Characterization and Fungicide Sensitivity of Fusaria Causing Collar Canker and Dieback in Tea

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ABSTRACT

A collar canker and associated dieback has emerged recently in tea in certain Up and Mid - Country tea growing regions of Sri Lanka. This study was aimed to identify and characterize the pathogens associated with the problem in Mid-Country and evaluate the efficacy of Bitertanol, Hexaconazole, Pyraclostrobin, Tebuconazole and Copper hydroxide fungicides against them. Of a total 96 isolates obtained from symptomatic tissues, randomly selected 09 isolates along with already characterized 4 isolates were used for molecular characterization. Phylogenetic analysis using concatenated ITS and *tef* sequences further confirmed the pathogens as the members of *Fusarium solani* species complex (FSSC) clade 3. Growth characterization was performed with 04 representative isolates. The optimum temperature range and pH for growth and sporulation of the isolates were 26 to 30°C and 5.5, respectively. Tebuconazole at 0.05 % (500 ppm) effectively controlled the disease in field. The findings of the study will help to intervene and reduce the economic impact of this recently emerged disease of tea.

Keywords: Fungicide screening, *Fusaruim solani* species complex, pathogenicity, phylogeny, tebuconozole

INTRODUCTION

Fusaruim solani species complex (FSSC) includes over 45 phylogenetically different species (O'Donnell *et al*. 2008) and generally referred as *F. solani*. *Fusarium solani* are ubiquitous soil fungi which cause disease in plants and humans (Aoki *et al*. 2014). The diseases caused by FSSC in plants include fruit rots, root rot, vascular wilt and cankers.

Stem cankers caused by *F. solani* have been reported on bitternut hickory (Park and Juzwik, 2012), citrus (Nemec, 1987), *Cedrelinga cateniformis* (Lombard *et al*. 2008), teak (Hocking, 1968), English walnut (Chen and Swart, 2000), sugar maple (Skelly and Wood, 1966), Poplars (Dochinger, 1967), *etc*. Cankers are localized dead areas of bark in trees and shrubs caused by fungal and bacterial infection. Outer bark effectively prevents the entrance of most canker causing pathogens. Therefore, these organisms usually enter and infect bark through wounds. Once canker pathogens breach the outer bark barrier, they may then colonize rest of the bark tissues. This process can reduce tree vigor, weaken the wood at the infection site, enhance the possibility of wood decay, and ultimately result in branch or trunk breakage and tree mortality. Canker pathogens can kill the phloem, disrupt nutrient movement, and starve portions of the tree.

In tea, *F. solani* was reported to cause rot diseases of tea cuttings in China (Olunloyo *et al*. 1987), dieback and seed decay in India (Barthakur, 2011). *Fusarium solani* as a canker causing agent was identified for the first time in the low elevation tea growing area of Sri Lanka in 2008 (Liyanage *et al*. 2013). Subsequently, in our previous study we reported FSSC associated with collar canker and associated bush dieback in tea fields located in Ruwanwella, Norton and Yatinuwara regions in Sri Lanka (Sinniah *et al*. 2017). Disease symptoms described were: infected bushes show scanty foliage, heavy flowering, fruiting and progressive dieback resulting in bare bushes (Figure 1A, B), prominent canker develops at collar region. Bark cracks and peels off at soil level or just below exposing discolored inner tissues (Figure 1C), Fibrous roots appear just above the cankered area. Canker can also be formed on the side branches leading to branch girdling. Under damp and moist conditions, reddish, tiny superficial perithecia are prominent (Figure 1D) (Sinniah *et al*. 2017). These disease symptoms were reported in young bushes of 2- 4 years as well as in mature bushes more than 16 years old. Death rate of bushes depended on the age and vigor of the plants and ranged 3-12 months after the expression of canopy symptoms.

Figure 1.
(A) Unthrifty and dead tea bush
(B) Attacked by collar canker
(C). Canker at the collar of a bush
(D). Reddish perithecia of *Fusarium solani* on an infected bush

Historically collar canker has been known to cause by *Phomopsis theae* and the disease was particularly confined to young tea fields in Up-Country region (Shanmuganathan, 1965). However, dieback due to *P. theae* is not very common (Balasuriya, 2008). Collar canker associated with FSSC clearly differs from *Phomopsis* collar canker in its ability to infect the tea bushes of a wide age category, cause rapid spread and death of tea bushes. These recent outbreaks are not only confined to Up-Country but also seen in Mid and Low-Country regions of Sri Lanka (Sinniah *et al.* 2017). Despite, the problem is becoming gradually spreading into tea fields, appropriate disease management strategies have not yet been developed. Thus, this study was carried out to further characterize the causative organism associated with collar canker and bush dieback at molecular level, to identify suitable conditions for growth of the pathogens and to screen selected fungicides for managing the disease.

MATERIALS AND METHODS

Study Locations

Symptomatic samples of collar canker and bush dieback were collected from fields of Tea Small Holdings at Hatale (N 7.382, E 80.722, WM 3b), Kalapitiya (N 7.044, E 80.647, WM 2a), Harangalagama (N 7.055, E 80.591, WM 2a) and Ukuwella (N 7.422, E 80.633, Intermediate Mid-Country, IM 2b). The fungal isolates obtained and already characterized in Sinniah *et al*. (2017) at Ruwanwella (ICMP 21513), Norton (ICMP 21515), and Yatinuwara (ICMP 21518, ICMP 21519) were also used.

Isolation of pathogen

Pieces of tissue (3-5 mm^3) from the margin of canker lesions of symptomatic tea bushes were separately plated on *Fusarium* selective Pentachloronitrobenzene (PCNB) medium, antibiotic enriched Potato Dextrose Agar (PDA, Oxoid), and antibiotic enriched Malt Extract Agar (MEA). Fungi were allowed to grow for seven days at room temperature (22±2 ^oC), sub-cultured and purified on PDA. The isolates identified as Fusaria were grown on Carnation Leaf Agar (CLA) at 27±1 °C to stimulate conidia development (Fisher *et al*. 1982).

Molecular characterization

Randomly selected 9 isolates from Ukuwella (ULM 1, ULM 2, ULM 3), Harangalagama (HLM 1, HLM 2), Hatale (HT 1) and Kalapitiya (KLM 1, KLM 2, KLM 3) were used for molecular characterization. The monoconidal cultures of the isolates were grown on PDA for 7-10 days. Mycelia were then scraped from the surfaces of the cultures, freeze-dried, and ground to powder in liquid nitrogen using a blunt pipette tip. DNA was extracted from the mycelial powder using DNeasy plant mini extraction kit (Qiagen) according to the manufactures protocol. The ITS region of rDNA and translation elongation factor (*tef* 1α) region of the 09 isolates were amplified using fungal specific ITS-1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primer pair and Ef 1 (5'ATGGGTAAGGA (A/G) GACAAGAC-3') and Ef 2 (5'GGA (G/A) GTACCAGT (G/C) ATCATGTT-3') primers respectively. PCR amplification of ITS region was performed in a 25 µl reaction mixture containing 1 µl of template DNA (~30 ng), 0.08 µM of each primer, 1× PCR buffer (Promega, USA), 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega, USA), and 1U of Taq polymerase (Promega, USA). PCR cycles were performed (BioRad MyCycler thermal cycler, USA) using the following conditions: an initial denaturation step of 95°C for 60s, denaturation step of 94 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 90s, followed by a final extension of 72 °C for 7 min. The PCR reaction for *tef* 1-α region was performed using conditions described in

O'Donnell *et al.* (1998). The amplified products were separated through electrophoresis in 1% agarose gels (Sigma, USA) and visualized under UV after staining the gels with ethidium bromide. A 100 bp ladder (Invitrogen, USA) was used as a size marker. Amplified PCR products of ITS (~ 560 bp) and *tef* 1- α (~700 bp) regions were sequence-characterized by submitting samples to Macrogen (Korea).

Phylogenetic analysis

The ITS and *tef* sequences of this study were analyzed together with DNA sequences of the fungal isolates published in Sinniah *et al.* (2017) (Table 1) and compared with published sequences available in the GneBank. Sequences were separately aligned using MAFFT http://mafft.cbrc.jp/alignment/server/index. html). The alignments were checked visually and improved manually where necessary. The individual and combined ITS and *tef* sequences were subjected to maximum likelihood (ML) analysis with raxmlGUI version 1.3.1 (Silvestro and Michalak, 2012). The optimal ML tree search was conducted with 1000 separate runs, using the default algorithm of the programme from a random starting tree for each run. The final tree was selected among suboptimal trees from each run under the GTRGAMMA substitution model. The resulting tree was edited and printed with Tree Graph 2 v. 2.11.1.

Table 1. Sequences details of FSSC used in the phylogenetic analysis

GenBank accession number		Isolate	Location
ITS	TEF		
KR707710	**KR707711**	**KLM 2**	**Kalapitiya**
KR707712	**KR707713**	**KLM 1**	**Kalapitiya**
KR707714	**–**	**KLM 3**	**Kalapitiya**
KR707715	**KR707716**	**HLM 2**	**Harangalagama**
KR707717	**KR707718**	**HT 1**	**Hathale**
KR707719	**KR707720**	**ULM 1**	**Ukuwela**
KR707721	**KR707722**	**ULM 2**	**Ukuwela**
KR707723	**KR707724**	**ULM 3**	**Ukuwela**
–	**KR707725**	**HLM 1**	**Harangalagama**
KJ009329	KR707726	ICMP 21519	Yatinuwara
KJ009327	KR707727	ICMP 21515	Norton
KJ009330	KR707728	ICMP 21518	Yatinuwara
KJ009328	KR707729	ICMP 21513	Ruwanwella

*Isolates 1-9 in bold are obtained from this study. Isolates 10-13 were already characterized in Sinniah *et al.* 2017

Effect of temperature and pH on growth and sporulation

Colony growth of four selected isolates, ICMP 21513, ICMP 21515, ICMP 21518 and ICMP 21519, was determined at 18, 22, 26, 30 and 34 ±1 °C. Mycelial discs of 5 mm in diameter were transferred from the margins of 7 days old growing colony of each test isolate to the center of PDA plates. Triplicate plates were maintained for each isolate at each temperature (4 isolates, 5 temperatures, 3 replicates). The colony diameter of each isolate was measured in mm in two perpendicular axes on the seventh day after

inoculations and then average colony diameter was calculated and recorded for each isolate.

Mycelial growth of the isolates ICMP 21513, ICMP 21515, ICMP 21518, and ICMP 21519 was studied at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7. The desired pH levels of PDA (Oxoid, USA) medium were maintained by adding the required amount of 1 N HCl or 1 N NaOH with the help of a digital pH meter (Orion, Model 420A). Then medium was sterilized in an autoclave at 120 °C for 15 min. About 20 ml of pH adjusted medium was poured in to each petri dish. Three replicate plates were maintained for each pH level. A mycelial disc (5 mm) was cut from the margin of the 7-day-old growing colony was transferred to the pH adjusted PDA plates and incubated at 25±1 °C. Colony diameters were measured as mentioned above.

Sporulation at different temperatures and pH was studied according to the method described in Gupta *et al.* (2010). Two discs (5 mm) were cut from the middle portion of each culture and put into two separate micro centrifuge tubes (1.5 mL) containing 1 mL of sterile distilled water and the tubes were vortexed for 1 min to dislodge conidia. The spore suspension was filtered through sterile glass wool and the number of conidia (mL^{-1}) were counted using a haemocytometer in the two duplicate tubes per plate. Average value of three replicate plates represented sporulation at a particular temperature. The experiment was repeated twice.

Testing fungicides against *Fusarium* isolates

***In vitro* screening of fungicides**

Systemic fungicides Bitertanol (Baycor EC 300 g/L), Hexaconazole (Contaf EC 50 g/L) Pyraclostrobin (Caprio EC1 250 g/L) and Tebuconazole (Folicur EC 250 g/L) and a contact fungicide Copper hydroxide (Champ DP 37.5% w/w) were used for *in vitro* screening. The test fungicides were suspended in sterile water and added to molten PDA medium to obtain final concentrations of 0.01, 0.1, 1, 10, 100 and 1000 ppm. PDA without fungicides served as the control. Tetracycline at 1 mL/L was added to the medium to prevent bacterial contaminations. After solidification of the medium, 5 mm disk from 7 days old pure culture of the selected isolates ICMP 21513, ICMP 21515, ICMP 21518, and ICMP 21519 were placed in the center of petri dishes. The inoculated plates were incubated at 26±2 ºC and radial colony growth was recorded on the 7^{th} day after inoculation. The experiment was conducted in Completely Randomized Block (RCBD) design having the isolates as blocks with three replicates per each treatment. The percent growth inhibition was calculated by using the following equation;

$$\textit{Percentage inhibition (\%)} = \frac{\text{Diameter of control} - \text{diameter of treatment}}{\text{Diameter of control}} \times 100$$

Effective concentration values representing reductions of mycelial growth by 50% (EC 50) was determined for each fungicide using non-linear dose-response curve fitted in GraphPad Prism 6.04.

***In vivo* (field) screening**

Based on the *in vitro* screening results, systemic fungicide Tebuconozole was selected for field screening. A collar canker infected tea field at Ruwanwella was selected for field screening of fungicide. The experiment was arranged in RCBD with two blocks, three treatments and two replicate plots in each treatment. A plot comprised 30-35 tea bushes. Tebuconazole at 100 ppm (0.01%), 500 ppm (0.05%) and 1000 ppm (0.1%) concentrations were treated as soil drench using a knapsack sprayer with a loosen nozzle. The treatments were applied 4 times at fortnight interval. Disease incidence and severity were estimated pre- and post-treatment. In each plot 15 bushes were randomly selected for the assessment of disease, based on the presence and the severity of cankers. The post treatment disease assessment was carried out 2 weeks after the final spray.

Disease incidence was calculated as number of diseased plants x 100/total plants observed. Disease severity was estimated on a scale of 0–4 where 0=healthy, 1=callused/superficial canker without foliar symptoms, 2=deep canker without foliar symptoms, 3= girdling with foliar symptoms and 4=complete death of bush. Disease severity and percentage decrease in disease severity after treatment were calculated according to the following formulae (McKinney, 1923):

$$\textit{Disease severity (\%)} = \frac{\text{Sum of all numerical ratings}}{\text{Total number of observation X Maximum rating}} \text{ X } 100$$

Sum of all numerical ratings= $(0 \text{ X } n_0) + (1 \text{ X } n_1) + \ldots + (4 \text{ X } n_4)$ where n_0-n_4 number of infected plants at each scale.

$$\textit{Reduction in disease severity (\%)} = \frac{\text{Disease severity before treatment} - \text{Disease severity after treatment}}{\text{Disease severity before treatment}} \text{ X } 100$$

Statistical analysis

Variation in the growth and sporulation at different temperature and pH and was analyzed using two-way ANOVA considering different temperature and pH as treatments and isolates as blocks. Effect of different concentrations of fungicide on disease severity was also analyzed using one-way and two-way ANOVA respectively for *in vitro* and *in vivo* experiments. All the statistical tests were performed in R statistical software (R version 3.0.2).

RESULTS AND DISCUSSION

Incidence of collar canker and dieback ranged from 50-85% in the studied fields. Tea cultivars TRI 2023, TRI 2026 and TRI 2025 were infected with collar canker and die back. All these cultivars were reported to be highly resistant to *P. theae* (Shanmuganathan, 1969). Variation in the infection in different tea cultivars was not observed even in the mixed cultivar fields. Ninety percent of a total of 96 isolates obtained from symptomatic tissues were Fusaria. *Botryodiplodia theobromae* and *Pestolotia* spp. represented > 5% of the total fungal isolate.

Characterization of the pathogens and phylogeny

The majority of the fungal isolates obtained from symptomatic plants were *Fusarium* spp. The morphological characters matched with the descriptions of *F. solani* (Leslie and Summerell, 2006). The isolates obtain from different regions also showed variations in morphology on PDA plates. Molecular phylogenetic analysis using concatenated ITS and *tef* 1-α sequences revealed that all 13 selected isolates from tea belonged to FSSC clade 3. The best scoring RAxML tree generated from maximum likelihood analysis is shown in Figure 2.

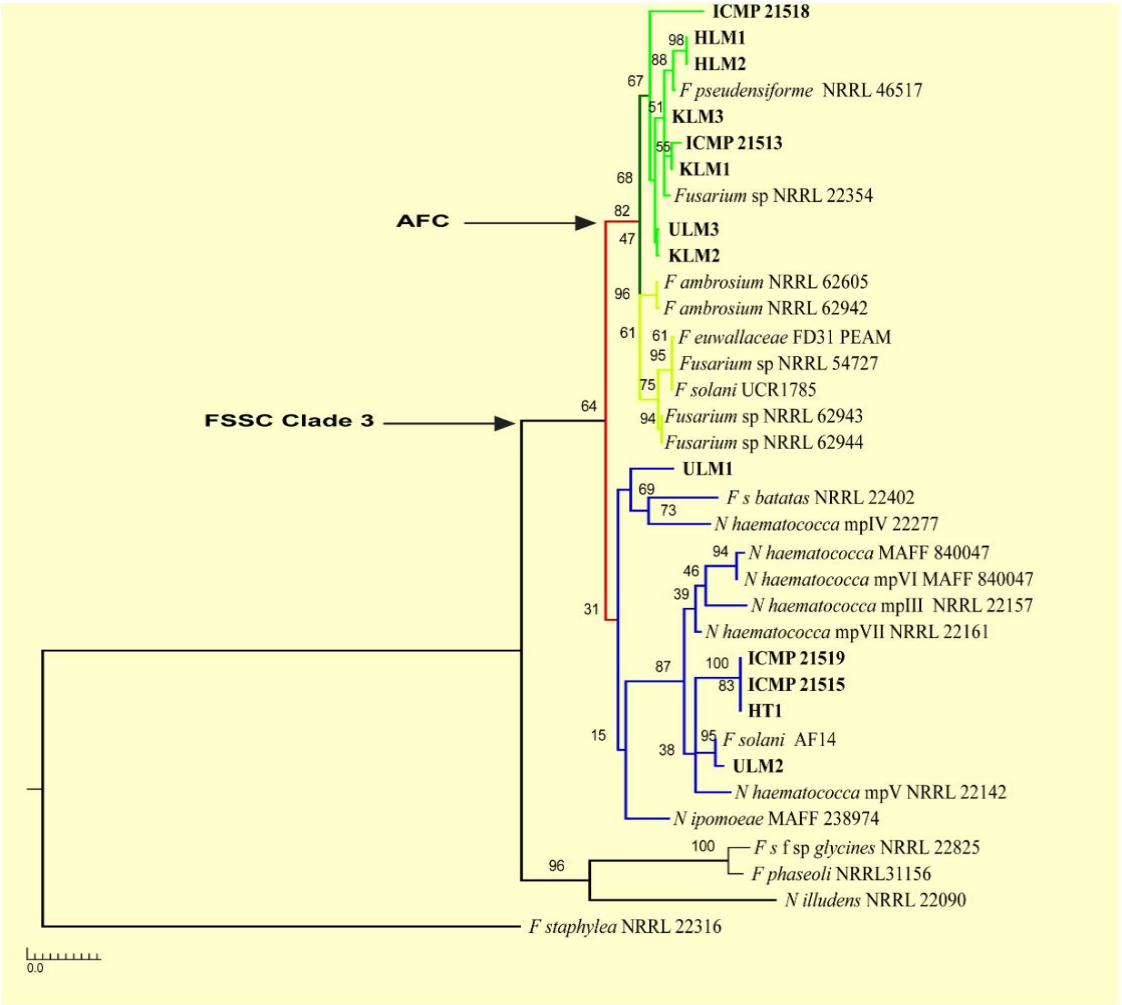

Figure 2. Best scoring RAxML tree of Fusaria using combined sequences alignment of ITS region of rDNA and *tef* 1-α region. Bootstrap replicate values from 1000 replicates are shown next to the branches. The isolates obtained in this study are in bold.

Eight of the 13 isolates (ICMP 21513, ICMP 21519, ULM 3, HLM 1, HLM 2, KLM 1, KLM 2, KLM 3) clustered together with *F. ambrosium* forming a sub-cluster within Ambrosia Fusaria Clade (AFC) with bootstrap support 71% together with the previously characterized GenBank isolate NRRL 22354 (FSSC 33 a). ULM 1 formed a separated group within FSSC clade 3. The individual *tef* 1-α sequence analysis also resulted in similar clustering (data not shown). The remaining four isolates, ICMP 21518, ICMP 21515, HT 1 and ULM 2, grouped within *Haematonectria haematococca* MP V together with the previously characterized GenBank isolate NRRL 22142. Thus the pathogens from all the study areas were

identified as the members of FSSC based on DNA sequences of ITS and *tef* 1-α regions.

Pathogenicity of all the isolates (9 isolates from this study and 4 already characterized isolates) was confirmed by artificial inoculation. Inoculation on nursery plants resulted in prominent canker development. Inoculated field plants and stem pieces showed clear internal tissue browning beneath the inoculated site and *F. solani* was constantly re-isolated. All the inoculated isolates were pathogenic and higher percentage of canker development was observed in mature tea plants which were inoculated with ICMP 21518 isolate (data not shown).

Suitable temperature and pH for the growth and sporulation of pathogens

Effect of temperature on growth and sporulation

Radial colony growth of ICMP 21515, ICMP 21518, and ICMP 21519 isolates at six different temperatures *viz*. 18 °C, 22 °C, 26 °C, 30 °C, and 34 °C showed an increasing trend with temperature up to 26 °C and maximum colony diameter was observed at this temperature (Figure 3A). Isolate ICMP 21513 showed maximum colony diameter at 30 °C. All isolates showed poor colony growth at temperature below 22 °C. Observed difference in radial growth was statistically significant at each temperature ($p<0.001$). Sporulation of the isolates increased with increasing temperature. There was a slight drop in sporulation at 22 °C in all the isolates tested. Except the isolate ICMP 21519 which showed highest sporulation at 34 °C, optimum sporulation was observed at 30 °C for rest of the tested isolates (Figure 3A).

Increase in temperature above 26 °C significantly affected the growth of ICMP 21518, ICMP 21519 and ICMP 21515, isolates. However, the ICMP 21513, isolate obtained from Ruwanwella showed optimum growth at 30 °C and peak sporulation at 34 °C. Ruwanwella comes under relatively warm climatic conditions (average annual temperature 27 °C) than Yatinuwara (average annual temperature 25 °C) and Norton (average annual temperature 20 °C).

These differences suggest the versatility and adaptability of the pathogen. It has been mentioned that temperature is a major factor affecting population dynamic and community structure of *Fusarium* spp. (Saremi and Burgess, 2000).

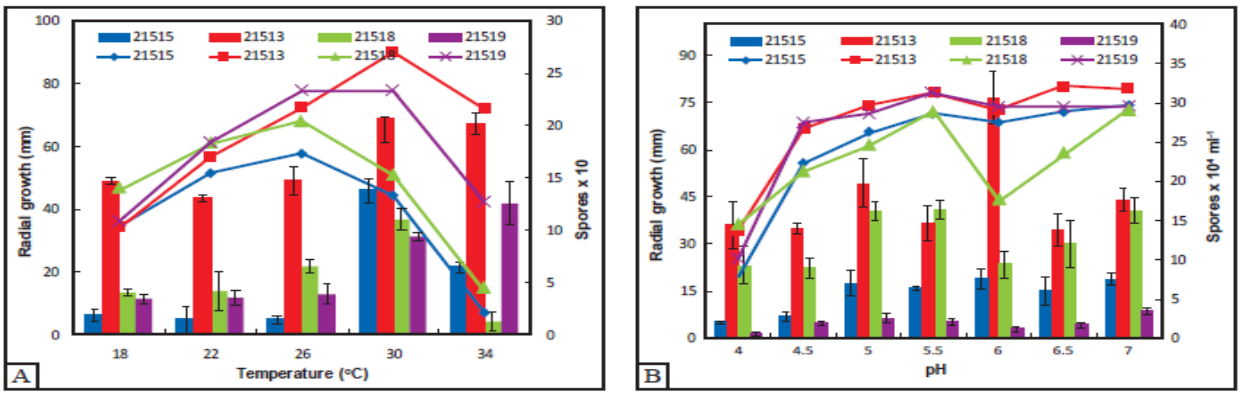

Figure 3. Radial colony growth (lines) and sporulation (bars) of *F. solani* isolates on PDA at different temperatures (A) and different pH levels (B). Error bars indicate Standard Error Mean.

Effect of pH on growth and sporulation

Colony growth in diameter significantly differed with increasing pH of the medium ($p<0.001$). Radial growth of the colonies increased with increasing pH above 4 and reached maximum growth at pH 5.5. There was a slight reduction in radial growth at pH 6.0 and again an increase at the neutral pH (Figure 3B). This trend is common to all the isolates tested. Conidia formation of the test isolates at different pH value varied significantly ($p=0.001$). Isolates, ICMP 21515 and ICMP 21513, showed maximum sporulation at pH 6 while ICMP 21518, ICMP 21519 showed two peaks at pH 7 and pH 5 to 5.5.

The results reveal that a suitable pH for maximum colony growth was at pH 5.5 and sporulation at pH 5-7 for all four *F. solani* isolates. Similarly, *F. solani* and *F. oxysporum* causing wilt in *Psidium guajava* showed maximum mycelia mass production at 5.5 followed by pH 5 (Gupta *et al.* 2010). Tea is cultivated in acidic soil with a pH range 4.5 to 5.5. This clearly shows the suitability of tea soil for *F. solani* to grow and sporulate as a soil inhabitant. Maintaining soil pH 4.5 to 5.0 would have an advantage in keeping the growth and sporulation of the fungi at lower levels.

Fungicide screening

In vitro screening

The radial colony growth of the isolate was reduced with the increase in fungicide concentration. Among the tested systemic fungicides, Tebuconozole was highly effective in inhibiting 100% of the radial growth of the isolate at 10 ppm. EC 50 values for Tebuconazole was 0.18 ppm. Hexaconazole and Pyrachlostrobin completely suppressed the colony growth at 1000 ppm. Bittertanol did not completely (100%) suppress the radial colony growth at any concentration. EC50 value of Hexaconazole, Bittertanol and Phyrochlostorbin was 0.60, 1.28 and 7.85 ppm respectively. The tested isolates showed less sensitivity to the contact fungicide copper hydroxide with EC 50 value 150.9 ppm. EC 50 value among the tested fungicides are statistically significant at 95% confidence interval ($p=0001$).

In vivo (Field) screening

The systemic fungicide Tebuconozole which showed highest efficiency *in vitro* test was used as soil drench at 100 ppm (0.01%), 500 ppm (0.05%) and 1000 ppm (0.1%) under field conditions. The pre-treatment disease incidence in the experimental blocks ranged from 94-100% and remained unchanged even after the treatment. The reduction in disease severity was 45.9±0.78%, 48.1±5.9% and 51.6±3.7% in Tebuconazole treatment at 100 ppm, 500 ppm and 1000 ppm, respectively. However, the reduction in disease severity with increasing fungicide concentration was not statistically significant at 95% confidence interval ($p>0.05$). Therefore, considering environmental safety and effective disease control, Tebuconazole at 500 ppm (0.05%) can be recommended to manage *Fusarium* collar canker and dieback in tea.

CONCLUSIONS

In consistent with previous findings, this study confirmed pathogens associated with collar canker and associated dieback in all the study locations as members of FSSC clade 3. Optimum temperature and pH for the growth and sporulation of the isolates were 26 ^{o}C to 30 ^{o}C and pH 5.5, respectively. Triazole fungicide Tebuconazole was found to be effective against FSSC collar canker and dieback.

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