Recent incidence of collar canker and dieback of tea (*Camellia sinensis*) caused by *Fusarium solani* species complex in Sri Lanka

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Received: 30 June 2016 / Accepted: 24 July 2017 / Published online: 19 August 2017
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Abstract A collar canker and dieback disease of tea has recently emerged in Sri Lanka. Isolation from cankers consistently yielded fusaria. Morphological characters and ITS and *tef1* combined sequence analysis identified the selected isolates as members of *Fusarium solani* Species Complex Clade 3. Artificial inoculation to young and mature tea plants confirmed pathogenicity.

Keywords Ambrosia *Fusarium* clade · *Fusarium ambrosium* · *Fusarium pseudensiforme* · Phylogenetic analysis · *tef1*

Tea (*Camellia sinensis*) is the major plantation crop in Sri Lanka contributing about 15% of total export earnings. Canker is the most widely prevalent stem disease of tea worldwide. Cankers are persistent, attacking twigs, stems and killing or girdling branches ultimately leading to death of tea bushes.

Phomopsis theae has been identified as the major pathogen causing stem canker disease in Japan (Kasai et al. 1965), India (Venkata Ram 1979), China (Chen and Chen 1982), Kenya (Onsando 1988) and Bangladesh (Ahmad et al. 2013). In Sri

Lanka, *Macrophoma theicola* and *Botryodiplodia theobromae* (Sabanayagam et al. 1974) are the major canker causing pathogens in the low (<600 m mean sea level) and mid-elevations (1200–600 m mean sea level) whereas *P. theae* is problematic in the high elevations (>1200 m mean sea level) (Balasuriya 2008). Liyanage et al. (2013) reported *Fusarium solani* as a canker causing agent for the first time in the low elevation tea growing areas of Sri Lanka. Recently in Sri Lanka, the incidence of collar canker and associated bush dieback has become widespread. This study aimed at identifying the causal organism in different tea growing regions.

Dieback incidence in affected fields ranged from 50 to 85%. The affected tea bushes had fewer and smaller leaves, yellowing of leaves and premature falling. Heavy flowering and fruiting commonly followed canopy thinning. Progressive dieback of branches resulted in bare bushes (Fig. 1a). Foliar symptoms were always associated with cankers having cracks and peeling of bark at the collar region. The exposed cambium and wood were discoloured, tan, brown or black. Cankers at the collar region were irregular in shape and extended to several centimetres in length and depth (Fig. 1b). Reddish, tiny superficial perithecia were seen under damp and moist conditions (Fig. 1c). Fibrous root formation just above the cankered area was also observed.

Symptomatic samples were collected from tea fields covering a range of rainfall and elevation during the period 2013–2014. Pieces (3–5 mm³) of tissue from the margin of canker lesions were separately plated on *Fusarium* selective pentachloronitrobenzene (PCNB) medium (Papavizas 1967), potato dextrose agar (PDA, Oxoid), and malt extract agar (MEA, Oxoid) supplemented with tetracycline (10 mg/ml). Fungi were allowed to grow for seven days in the dark at room temperature (22 ± 2 °C), sub-cultured and purified on PDA. The isolates identified as fusaria were grown on carnation leaf agar (CLA) at 27 °C to stimulate conidia development (Fisher

Electronic supplementary material The online version of this article (doi:10.1007/s13314-017-0262-5) contains supplementary material, which is available to authorized users.

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Fig. 1 **a** Tea bush naturally affected by collar canker and dieback **(b)** natural infection showing the prominent canker at the collar region **(c)** perithecia on an infected tea bush **(d)** necrotic tissue beneath the point of artificial inoculation (note the bark has been removed to expose internal tissue browning)

et al. 1982). The colony characters of *Fusarium* isolates were compared with diagnostic characteristics specified by Leslie and Summerell (2006) and Nirenberg and O'Donnell (1998). Selected isolates were deposited at the International Collection of Microorganisms from Plants (ICMP).

Four isolates were selected for molecular characterisation, being one from Ruwanwella (ICMP 21513), one from Norton (ICMP 21515), and two from Yatinuwara (ICMP 21518, ICMP 21519). DNA was extracted using a DNeasy plant mini extraction kit (Qiagen) from mycelium of 7–10 days old monoconidial cultures grown on PDA. Internal transcribed spacer regions and intervening 5.8S rRNA gene (ITS) of the rDNA operon were amplified using the primer pair ITS-1F (5'-CTTGGTCATTAGAGGAAGTAA-3') / ITS-4 (5'-

TCCTCCGCTTATTGATATGC-3'), and PCR conditions of White et al. (1990). Translation elongation factor 1- α (*tef1*) was amplified using the primer pair EF-1 (5'-ATGG GTAAGGA(A/G)GACAAGAC-3') / EF-2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'), following PCR conditions described by O'Donnell et al. (1998). Amplicons of about 560 bp and 700 bp (ITS and *tef1*, respectively) were sequenced through Macrogen (Korea) and deposited in GenBank (Accession No KJ009327 to KJ009330, KR707726 to KR707729).

Sequences generated in this study and others obtained from GenBank (Table 1), composed two datasets (ITS and *tef1*) which were separately aligned using Mafft v.7 (<http://mafft.cbrc.jp/alignment/server/index.html>). Ambiguous regions

Table 1 Sequences used in the phylogenetic analysis, newly generated sequences are in bold

GenBank accession number		Species	Isolate/strain*	Host	Reference
ITS	TEF				
KJ009328	KR707729	<i>Fusarium</i> sp.	ICMP 21513	<i>Camellia sinensis</i>	In this study
KJ009327	KR707727	<i>Fusarium</i> sp.	ICMP 21515	<i>Camellia sinensis</i>	In this study
KJ009330	KR707728	<i>Fusarium</i> sp.	ICMP 21518	<i>Camellia sinensis</i>	In this study
KJ009329	KR707726	<i>Fusarium</i> sp.	ICMP 21519	<i>Camellia sinensis</i>	In this study
JX173101	—	<i>F. solani</i>	AF14	<i>Aquilaria malaccensis</i>	Premalatha and Kalra 2013.
JQ723751	JQ723758	<i>F. solani</i>	UCR1785	<i>Persea americana</i>	Eskalen et al. 2012
AF178408	AF178344	<i>F. solani</i> f. sp. <i>batatas</i>	NRRL 22402	Unknown	O'Donnell 2000
AF178419	AF178357	<i>F. solani</i> f. sp. <i>glycines</i>	NRRL 22825	Unknown	O'Donnell 2000
KM406631	KM406624	<i>F. ambrosium</i>	NRRL 62942	Tea Shot Hole borer	O'Donnell et al. 2015
KC691559	KC691531	<i>F. ambrosium</i>	NRRL 62605	Tea Shot Hole borer	Kasson et al. 2013
JX891747	JX891859	<i>F. euwallaceae</i>	FD31 PEAM	<i>Persea americana</i>	Eskalen et al. 2013
AY220236	AY220187	<i>F. phaseoli</i>	NRRL31156	<i>Glycine max</i>	Aoki et al. 2003
KM406635	KM406628	<i>Fusarium</i> sp.	NRRL 62943	<i>Camellia sinensis</i>	O'Donnell et al. 2015
KM406634	KM406627	<i>Fusarium</i> sp.	NRRL 62944	<i>Camellia sinensis</i>	O'Donnell et al. 2015
JQ038019	JQ038012	<i>Fusarium</i> sp.	NRRL 54727	<i>Persea americana</i>	Freeman et al. 2013
AF178402	AF178338	<i>F. cf. ensiforme</i>	NRRL 22354	Unknown	Kasson et al. 2013
KC691584	KC691555	<i>F. pseudensiforme</i>	NRRL 46517	Unknown	Kasson et al. 2013
AF178423	AF178361	<i>F. staphyleae</i>	NRRL 22316	Unknown	O'Donnell 2000
AF178421	AF178359	<i>Nectria haematococca</i> MPIII	NRRL 22157	Unknown	O'Donnell 2000
AF178401	AF178336	<i>N. haematococca</i> MPIV	NRRL 22277	<i>Zanthoxylum</i> sp.	O'Donnell 2000
AF178411	AF178347	<i>N. haematococca</i> MPV	NRRL 22142	<i>Cucurbita</i> sp.	O'Donnell 2000
AB513852	AB513842	<i>N. haematococca</i> MPVI	MAFF 840047	<i>Morus alba</i>	Nakayama and Aoki 2010
AF178395	AF178330	<i>N. haematococca</i> MPVII	NRRL 22161	<i>Robinia pseudoacacia</i>	O'Donnell 2000
AB513849	AB513845	<i>N. ipomoeae</i>	MAFF 238974	<i>Passiflora edulis</i>	Nakayama and Aoki 2010
AF178393	AF178326	<i>N. illudens</i>	NRRL 22090	Unknown	O'Donnell 2000

*NRRL Culture collection of Agricultural Research Service, USA

ICMP International Collection of Microorganism from Plants

MAFF Culture collection of Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan

Others indicate collectors' codes

were removed and, whenever necessary, the alignments were manually improved. The individual and combined ITS and *tef1* sequences were subjected to a Maximum Likelihood analysis (ML) using RaxMLGUI version 1.3.1 (Silvestro and Michalak 2012). The optimal ML tree search was conducted with 1000 separated runs, using the default algorithm of the program, from a random starting tree for each run. The final tree was selected among sub optimal trees, from each run, under the GTR + G substitution model. The resulting tree was edited and printed with TreeGraph 2 v. 2.11.1 (Stöver and Müller 2010).

Pathogenicity tests were carried out on vegetatively propagated 1 year and 10 years old tea plants (cv TRI 2023) with the selected isolates, ICMP 21513, ICMP 21515, ICMP 21518 and ICMP 21519. Each isolate/control was inoculated

on to ten replicate plants. A wound (5 mm diameter) was made on the main stems of test and control plants by removing the bark with a cork borer. Test plants were inoculated by placing a mycelia plug (5 mm in diameter) cut from the margins of seven-day-old monoconidial cultures of each isolate, on the wounds. Control plants were inoculated with sterile PDA plugs without mycelia. The plants were maintained under recommended nursery or field conditions for tea plants. The pathogen was re-isolated from lesions after development of symptoms.

Ninety percent of 96 isolates obtained from symptomatic tissues were fusaria. The 4 selected isolates were identified as the members of *Fusarium solani* species complex (FSSC) based on morphological characters (Fig. 2 A-F, supplementary data) and analysis of two DNA loci (ITS and *tef1*). The

Fig. 2 Colony on PDA of (a) ICMP 21515 (b) ICMP 21518 (c) ICMP 21513 and (d) ICMP 21519, (e) micro and macro conidia of ICMP 21519 (f) perithecia of ICMP 21513 on PDA

combined phylogenetic analysis (Fig. 3) revealed that all 4 isolates from tea belonged to FSSC clade 3 (O'Donnell 2000). Two isolates (ICMP 21513 and ICMP 21518) formed a sub-cluster within the Ambrosia *Fusarium* Clade (AFC), together with *F. pseudensiforme* NRRL 46517 (Kasson et al. 2013) and *F. cf. ensiforme* NRRL 22354 (Kasson et al. 2013). The isolates ICMP 21519 and ICMP 21515 clustered on a distinct clade, together with *F. solani* AF14 and *Nectria haematococca* (Fig. 3).

The AFC includes symbiont fusaria associated or not with ambrosia beetles (Kasson et al. 2013). *Fusarium ambrosium* is a symbiont of the Tea shot-hole bore (TSHB), *Euwallacea fornicatus* (= *Xyleborus fornicatus*), a serious pest of tea in India and Sri Lanka (Gadd and Loos 1947). *Fusarium pseudensiforme* and *F. cf. ensiforme* are not known to be associated with ambrosia beetle (Nalim et al. 2011; Kasson et al.

2013). These two species and the isolates associated with collar canker and dieback of tea differ from *F. ambrosium* by having typical fusiform macroconidia, the latter produces a clavate macroconidia (Gadd and Loos 1947). Members of *N. haematococca* are also economically important plant pathogens causing root rot, fruit rot and dieback in important plant species (O'Donnell 2000).

The occurrence of isolates belonging to the AFC and *N. haematococca* clade indicate the genetic variability of the casual agents of collar canker and dieback of tea. Artificial inoculation of the selected isolates from each group resulted in prominent cankers in 50–80% of the nursery plants and 70–90% of the field plants, confirming its pathogenicity (Fig. 1d). This study identified causal agents of collar canker and dieback of tea in Sri Lanka to be members of FSSC clade 3 and established the existence of diversity among the isolates. The

Fig. 3 Best scoring RAXML tree of *Fusaria* obtained from concatenate sequence alignment of ITS and *tef1* region. The scale bar shows 0.02 substitutions per site and bootstrap replicate values from 1000 replicates are shown next to the branches. Isolates obtained in this study are in bold

information generated will help to develop intervention strategies and efforts to reduce the economic impact of this recently emerged disease of tea.

Acknowledgements We sincerely acknowledge the support given by the Tea small holders of the study sites. We also thank Mr. JCK Rajasinghe, Principle Advisory Officer, Tea Research Institute for the support and valuable suggestions and Drs. S Maharachchikumbura and KPRN Athanayake for the assistance in the analysis of molecular data. Tea Research Institute of Sri Lanka is acknowledged for the funding of project D/PI/Pa/1.

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