IDENTIFICATION AND CHARACTERIZATION OF PATHOGEN CAUSAL OF RED ROOT DISEASE OF TEA PLANT AT SABAH TEA PLANTATION

Chin, C. F. S. 1*, Markus, A. 1 and Wong, N.K. 2

1 School of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan Campus, Mile 10, Batang River Road, 90000 Sandakan, Sabah, Malaysia
2 School of Science and Technology, Universiti Malaysia Sabah, UMS Road, 88400 Kota Kinabalu, Sabah, Malaysia

* Corresponding author email: clament@ums.edu.my

Abstract

Sustainability of organic tea production at Sabah Tea Plantation is currently hampered by the presence of a root disease. Infected plants gradually showed wilt of above ground parts and rot in the underground roots. This study is aimed to identify the pathogen causal of red root disease at Sabah Tea Plantation and to provide basic descriptions of its macro- and micromorphological features. A basidiomycete fungus was isolated from infected tea roots and its pathogenicity on tea plant was confirmed by Koch’s Postulate. Basidiomata formed on several infected tea roots in field was examined and found in resupinate form, annual, yellow when fresh and gradually become cream to pale tan upon drying. Basidia and basidiospores were not seen. Hyphal system was present in pseudodimitic form, lightly to heavily encrust with loosely attached small hyaline crystals that dissolve in 5% KOH. Clamps connection was present at all septa together with yellowish variably branched skeletal-like hyphae. Cystidia present in sphaeropedunculate shape with a long stalk. On media, the fungus grew rapidly with a mean growth rate of 0.42 cm/day. Rhizomorph structure was present in field and on artificial growing media. The pathogen causal of red root disease at Sabah Tea Plantation is suggested to be Schizopora flavipora (synonym Poria hypolateritia) based on conventional mycological examination and literatures research.

Introduction

Tea (Camellia sinensis var. assamica) is one of the valuable commodities in Malaysia due to consumer preference and high economic viability. Sabah Tea Plantation (STP) is located at Nalapak Village (5° 55’ 58.53” North, 116° 46’ 22.44” East) of Ranau, covering 2,480 hectares of the ancient rainforest land at 830 m elevation. It is one of the very few tea plantations worldwide that have received SKAL International, Netherlands certification for producing organic tea. No additional pesticides control or synthetic fertilizers is used instead tea waste from factory is scattered back into the field as a source of organic fertilizer.

Intensive monoculture practice during the last three decades (1976-2011) over an intensive area in STP, however, has formed a stable tea ecosystem and provided unlimited opportunity for perpetuation and spread of endemic disease. Currently, sustainable production of organic tea is hampered by the presence of a root disease. The disease is characterized by distinct dark red rhizomorph batches spread on infected roots, with wilting symptom and semi-detached leaves seen on infected plants. This study was conducted to isolate and to describe in details the morphological features of the pathogen causal of red root disease (RRD) at STP.
Materials and methods

Plant material
Tea (C. sinensis var. assamica) seedlings in the age of two-year-old were obtained from STP. They were originally sown in 2 kg of topsoil in polybag under a net house (28 °C, 71% humidity) and water regularly twice a day. Several infected tea bushes in the field were uprooted for root samplings and basidiomata collection. Microscopic features were examined under a Zeiss Axioplan compound microscope using wet-mount technique with or without cotton blue.

Isolation of tea root pathogen
Diseased bushes were uprooted from STP and the roots were washed thoroughly with running tap water before brought back to laboratory. Infected rootlets showing sign of diseased symptoms were detached and cut laterally into a length of 1 cm long using a sterile razor blade. Rootlets were surface-sterilized by using 0.1% solution of mercury chloride, followed by 75% ethanol aqueous and rinsed thoroughly with sterilized distilled water. Disinfected rootlets were blotted dry and aseptically transferred onto potato dextrose agar (PDA). Cultures were incubated in the dark at 28 °C and examined every two days interval. Subcultures were carried out when necessary to obtain pure culture of the isolate pathogen.

Pathogenicity test
Pathogenicity test was done according to Oliveira et al. (2008) with minor modification. A 7 mm mycelia plug from five-day-old culture was first inoculated to a commercial medium used for cultivate edible oyster (Pleurotus ostreatus) mushroom and incubated for two weeks prior inoculation assay. Inoculation of seedlings were done by cutting the underneath of each polybag to a height of 10 cm. Twenty grams of inoculum were placed in contact with the roots in a new plastic pot and the removed soil was put back. Total of five inoculated seedlings were used, maintaining an equal number of seedlings without inoculum that serve as controls. All seedlings were uprooted for re-isolation of the pathogen at six months post-inoculation.

Culture description
Colony growth and morphological characteristics on PDA were observed over a period of 30 days. A consistent measurement of colony size was taken along the vertical and horizontal sides of the Petri dishes in every two days interval for determination of daily means growth (mm.day⁻¹). Colony colours were described by referring to MUNSELL® colour chart for plant tissues.

Extracellular Polyphenolic Oxidases Test
Extracellular polyphenolic oxidase (EPO) activities were studied using 0.5% of gallic acid (GA) and tannic acid (TA) methods as described by Davidson et al. (1938), with the exception PDA was used instead of malt agar.

Rhizomorphs produced by woody Inocula
Production of rhizomorphs in vitro was attempted in a 200 mL conical flasks filled with mushroom cultivation medium. Tea rootlet length about 5 cm and 2 cm in diameter that has been previously colonized by the pathogen was aseptically transferred into the medium flasks. The inoculated flask was then placed in a 20 cm x 10 cm sealable plastic bag, and small holes were made by sterile needle for aeration. The flask was incubated at 28 °C in dark for six months and observations were made of the growth patterns of any rhizomorphs sent out from the medium.
Results and discussions

Macroscopic features
When natural infected tea plant was uprooted in the field and cleaned for examination, red (5YR 5/10 – 10R 4/10) shiny rhizomorphs were observed on the root surface. Pure culture of the pathogen was obtained and the appearance of some cultures was analog to the symptom showed in plant (Fig. 1a). Basidiomata were found in resupinate forms, forming as an irregular lumpy sheet or crust on the substratum, pale tan (2.5Y 8/6) in centre with cream whitish margin. The presence of small, angular and shallow pores (3-5/mm) on the basidiomata indicates the pathogen is from the family Polyporaceae.

Cultural morphology and extracellular polyphenolic oxidase activity
Fungal growth was moderate on PDA and covered culture dishes in three weeks, with a mean growth rate of 4.3 ± 0.1 mm.day⁻¹. Colony radius was 12-15 mm in the first week, cottony to fluffy, and remains whitish with advancing zone fairly even. In the second week center of the culture turned from pale yellow (5Y 8/10) to orange (7.5YR 7/10) colour and colony radius reached 23-29 mm. In the third week, colony radius was 34-40 mm, with an orange red (7.5YR 6/10) colour and aerial mycelium collapses towards growth margins. When the colony radius reaches the edge of culture dishes on the fourth week, further extension growth was obstructed. At this stage, the culture has a red (5YR 5/10) to brownish red (5YR 4/8) leathery texture that appeared and touched like a root bark (Fig. 2). The culture contains no distinct odor and no agar discoloration occurs up to six weeks. Test was positive for EPO activity and the reaction was strong (+++++) with light to dark brown diffusion zone extending a great distance beyond the margin of the hyphal malt about 5.03 ± 0.41 cm diameter in gallic acid and 5.51 ± 0.04 cm diameter in TA amended agars, suggesting the pathogen belonged to the distinct white rotter group.

Fig. 2: Culture characteristics of pathogen causal of red root disease of tea plant. Culture dishes (a) to (d) showed the morphological changes took place on PDA media in four weeks.
Pathogenicity test

All two-year-old tea seedlings inoculated with the isolated fungus exhibited symptoms of wilting and defoliation starting at two months post-inoculation. When inoculated seedlings were examined, tap roots were found heavily encrusted by hyphal mat and the root barks were visually appeared as leathery dark red (2.5YR 3/6) at the inoculum site, with sulphur yellow (2.5Y 8/10) to whitish in the new invasion site (Fig. 3). It was revealed the reason of having reddish discolouration of infected roots were due to the coverage of the fungus mycelia strand, which can be sometimes misled as root bark if not properly examined.

![Fig. 3: External symptoms of red root disease in the above-ground part. (a) Healthy and (b) infected tea bushes in field, (c) pre- and (d) post-inoculated seedling at six months, (e) severe infected tea root by the red root pathogen (bar = 5 mm).](image1)

Rhizomorph formation

Rhizomorph structures were produced by the pathogen in both inoculated PDA and mushroom cultivation media (Fig. 4). On agar medium, rhizomorphic colonies often turned into a network of rhizomorphs with only a small mycelial center. The rhizomorphs were compact or open in appearance depends to the frequency of branching, submerged and aerial, cylindrical or flat. Rhizomorphs grown out from mushroom cultivation medium were crustier and cylindrical with diameter range from 0.2 to 2.0 cm and 15 to 20 cm in length that overall has a soil-coloured rind. Beneath the reddish (7.5R 3/8) leathery skin was a cream to white interior tissue that has both texture and odour similar to mushroom fleshes, and remains elastic upon drying. Yellow (2.5Y 8/8) fimbriate dissepiments hyphal arose from the red crusty surface were found to present randomly on rhizomorphs, which were also found on natural infected tea roots. However, the function of this yellowish hyphal was unknown.

![Fig. 4: Rhizomorphs of pathogen causal of red root disease. (a) Rhizomorphs produced in artificial culture; (b) a network of the rhizomorphs produced in woody material (bar = 30 mm); (c) a close-up of the rhizomorphs showing red crested skin with white interior fresh (bar = 5 mm), and (d) yellow fimbriate hyphae that present on rhizomorphs (magnification = 10X).](image2)

Microscopic features

Microscopic features of the isolated fungal species (Fig. 5) are important characteristics used in conventional mycology study. Hyphal system apparently pseudomitic with yellowish variably branched generative hyphae. Hemispheric clamp connection (15.5 x 12.4 μm) was present at most septa of generative hyphae, indicates the pathogen is from the phylum basidiomycota. Hyphae tips were found...
lightly to heavily encrust with loosely attached small hyaline crystals that dissolve in 5% potassium hydroxide (KOH). Cystidia are microstructures found in very few genera of polypores. The presence of capitate cystidia (10.5-15.5 x 9.0-12.7 μm) with a long stalk in the tramal tissue of the basidiomata (Langer, 1994) and drepanocysts in the pure culture (David and Rajchenberg, 1992) are unique and taxonomically important microscopic features for the genus and species of *Hyphodontia* or *Schizopora*. No true basidia or basidiospores were found. In PDA medium, clamydospore-like cells (3.5-8 x 1.5-2 μm) were found abundantly produced on aerial mycelium, while interlocking hyphae and cuticular cell were produced in pure culture as colony aged, given the fungus a leathery-like texture.

![Fig. 5: Microscopic features of pathogen causal of red root disease of tea plant. (a) Variable branched generative hyphae; (b) generative hyphae with clamp connection; (c) hyphae encrusted with hyaline crystals; (d) capitate cystidia or allocysts with swollen hyphae tips; (e) drepanocysts; (f) clamydospore-like cells; (g) interlocking hyphae and (h) cuticular cell.](image)

**Identification of pathogen causal of red root disease of tea plant**

On the basis of the characteristics of basidiomata, pores size and microscopic features, *Schizopora flavipora* (Berkeley and M. A. Curtis ex Cooke) Ryvarden, which is synonymous to *Poria hypolateritia* (Berk) (Baby et al., 2004) is suggested to be the pathogen causal of RRD of tea at STP. The issue whether to address the fungus as *S. flavipora* or *H. flavipora* remains undetermined, and both names were accepted and considered synonymous to each other (Langer, 1998). Details of its macro- and micromorphological features were described. The pathogen was found to spread chiefly by vegetative hyphal and rhizomorph growth underneath the soil. Numerous clamydospore-like cells were also seen on the aerial mycelia. This finding lead to a new suggestion that they are potential means of disease dispersion tool used by the root pathogen as each of the cell is capable to form new hyphal mat and rhizomorph structures, which further invade the roots present nearby resulting death of tea bushes that occurred in patches as seen in STP.

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