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In Vitro and *In Vivo* Tests for Parasitism of *Verticillium psalliotae* Treschow on *Hemileia Vastatrix* BERK. and BR.

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ABSTRACT

The fungus *Verticillium psalliotae* Treschow is known to grow on the rust sori masses of *Hemileia vastatrix* in coffee plantings in Malaysia. However, published reports on this parasite or other biocontrol organisms present naturally in the coffee ecosystems in Malaysia are lacking. *In vitro* and *in vivo* studies were conducted to elucidate evidence of parasitism of *V. psalliotae* on *H. vastatrix.* The host-mycoparasite interaction conducted *in vitro* resulted a significant reduction in germination of *Hemileia* uredespores and an increase in germination of *Verticillium* conidia. A significant reduction in leaf rust severity by the presence of *V. psalliotae*, particularly when *V. psalliotae* was applied 24 hours before the inoculation of *H. vastatrix*, demonstrated an evidence parasitism *in vivo*. The evidences were supported by SEM observation of penetration and damage to *Hemileia* uredospores by hyphae of *V. psalliotae*.

Keywords: *Verticillium psalliotae*, mycoparasite, *in vitro, in vivo, Hemileia vastatrix*

INTRODUCTION

Leaf rust disease caused by *H. vastatrix* Berk. And Br. is a serious problem in coffee plantings in Malaysia. Since rust produces external fructifications as a secondary inoculum, helping the disease to spread, it can be controlled more effectively by mycoparasite than other disease such as leaf spots (Sharma and Sankaran, 1986). *Verticillium psalliotae* is a mycoparasite on rust fungi (Jeffries and Young, 1994). It was found as a mycoparasite on uredosori of the soybean rust (*Phakopsora pachyrhizi* Syd.) in Thailand and Taiwan (Saksirirat and Hoppe, 1991). The strains of *V. psalliotae* originally isolated from soybean rust grew rapidly on *Puccinia coronata* and *H. vastatrix* (Saksirirat and Hoppe, 1990). In Malaysia, *V. psalliotae* grew vigorously between the warts on the surfaces of the uredospores of *H. vastatrix*, the causal organism of leaf rust, the most destructive disease in coffee plantings (Lim and Nik, 1983). When a mycoparasite is found naturally on a plant pathogen in a commercial crop plantation, an examination of its potential in disease control should immediately be considered (Spencer, 1980), by *in vitro* and/or *in vivo* tests (Campbell, 1990). According to Sivan and Chet (1992), elucidation of mechanisms involved in biological control activity is considered to be one of the key factors in the development of useful biological control agents.

In the *in vitro* test, spores of the mycoparasite and test pathogen are mixed together in sterile distilled water, and incubated at room temperature (Dhingra and Sinclair, 1995). The degree of inhibition of pathogen growth is used as a measure of effectiveness. *In vivo* tests are usually the first choice as they most closely imitate the conditions under which the control agent will eventually have to operate (Campbell, 1990). The effect of the leaf mycoparasite on a pathogen can be tested on detached leaves as well as on seedlings growing in the glasshouse (Dhingra and Sinclair, 1995) by using the droplet inoculation assay (Andrews, 1985). The inoculation of either a mixture of both organisms or each organism separately on the leaf surface is used in the test (Dhingra and Sinclair, 1995). Measurement of the effect of the mycoparasite is based on disease severity (Andrews, 1985), which is measured regardless of the mode of action (mycoparasitism) (Campbell, 1990). The objective of this study was to evaluate the mode of mycoparasitism by *V. psalliotae* on rust pathogen *H. vastatrix.* This paper reports evidence of parasitism based on *in vitro* and *in vivo* tests.

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MATERIALS AND METHODS

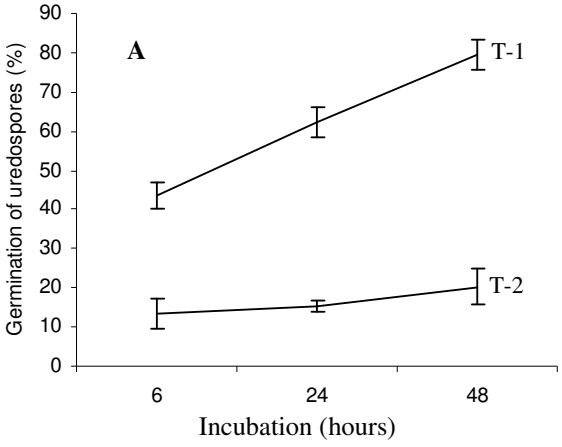
Uredospores of *Hemileia vastatrix* and mycoparasite *V. psalliotae* were collected from naturally infected leaves of *Coffea liberica* at smallholder's plots in the Kuala Langat district of Selangor. *H. vastatrix* uredospores suspension (1.6×10^5 uredospores ml^{-1}) was obtained by mixing 10 mg uredospores in 10 ml distilled water. Conidia of the mycoparasite *V. psalliotae* (1.8×10^6 conidia ml^{-1}) were prepared from two-week-old pure culture on oatmeal extract agar (OMEA). In *in vitro* test, 10 mg of *Hemileia* uredospores were mixed with 10 ml of *Verticillium* conidia suspension and incubated in the dark at ambient temperature. Percentage germination of *Hemileia* uredospores and *Verticillium* conidia as mixtures assessed at 6, 24 and 48 hours of incubation and compared to the control (in sterile water only). Significant

difference between treatments were analyzed using t-test at $p \leq 0.05$. In *in vivo* test, the mycoparasite conidia suspension was applied to the leaves simultaneously with *H. vastatrix,* or before and after inoculation of *H. vastatrix*. Inoculation was done in the evening using a micropipette. One droplet of about 25 µl was placed on the under surface of four young leaves of each 4-month-old coffee seedlings. After inoculation, the seedlings were incubated in the dark for 48 hours before being placed in a glasshouse. Parasitism assessed was based on disease severity on a scale of 0 to 9 modified from Eskes and Toma-Braghini (1981). For elaboration of the result, statistical analysis has been applied. Scanning electron microscope (SEM) observations were done to show the effect of mycoparasitism on the uredospores.

RESULTS AND DISCUSSION

In Vitro Test

Germination of uredospores was significantly reduced ($p \leq 0.05$) at all intervals of incubation in the presence of the mycoparasite in sterile water. In a mixed suspension of uredospores and mycoparasite conidia (T-2), percentage of uredospores germination was 13.4, 15.4 and 20.3% at 6, 24 and 48 hours of incubation respectively. This was significantly lower ($p \leq 0.05$) than in control (T-1) where germination was 43.7, 62.3 and 79.6% during the same incubation period. In contrast, germination of the mycoparasite conidia was significantly enhanced ($p \leq 0.05$) in the presence of *Hemileia* uredospores. Conidial germination in the presence of *Hemileia* uredospores was 10.9, 29.1 and 91.2% at 6, 24 and 48 hours incubation respectively, while in the control this was 2.9, 27.5 and 39.9% for the same period (Figure 1).



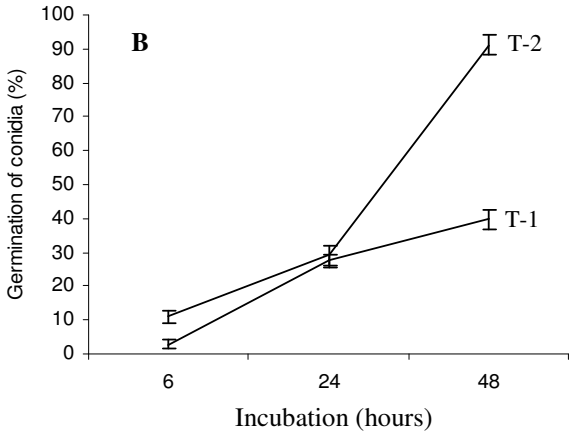


Figure 1:Mean germination progress of *Hemileia* uredospores (A) and *Verticillium* conidia (B) in sterile water (T-1, control) and in a mixed suspension of both uredospores and conidia (T-2);
Data were arcsin transformed before statistical analysis; vertical bars represent $\pm$ SD (n = 12)

The number of spores produced, and their viability, are significantly influenced by the presence of hyperparasites. Spores contaminated with hyperparasite *V. psalliotae* show reduced germination (Lim and Nik, 1983). Some authors reported the same result on other species. *Hemileia* uredospores contaminated with hyperparasite *V. lecanii* show a reduced germination (Kushalappa and Eskes, 1989). Eskes *et al* (1991) also reported that the germination of *V. lecanii* and *V. leptobactrum* was enhanced by the presence of uredospores of *H. vastatrix,* and there was also an increase in the length of the germination tube. This was observed to be especially true for the conidia of both *Verticillium* species in close distance to uredospores, whereas conidia that were at some distance from the uredospores often did not germinate or germinated more slowly. When *V. lecanii* suspensions were added to uredospores of *H. vastatrix*, uredospore germination was reduced by about 50% (Eskes, 1989).

The present laboratory studies demonstrated that the germination of *Hemileia* uredospores was reduced by the presence of the mycoparasite conidia, which suggests the action of a limiting factor from the mycoparasite. In contrast, the germination of *V. psalliotae* conidia was increased by the presence of *Hemileia* uredospores, which suggested the action of a stimulating factor from the rust uredospores. This same phenomenon occurred in the mycoparasitism by *V. lecanii* on *H. vastatrix* (Eskes *et al*., 1991). Besides the hyperparasitic action, Eskes (1989) concluded that this *Verticillium* was antagonistic to uredospore germination.

Saksirirat and Hoppe (1991) reported that *Verticillium* grows rapidly in a liquid culture on autoclave uredospores of soybean rust fungus, and is probably based primarily on nutrients made available to the mycoparasite by activities of β-1.3-glucanases, chitinases and proteases.

Their rapid growth is primarily based on the secretion of lytic enzymes, which made nutrients available to the mycoparasite.

In Vivo Test

The first evidence of mycoparasitism began to appear two weeks after inoculation of *V. psalliotae*. These were in the form of small white spots at the center of the rust sori. The spots gradually enlarge; the cotton-like, white coloured mycelium of the mycoparasite covered the rust sori. The development of the mycoparasite was restricted to the rust infected leaf parts, but never grew to over the entire width of the rust lesions. Healthy leaf tissues were also never invaded. This means that application of *V. psalliotae* on coffee leaves infected by rust did not appear to cause any adverse effects on the plant. This characteristic indicated that *V. psalliotae* might be of value in controlling rust infections (Garcia-Acha *et al*., 1965). Necrosis of parasitized rust lesions often occurred, but could not always be related to the presence of the mycoparasite.

The observations of this experiment were terminated 60 days after inoculation of *H. vastatrix* as some inoculated leaves had shown maximum disease severity (severity scale 9). The experiment revealed that the level of rust severity was significantly reduced ($P \leq 0.05$) when *V. psalliotae* conidia were applied 24 hours before the inoculation of *H. vastatrix* (T-1), simultaneously with the inoculation of *H. vastatrix* (T-2) and 24 hours after the inoculation of *H. vastatrix* (T-3). Application of *V. psalliotae* one week after the uredia of *H. vastatrix* erupted (T-4) did not reduce leaf rust severity. The lowest leaf rust severity was demonstrated by T-1, and was significantly different ($P \leq 0.05$) from all other treatments (Figure 2).

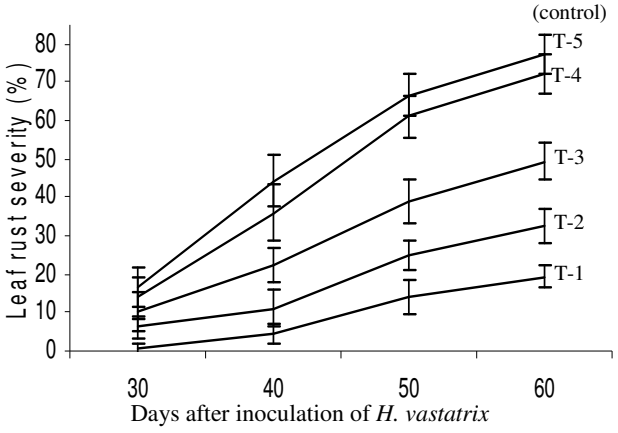


Figure 2: Leaf rust severity progress on coffee seedling applied with *V. psalliotae* conidia at 24 hours before inoculation of *H. vastatrix* uredospores (T-1), simultaneously with uredospore (T-2), 24 hours after inoculation of uredospore (T-3), one week after eruption of *H. vastatrix* uredia (T-4), and inoculation with *H. vastatrix* uredospore only (T-5, control); data were arcsin transformed before statistical analysis; vertical bars represent $\pm$ SD (n = 5)

This experiment provided evidence to support that *V. psalliotae* was effective as a biological control agent when applied 24 hours before the inoculation of *Hemileia* uredospores.

The infection process by the coffee leaf rust pathogen began with the germination of uredospores (Kushalappa and Eskes, 1989). Coutinho *et al* (1993) reported that germinating uredospores of rust fungi did not penetrate the host directly, but through formation of appressoria which penetrated through the stomata. The lowest disease severity associated with T-1 (*V. psalliotae* was applied 24 hours before the inoculation of *H. vastatrix*) was presumably due to the reduction in germination of uredospores caused by the presence of *V. psalliotae* on the leaf surface before the appressorium of *H. vastatrix* formed which decreased the infection level of *H. vastatrix*. Based on *in vitro* study, it was assumed that the optimum germination of *Verticillium* conidia occurred 24 hours after incubation in sterile water, and significantly reduced the germination of uredospores when the uredospores was mixed in the suspension of *Verticillium* conidia. Similar results had been reported for the interaction between *V. lecanii* and *V. leptobactrum* with coffee leaf rust uredospores (Eskes, 1989). A hyperparasitic fungus such as *V. psalliotae* was frequently associated with lesions, and reduced the viability of *H. vastatrix* uredospores significantly (Ferreira and Boley, 1991). A reduction of the primary inoculum by antagonists was considered effective in controlling a polycyclic disease (Kohl and Fokkema, 1994).

The lowest disease severity was achieved by applying *V. psalliotae* 24 hours before the inoculation of *H. vastatrix* suggesting the role of the mycoparasite as more of a prophylactic than as a therapeutic in biological control. This observation is supported by Spencer (1980) that when a mycoparasite was applied to the crop as a means of biological control the indications were that it would be used as a prophylactic rather than as a therapeutic since the conidia applied to the plants after inoculation with rust gave a few benefits. It appears therefore that conidia were either inherently short-lived or are sensitive to environmental stresses.

SEM Observation

SEM observation revealed that hyphae of *V. psalliotae* grew inside and outside the uredospores, and penetrated through the warted convex surface (WCS) and smooth concave surface (SCS) of uredospore. Often a single spore was penetrated by more one hyphae. No appressoria like structures were observed. Signs of damage were observed on most uredospores (Figure 3).

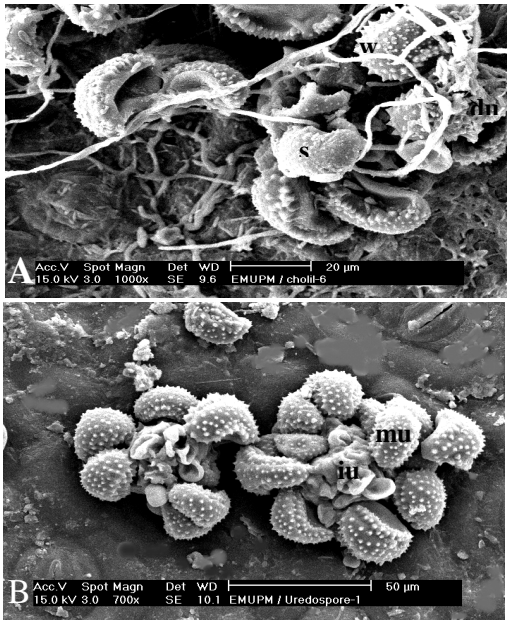


Figure 3: SEM of uredospores being parasitized by *V. psalliotae* (A), and healthy uredospores (B); mycoparasite hyphae penetrated uredospores through SCS (s) and WCS (w); du = damaged uredospore; iu = immature uredospores; mu = mature uredospores

Jeffries and Young (1994) observed that the hyphae of *V. psalliotae* that penetrate the uredospores destroyed the cytoplasm and rendered the uredospores non-viable. Lim and Nik (1983) reported that the hyphae of *V. psalliotae* grew closely oppressed to the uredospore surface between the warts. The formation of conidiophores and conidia by *V. psalliotae* occurred frequently on both the strong warted convex surface and the smooth concave surface of the rust uredospores. The base of the conidiophore appeared to be firmly anchored to the uredospore surface by means of a radiate clasping structure. The mycoparasite was also observed to penetrate living uredospores. However, there was no evidence of any toxin secretion or break down of host tissue in advance of infection. The hyphae of the mycoparasite grew inside the uredospore, eventually filling the spores and obliterating host cytoplasm, leaving the host oil bodies intact. The uredospores infected internally by the mycoparasite were rendered non-viable and did not germinate. Sporulation of *V. psalliotae* also occurred inside the uredospores. However, penetration of the germ-tubes and mycelia of *Hemileia* by *V. psalliotae* was not observed. Even after 48 hours incubation, lysis of rust germ-tubes was not observed, but the complete obliteration of the uredospores by the vigorous growth of *V. psalliotae* was observed.

The specialized hyperparasites affect plant pathogens in two main ways: (a) the penetration of fungal tissues and production of metabolic substances which result in destruction, by lysis, of spores, sori, or hyphae and (b) the displacement of tissues of the pathogen by the hyperparasite either within pustules or by the formation of crusts of mycelium which overlay fruiting structures (Blakeman and Fokkema, 1982). Barnett and Binder (1973) separated the mycoparasites into a necrotrophic and a biotrophic group based on their mode of parasitism. Neotrophic parasites destroy their host, while biotrophic parasites act by means of haustoria or through indirect reaction, by which the host is less obviously harmed (Spencer, 1980). The infection of living uredospores of *Hemileia* by *V. psalliotae* does not show signs of damage, such as lysis of germinating uredospores (Leal and Villanueva, 1967) or bursting of uredospores (Eskes, 1989). A similar finding was obtained by Allen (1982) while studying the parasitism of *V. lecanii* on bean rust (*U. appendiculatus*), and it was reported that the hyphae of *V. leccan*i penetrated and invaded the uredospores of bean rust, but did not lyse them. The presence of mycoparasite hyphae penetrating *H. vastatrix* uredospores found in this investigation concured with the observations by Lim and Nik (1983) suggesting that *V. psalliotae* behaved more as a biotrophic than a neotrophic parasite of *H. vastatrix.*

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