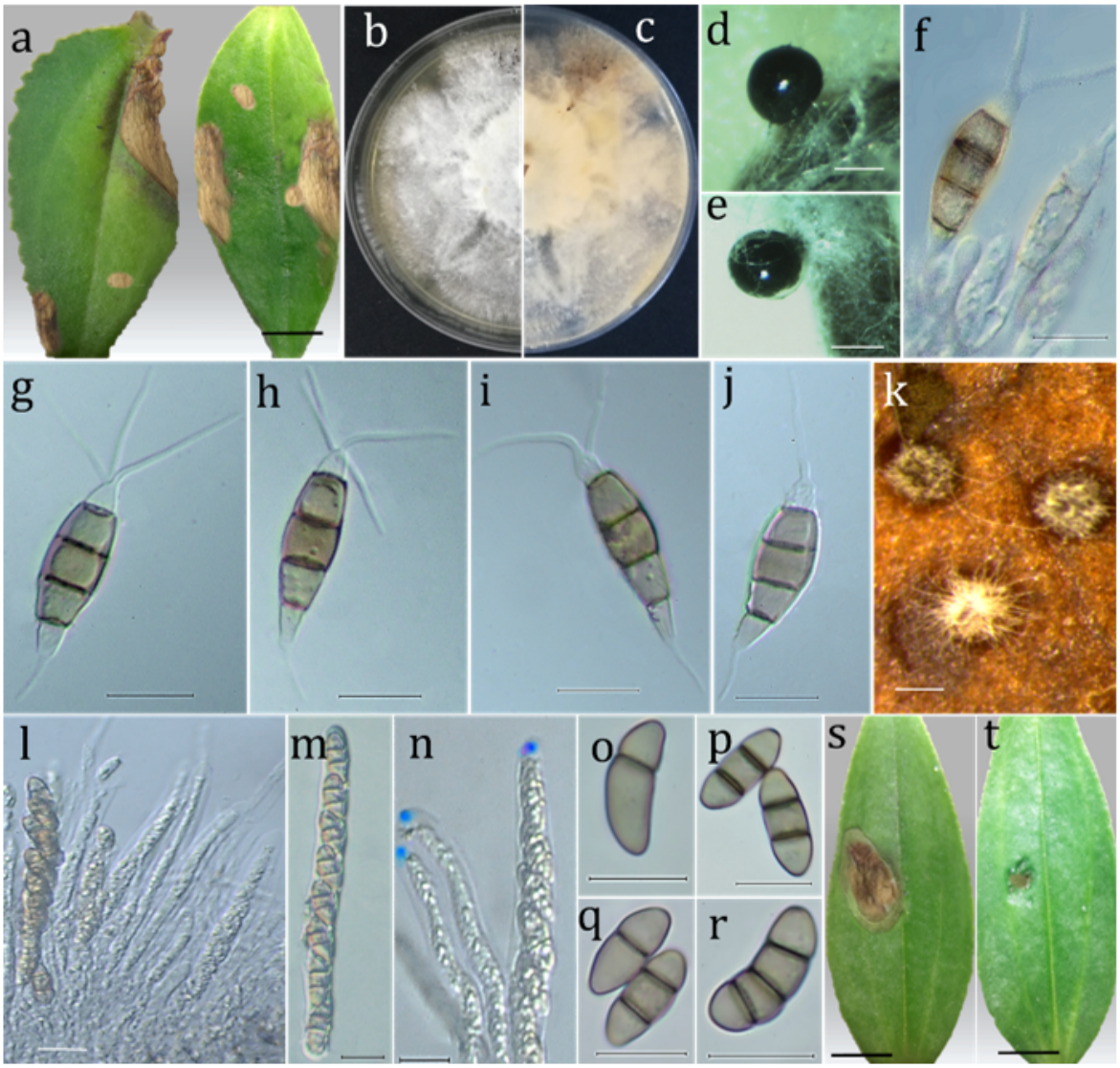

Figure 2

Symptoms and pathogen morphology of leaf spot in Gentiana rhodantha caused by Pestalotiopsis trachicarpicola. a Symptoms of leaf spots on leaf of G. rhodantha, scale bar=5.0 mm. b–c Front and back view of colony on PDA. d–e Acervuli on double–autoclaved pine needle, scale bars=200 μm. f Conidiophore, conidiogenous cells and developing conidia. g–j Conidia, scale bars=10 μm. k Ascomatas on double–autoclaved Taxus chinensis needle with SNA medium, scale bar=200 μm. l–n Mature and immature unitunicate asci and amyloid ring on apical apparatus, scale bars=10 μm. o–r Ascospores, scale bars=10 μm. s Symptoms after inoculation with Pestalotiopsis trachicarpicola, and t Control at 7th days, scale bar=5.0 mm.

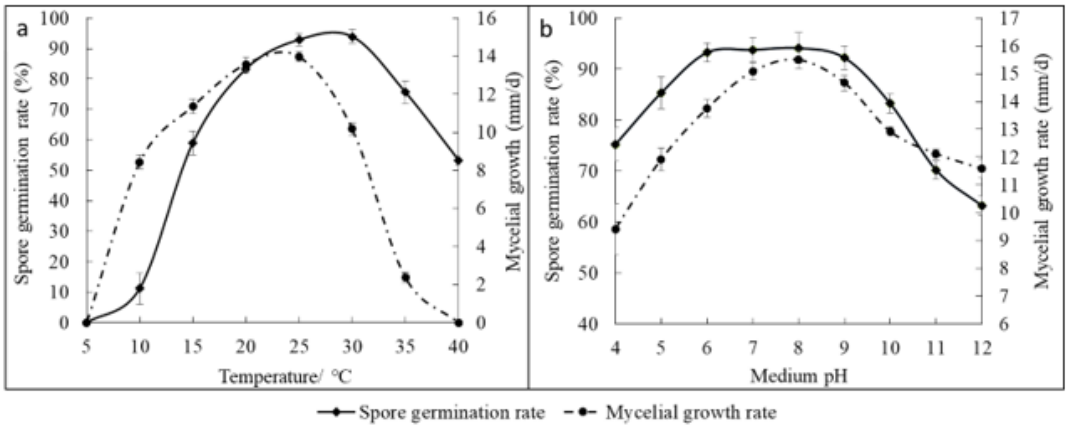

Figure 3

Effect of temperature and pH value on Pestalotiopsis trachicarpicola mycelial growth and spore germination