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Causal agent, symptoms and environmental factors of root rot disease of organic assam tea in Mae Taeng district, Chiang Mai province

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During January to December 2012, root rot disease was observed in an organic Assam tea plantation located at 19° 16' 2.30" N latitude and 98° 55' 17.14" E longitude. The plants showed symptoms of stunting, yellowing or browning of the foliage, leaf drop and death. Temperature and humidity were major environmental factors that correlated with the symptom severities. The infected plants were analyzed by fungal isolation techniques. Six fungal genera were found and three were selected for characterizations since of their mycelial morphologies were similar to fungal mats found in association with the underground symptoms. The selected isolates were shown to produce pathogenicity-related factors associated with *Ganoderma* spp., cellulase and ligninolytic enzymes, on CMC agar and Poly R-478 agar, respectively. After 14 days, the isolates produced 1.43 and 1.88 cm-diam. clear zones on CMC agar and Poly R-478 agar, respectively. Pathogenicity tests were performed on 2-year-old seedlings of *Camellia sinensis* var. *assamica* which were wounded and inoculated with fungal mycelia at the stem bases. Six months after inoculation, all of the tested plants showed necrosis typical of fungal infection. Plant vascular tissue destroyed by fungal infection was sectioned using a freezing microtome and microscopically compared with the control. The pathogen was re-isolated and identified by molecular analyses, using PCR to amplify the 18s rRNA gene with the F63-forward and LR3-reverse primers. Nucleotide sequences showed maximum identity at 99 % to the large rRNA subunit gene of *Ganoderma australe* (acc. No. JN048792.1) retrieved from GenBank.

Keywords: Root rot, *Ganoderma australe*, Environmental factors, *Camellia sinensis*, Organic Assam tea, Chiang Mai

Introduction

Assam tea (*Camellia sinensis* var. *assamica*) is an economically important crop in Northern Thailand. The plant is susceptible to a high number of diseases, particularly fungal diseases such as anthracnose (*Colletotrichum*

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theae-sinensis), blister blight (*Exobasidium reticulum*), grey blight (*Pestalotia theae*), brown blight (*Colletotrichum camilliae*), stem canker (*Macrophoma theicola*), charcoal root disease (*Ustulina deusta*), red root disease (*Poria hypolateritia*), brown rot disease (*Fomes noxius*), root splitting disease (*Armillaria mellea*) and root rot (*Ganoderma pseudoferreum*) (Schoorel and Van, 2000). In an organic plantation system, control of pathogens is very difficult because there are many factors involved (Koike *et al*., 2000).

Moreover, this system is found to generate new disease occurrences since pathogens constantly change or mutate usually resulting in new strains and new challenges to growers. Accurate diagnosis of the pathogen may be very difficult and require specialized equipment Hanna *et al*., 2008). Root rot diseases are a major problem for organic tea plantations. This disease type is difficult to identify (Lee, 2002) since most common root diseases are caused by many genera of fungi. Root rot diseases are often associated with such nonliving stress factors as flooding or soil compactions which destroy the root system, resulting in growth reduction and wood decay (Mohd *et al*., 2005; Liang *et al.*, 2010). Foliar yellowing of diseased plants is commonly related to destruction of the root system and the subsequent reduced supply of water and nutrients to the foliage (Ann *et al.* 2002). Groups of trees are commonly infected by root to root spread of the pathogen from diseased plants to healthy plants. Fungal pathogens can survive in roots for decades after the infected tree has died (Brooks, 2002). The objective of this study was to diagnose and identify the pathogen causing root rot disease of organic Assam tea in Mae Taeng district, Chiang Mai province, Thailand.

Materials and methods

Disease Surveys

Monthly root rot incidence surveys were conducted in forestry plantations at the organic Assam tea orchard belonging to the Raming Company, Mae Taeng district, Chiang Mai province, located at 19° 16' 2.30" N latitude and 98° 55' 17.14" E longitude, comprised of a total area of 7,875 m^2. Six hundred plants were selected for disease incidence studies. Rating of disease severity level of above-ground symptom was recorded as follows:

rate 0= healthy or leaf yellowing and defoliation less than 20%
rate 1= leaf yellowing and defoliation between 20-50%
rate 2= leaf yellowing and defoliation between 50-80%,
rate 3= leaf yellowing and defoliation more than 80% or plant death

Then, the disease index was calculated using the following formula:

$$\text{Disease index} = \frac{\text{No. plants in each disease severity level}}{\text{Total no. plants examined}} \times 100$$

Environmental factors, i.e., temperature and relative humidity, were also recorded during January to December 2012. Number of diseased plants counted in each disease severity level in the total of 600 plants and environmental factors were correlated using the program Eview version 5 by least squares method at $P < 0.05$ level of probability according to Gary (2010).

Fungal Isolation

Roots of diseased plants were sampled and tissues exhibiting rot were rinsed in running tap water for 30 min until free from soil particles as described by Agnihothrudu (1962). Thereafter, plant tissue was soaked in 70% ethanol for 15 min, treated with 10% sodium hypochlorite for 15 min, rinsed in sterilized water, then cut into several pieces of approximately 0.5 x 0.5 cm. The disinfested plant tissue was plated onto Potato Dextrose Agar (PDA) and incubated at room temperature (28 ^{o}C ± 2 ^{o}C) for fungal growth. The pure cultures were transferred onto Malt Extract Agar (MEA) for morphological studies (Agrios, 2005).

Cellulase and Ligninolytic enzyme production

Cellulase enzyme production was tested, using modified CMC agar medium according to Han (1968). Fungi isolated from diseased tissue were grown on CMC agar which contained KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.5g; NaCl, 0.5g; $FeSO_4.7H_2O$, 0.01g; $MnSO_4.H_2O$, 0.01g; NH_4NO_3, 0.3g; CMC, 10g; agar, 15g; and distilled water, 1 liter. The CMC agar plates were incubated at 25°C for 14 d, then agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The plates were further treated by flooding with 1M NaCl for 15 min. The formation of a clear zone of hydrolysis which indicated cellulose degradation was measured. Evaluation of ligninolytic enzyme production was done using a modified method of Bumpus *et al.* (1985), Kirk and Farrell (1987) and Eriksson *et al*. (1990). The same fungi tested on CMC agar were grown on modified Poly R-478 agar medium which contained malt extract, 20 g; peptone, 1 g; dextrose. 20 g; agar, 15 g; polymeric dye (Poly R-478), 0.2g; and distilled water, *1* liter. The Poly R-478 agar plates were incubated at room temperature (28 ^{o}C ± 2 ^{o}C) for 14 days before clear zones

were measured. The plates were arranged using a CRD and clear zone diameters were significantly different using the Statistix 8 program.

Pathogenicity Tests

Pathogenicity tests were performed using 2-year-old seedlings of Assam tea grown in 4-inch pots containing sterile soil and kept under greenhouse conditions. Fungi that were positive for cellulase and ligninolytic enzyme production were grown on MEA for 1 month. Mycelial pieces were then used as inoculum. Soil around basal stem of seedlings was removed to a depth of 1 cm, the stems were wounded with a scalpel, and a 1 x 1 cm mycelial piece was placed on the wound and wrapped with a clear plastic sheet. Inoculation with each fungal isolate and control (inoculated with an agar piece without fungi)were replicated 4 times. Plants were periodically examined for disease development such as yellowing of leaves, defoliation and wilting (Mohd *et al*., 2005). All wilted plants were examined for vascular degradation under microscope after the inoculated stems were sectioned with freezing microtome, and reisolation of the fungi was performed as indicated above.

Pathogenic fungi identification

Fungi which produced a high level of cellulase and ligninolytic enzymes and were positive for pathogenicity were re-isolated from inoculated plant tissue and grown for 14 days on MEA medium. Sequencing of the fungus was done using the 18s rRNA gene at the Mahidol University–Osaka University Collaborative Research Center for Bioscience and Biotechnology (MU-OU: CRC), using primers F63-Forward (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and LR3-Reverse (5'-GGT CCG TGT TTC AAG ACG G -3'). The sequencing data were analyzed with Chromas version 1.45 (32-bit) software, and then the alignments were preceded in BLASTn (Basic Local Alignment Search Tool for nucleotides) according to Altschul *et al.* (1990) to identify the fungal species.

Results

Disease survey and above-ground symptoms

Above-ground symptoms of fungal root rot disease in Assam tea including leaf drop, yellowing or browning of leaves, defoliation, stunting, decline of vigor, twig and branch dieback, and plant death (Fig. 1-A) were generally found in the survey area. Underground trunks and roots of dead tree were characterized by formation of white to dark-brown mycelia mats on the plant surfaces (Fig. 1-B, 1-C) after soil was removed over 1 foot depth.

Fig. 1. Root rot symptoms found in an Assam tea pant at an organic tea plantation at Mae Taeng district, Chiang Mai province, during January to December 2012, Above-ground symptoms: 1-A; underground symptoms: 1-B, mycelium covering the root surface; 1- C: mycelia mat covering the root surface.

We observed that the number of plants that were infected and killed by root rot which gradually increased over time (Fig. 2). The percentage of dead plants (disease index 3) clearly increased over time, especially in the last month of December, when it reached 6.3 %. Disease index percentage of 0 (healthy plants) showed decreasing of healthy plant numbers showed a corresponding decrease. Environmental factors influenced root rot disease; temperature was positively correlated ($P<0.05$, $n =12$) with disease index 1 (Fig. 3-A), while humidity showed a negative correlation ($P < 0.05$, $n = 12$) with disease index 3 (Fig. 3-B).

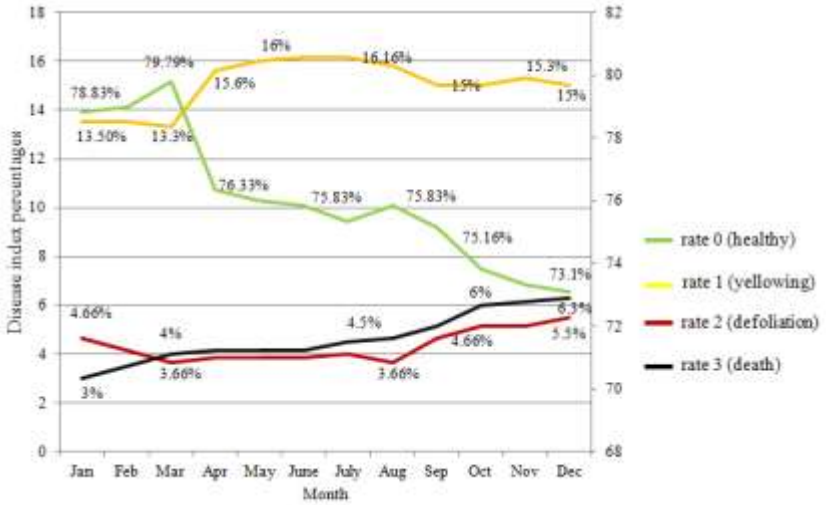

Fig. 2. Root rot disease indices found in an organic Assam tea plantation in Mae Taeng district, Chiang Mai province during January to December 2012.

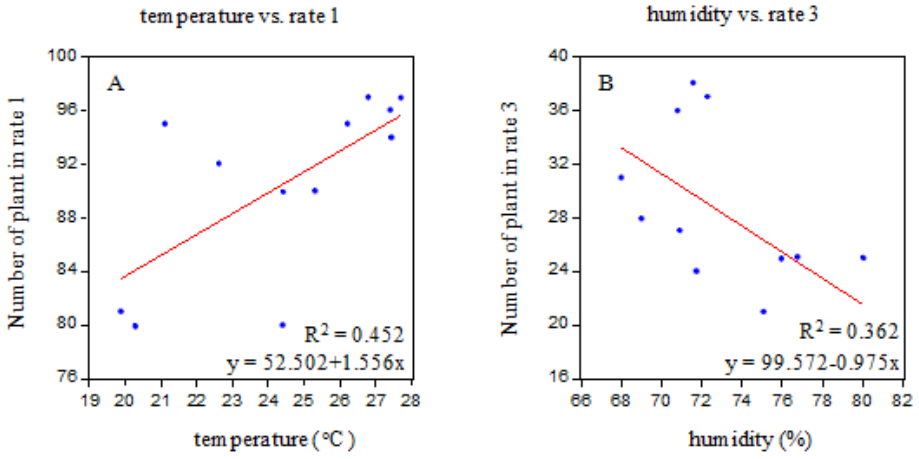

Fig. 3. Correlation of environmental factors and disease index percentage of indices of 1 and 3 of root rot disease of Assam tea, surveyed from January to December 2012; A: positive correlation between temperature and disease index 1, B: negative correlation between humidity and disease index 3 ($P < 0.05$).

Fungal isolation

Six fungal genera were isolated from the roots of infected plants including: *Aspergillus* sp., *Fusarium* sp., *Ganoderma* sp, *Paecilomyces* sp, *Pennicillium* sp, and *Trichoderma* sp., based on morphological characteristics, mycelial growth rate, color of mycelial mat, mycelial texture and conidia production. Three genera, *Fusarium* sp. *Ganoderma* sp. and *Paecilomyces* sp.

respectively, were selected for pathogenicity since of their white color and mycelial texture were similar to mycelial mats found in association with underground symptom of the diseased specimens.

The isolate of *Fusarium* sp. grew rapidly on PDA covering the plate in 10 d with creamy white *and* fluffy *mycelia*, while the growth rate of *Ganoderma* sp., and was comparatively slower, covering the plate in 3-4 weeks with mycelium that was white then gradually turned brown over time. The texture of the mycelia mat of *Ganoderma* sp.became crusty with age. *Paecilomyces* sp. showed had a fast growth rate similar to *Fusarium* sp., producing white colonies with a powdery and velvety *mycelial texture in the* initial culture of 3-4 days, which then became gray with age (Fig. 4).

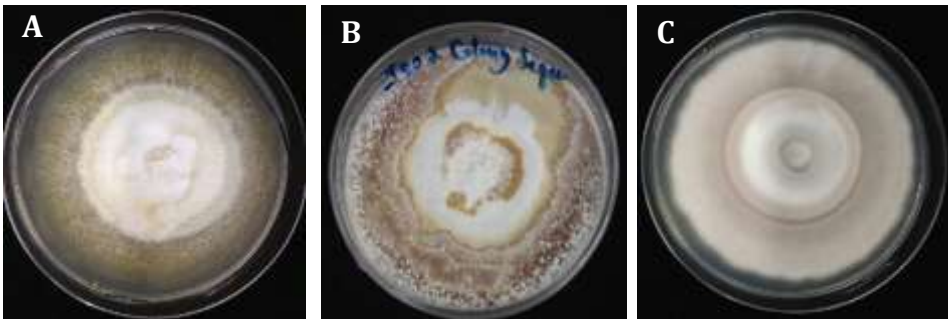

Fig. 4. Colony characteristics of *Fusarium* sp., *Ganoderma* sp. and *Paecilomyces* sp. isolated from inner tissue of infected root of Assam tea; A: colony of *Fusarium* sp. grown on PDA for 10 days, B: colony of *Ganoderma* sp. grown on MEA for 30 days, C: colony of *Paecilomyces* sp. grown on PDA for 10 days.

Cellulase and Ligninolytic enzyme production

The ability of the three species isolated from Assam tea to producecellulase and ligninolytic enzymes was based on the extent of clear zones on specialized agar. Fourteen days after incubation, *Ganoderma* sp. produced the largest clear zone diameters at measuring1.43 and 1.88 cm for cellulase and ligninolytic enzymes, respectively (Table 2).. Ganoderma not only produced more of the enzymes but did so more rapidly; initial clearing could be observed within 3-4 d.

Table 2. Production of cellulase and ligninolytic enzymes by three fungi isolated from diseased roots of Assam tea

Isolate	Cellulase Enzyme[1]	Ligninolytic Enzyme[2]
	Clear zone diameter (cm)[3]	Clear zone diameter (cm)[3]
Fusarium sp.	0.00[c4]	0.18[b4]
Ganoderma sp.	1.42[a]	1.88[a]
Paecilomyces sp.	1.02[b]	0.00[c]
%CV	24.32	9.13

[1]Fungi were grown on CMC agar for 14 d[2] Fungi were grown on Poly R-478 agar for 14 d
[3]Average from 4 replications[4]Means in a column followed by the same letter are not significantly different ($P < 0.05$)

Pathogenicity Test: Two-year-old Assam tea seedlings inoculated with *Ganoderma* sp. Showed above ground symptoms similar to those observed on the affected trees in the forest plantations area; yellowing of leaves, wilting and defoliation were among the major symptoms observed. However, the early stages of infection could not be distinguished and were difficult to diagnose. Six months after inoculation, all four of the inoculated plants showed collar rot and lesions on the basal stems. Microscopic examination of affected tissues following freezing microtome sectioning and staining revealed tissue destruction, particularly vascular tissue. Stained fungal hyphae were seen first in the phoem before spreading to xylem. (Fig. 5)

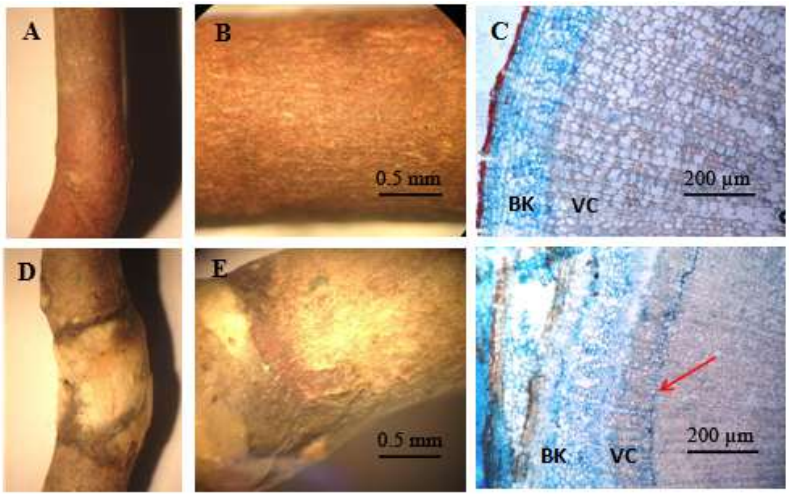

Fig. 5. Pathogenicity testing of fungal *Ganoderma* sp. conducted on 2-year-old organic Assam tea seedlings; A, B and C: Non-inoculated control examined by stereomicroscope, and after staining with cotton blue, examined by light microscope, respectively. D, E and F: inoculated stem by *Ganoderma* sp., necrotic *lesion* and plant bark (BK) was destroyed by blue-staining fungal mycelium (arrow) beyond the vascular cambium (VC), respectively.

Pathogenic fungi identification

Fungal re-isolation and identification of 14-day-old mycelium of *Ganoderma* sp. using the 18s rRNA sequencing technique showed query cover 100% and maximum identity 99% with the large subunit ribosomal RNA gene of *Ganoderma australe* (acc. No. JN048792.1) retrieved from GenBank.

Discussion

A fungal root rot pathogen of organic Assam tea, observed in Mae Taeng district, Chiang Mai province, was identified as *Ganoderma australe* by its ability to invade and destroy vascular tissue and production of putative pathogenicity enzymes (Martinez *et al.*, 1995; Schwarze and Ferner, 2003; Vuledzani, 2009). The fungus apparently moved from the roots to the woody trunk tissue where it destroyed the wood and xylem. Early stages of infection were mostly indistinguishable from healthy trees while advanced stages of the infection were obviously visible as indicated by bark depression and rotting at the root collar (Trevor *et al.*, 1999; Paterson, 2007).

Ganoderma was reported to destroy many agricultural crops (Likhitekaraj and Tummakate, 2000; Seo and Kirk, 2000; Sankaran *et al.*, 2005; Zakaria *et al.*, 2009). Primary aboveground symptoms observed in infected Assam tea in northern Thailand matched those reported on tea from Singapore which included: leaf drop, stunting, yellowing or browning of the foliage, and a general decline in the vigor (Corner, 1932). In addition, the disease survey results showed a significant positive correlation between the primary yellowing stage and high temperature. It appears that the damaged roots were non-functional for water uptake while the transpiration rate was increased by high temperature. On the other hand, rate of plant death showed a negative correlation with high humidity level, most likely due to reduced transpiration in a humid environment. Hennessy and Daly (2007) also reported that *Ganoderma* disease development was affected by environmental factors, the death could be either slow or rapid depending on water availability and temperature. During the dry season in December, mountain areas of northern Thailand sometimes face very low humidity and high temperature in the daytime; the highest incidence of dead tea plants (6.3%) occurred during this period of severe water stress. However, personal communication from Raming Company reported that the incident of root rot has occurred year by year. Re-plantation of healthy seedlings was not a success during the past 30 years, as was the case. with root rot disease in California oaks (Swiecki and Bernhardt, 2006). Future directions for research on *Ganoderma* in organic Assam tea production could include

screening the Assam tea population for resistant individuals, grafting on to resistant rootstock, reducing water stress and biological control.

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