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ATTACK ON TEA BY *XYLEBORUS FORNICATUS*: INHIBITION OF THE SYMBIOTE, *MONACROSPORIUM AMBROSIUM*, BY CAFFEINE

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Key Word Index—*Camellia sinensis*; Theaceae; tea; *Xyleborus fornicatus*; shot-hole borer beetle; *Monacrosporium ambrosium*; fungus; caffeine; antifungal effects.

Abstract—Methylene chloride extracts of tea (*Camellia sinensis*), stems from two clones, TRI 2023 and TRI 2025, inhibited growth of the fungus, *Monacrosporium ambrosium*, the symbiote of the shot-hole borer beetle, *Xyleborus fornicatus*. Activity-guided fractionation of the extracts yielded caffeine as the major antifungal compound. The caffeine content of healthy pencil-thick stems of the two clones was estimated and compared with the caffeine content of pencil-thick stems infected by the beetle. Healthy stems of clone TRI 2023 had a very low caffeine content, but accumulation of caffeine was found to have occurred to a greater extent in infected samples of stems from this clone. Infected stems from both clones had a higher caffeine content than samples of healthy stems. The effect of caffeine on mycelial growth, sporulation and spore germination of *M. ambrosium* in liquid and agar media was also studied. Inhibition of mycelial growth was observed with 5000 ppm of caffeine, while 500 ppm resulted in 21% inhibition in the liquid medium. In the agar medium, colony-size was reduced by 500 ppm of caffeine, while no growth took place with 5000 ppm of caffeine. Inhibition of sporulation was observed with 2000 ppm of caffeine. Germination of conidia was inhibited completely with 3750 ppm of caffeine; inhibition was also observed with 500–2500 ppm of caffeine.

INTRODUCTION

Camellia sinensis var. *assamica* (tea) is attacked by adult female shot-hole borer beetles, *Xyleborus fornicatus*, which bore galleries into tea stems. Attack of the tea plant by this beetle results in damage to the frame of the tea bush, a loss in yield of the valuable leaf and also makes the bushes vulnerable to attack by other pests, such as termites [1]. Studies carried out at the Tea Research Institute (Talawakelle, Sri Lanka), have shown that of 77 clones, clone 2025 is the most susceptible, while clone TRI is least susceptible to attack by the shot-hole borer beetle [2]. The beetle also prefers to attack stems of pencil thickness (10–12 mm) rather than thicker older stems. Most shot-hole borer galleries spread longitudinally along the pith tissue in both directions to varying distances. Sometimes, these galleries traverse around the xylem cylinder forming circular rings.

Spores of *Monacrosporium ambrosium* (ambrosia fungus) are introduced into the gallery by the adult female beetle. The beetle lays eggs inside the gallery, the beetle larvae feed on fungal conidia and metamorphose into adult beetles [3]. It has been suggested that the beetle obtains its requirements of steroidal moulting hormone from the fungus which is therefore essential for the completion of the life-cycle of the beetle [2]. Hence, factors which affect the growth of the fungus may have a bearing on the tolerance/susceptibility of tea clones.

Secondary metabolites, such as the alkaloids caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine), may be involved in chemical defence strategies of plants [4]. Caffeine has been demonstrated to be a naturally occurring insecticide and is also larvicidal and antifungal at concentrations found in plants [5]. A fungitoxic effect was also demonstrated on species of *Aspergillus* and *Penicillium* [6]. At higher concentrations (1.5%), caffeine caused 100% sterility in *Callosobruchus chinensis* [7].

The present paper reports on the effects of caffeine on mycelial growth, sporulation and spore germination of the ambrosia fungus.

RESULTS AND DISCUSSION

The shot-hole borer beetle attacks cause most damage in tea plantations at elevations of 150–1300 m in Sri Lanka [1], but variations have not been observed in the chemical composition of tea bushes growing at different elevations [2]. However, shot-hole borer attack was found to be most frequent at altitudes which provide the optimal temperature for the growth of its fungal symbiote. The same observation has been made in the case of the related scolytid beetles, *X. germanus* and *X. compactus* [9]. Clearly, the development of the shot-hole borer

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beetle is governed by the growth of the associated fungus. Interaction between the tea plant and the fungus may eventually determine the degree of infestation by the insect. Factors which either inhibit or curtail the growth of the fungus may therefore provide a method to control moderate attack by the shot-hole borer beetle.

An investigation of CH_2Cl_2 extracts of the two clones was carried out in order to detect the possible presence of chemical defence compounds, such as antifungal compounds, in tea stems. The CH_2Cl_2 extracts of pencil-thick pieces of stem from both tea clones 2023 and 2025 were found to be active against *M. ambrosium* in a TLC bioassay. Extracts were fractionated by chromatographic methods and the antifungal activity of each fraction was tested by the TLC bioassay method. Activity-guided fractionation led to the isolation of the major antifungal compound as a crystalline solid which was identified as the alkaloid, caffeine. Bioassay also showed the presence of very small amounts of other less polar antifungal compounds in the CH_2Cl_2 extracts of tea stems, but these were not isolated because their activity was lost during the process of purification.

The caffeine content of healthy and infected pencil-thick tea stems was determined using HPLC. The caffeine concentration was highest (1710 ppm, dry wt) in infected stems of clone 2023 and lowest (40 ppm, dry wt) in healthy stems of the same clone. The difference in caffeine contents from extracts obtained from healthy and infected stems of clone 2025 was less pronounced (631 and 1315 ppm, respectively). It is possible that caffeine accumulates at sites of infection and is being used as a plant defence strategy to prevent further colonization of beetle galleries by the fungus. This may account for the observation that there is a greater difference in caffeine contents in extracts from the more tolerant clone, 2023.

It has been suggested that conditions of stress could affect the secondary metabolism of qualitative defence substances, such as alkaloids, cyanogenic glucosides and other compounds [10]. Hence, tea clones which are able to accumulate caffeine by some mechanism may show a greater degree of resistance to shot-hole borer disease. There is a report that caffeine production in cell cultures of *Coffea arabica* was stimulated by the application of salt and light stress [11].

The effect of caffeine on mycelial growth, sporulation and germination of *M. ambrosium* was then studied. Growth of the fungus was completely inhibited in the presence of 5000 ppm of caffeine, while the diameter of colonies was reduced by 19, 36 and 70% with 500, 1000 and 2000 ppm, respectively, of caffeine (Fig. 1).

Mycelial growth in a liquid medium was reduced in the presence of caffeine; the fungicide, Benlate, was more effective in inhibiting mycelial growth. When 500 ppm of Benlate was used, a 65% reduction in weight was observed, whereas 500 ppm of caffeine resulted in only a 22% reduction of weight. Also, 2000 ppm of Benlate caused inhibition of mycelial growth, while 2000 ppm of caffeine resulted in a 72% reduction of mycelial growth (Fig. 2). In our experiments, mycelial mass was determined three days after introducing a spore suspension

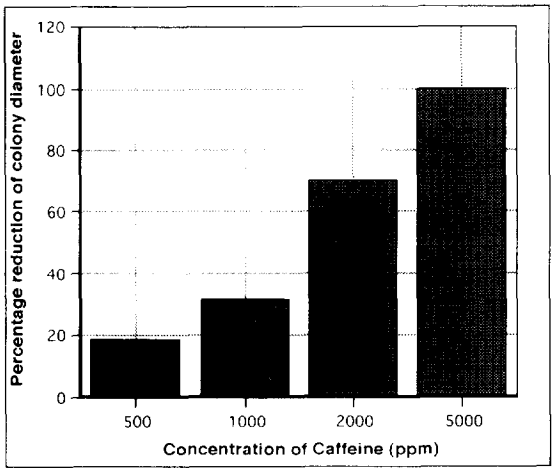

Fig. 1. Effect of caffeine on growth of colonies of *Monacrosporium ambrosium*.

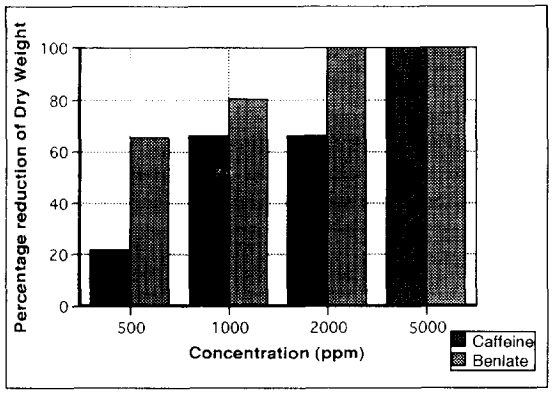

Fig. 2. Effect of caffeine on mycelial growth of *Monacrosporium ambrosium*.

into the liquid medium. Buchanan *et al.* [6] have reported that caffeine had a only small effect on the initial growth of six mycotoxigenic species of *Aspergillus* and *Penicillium*, but there was a dose-related decrease in the mycelial mass in older cultures. From the growth data for *A. ochraceus* it was suggested that inhibition of mycelial growth by caffeine is a result of the inhibition of lipid accumulation. Caffeine was also found to have a pronounced inhibitory effect on mycotoxin synthesis [6].

The average number of conidia in colonies grown on agar in the absence of caffeine was found to be 825 cm^{-2}. This number was reduced to 168 cm^{-2} in the presence of 500 ppm of caffeine and to 51 cm^{-2} with 1000 ppm of caffeine. Sporulation did not occur in the presence of 2000 ppm of caffeine.

Spore germination was also inhibited in the presence of 3750 ppm of caffeine. In the presence of 500 ppm of caffeine, 60% of spores germinated, whereas 50% germination was observed with 500 ppm of Benlate. Spore germination did not take place with 2500 ppm of Benlate

and 3750 ppm of caffeine. These results indicate that 500–1000 ppm of caffeine has a pronounced inhibitory effect on the growth sporulation and spore germination of *M. ambrosium*. It is significant that after attack by the beetle the infected clone 2023 has an increased caffeine concentration of 1710 ppm, which is *ca* 800–900 ppm of the fresh weight. *In vitro* results suggest that this amount of caffeine should be sufficient to inhibit growth of the fungus and to prevent the colonization of beetle galleries by the fungus. The caffeine concentration of the infected more susceptible clone 2025 is less (600–650 ppm, fresh wt) and is probably not sufficient to inhibit growth of the fungus.

The greater difference in caffeine concentration observed between the healthy and infected stems of the less susceptible clone 2023 suggests that the ability to accumulate caffeine after attack by the beetle, may be one of the factors which determines the resistance of tea clones to the shot-hole borer beetle. Caffeine may well be involved in a defence strategy which controls the growth of the fungus inside beetle galleries and, thereby, reduces attacks by the shot-hole borer beetle. Synergistic effects of two or more substances may also be responsible for variations in susceptibility of different tea clones and make certain tea clones more/less favourable for the germination and development of fungal spores.

EXPERIMENTAL

Isolation of ambrosia fungus. Adult female shot-hole borer beetles were removed from a gallery in a tea stem, and surface-sterilized by immersing in 0.1% $HgCl_2$ for 1 min, followed by three serial washings with sterilized dist. H_2O. After removing H_2O using a sterilized filter paper, beetles were placed on quarter-strength Cooks No. 2 agar (4) medium and plates incubated at 26 ± 2° until the growth of mycelia was observed. Mycelia from growing colonies were transferred into fr. medium to obtain a pure culture.

Extraction of tea stems. Pencil-thick pieces (6–10 mm diameter and 15–20 mm length) from bushes belonging to the same age group of healthy and infected stems of the tea clones, TRI 2025 and TRI 2023, were collected separately from the TRI substation at Hantana, Kandy. Air-dried and powdered stems were sequentially extracted with CH_2Cl_2 and MeOH.

Isolation and identification of caffeine. A portion (2 g) of the CH_2Cl_2 extract was fractionated using flash CC (3% MeOH in CH_2Cl_2). Antifungal activity of frs was tested by the TLC method described below and the highly active frs (4, 5, 6 and 7, 500 mg) were fractionated further by HPLC (45% CH_2Cl_2 in hexane to 4% MeOH in CH_2Cl_2). The antifungal activity of each fr. was tested and the most active crystalline fr. (54 mg, mp 221°) was identified as caffeine, lit. mp 235° (anhydrous) [5]. δ_H ($CDCl_3$), 7.5 (1H, *s*, CH), 4 (3H, *s*, NCH_3), 3.6 (3H, *s*, NCH_3), 3.4 (3H, *s*, NCH_3); δ_C ($CDCl_3$) 140 (CH), 24 (NCH_3), 25 (NCH_3), 27 (NCH_3). MS, *m/z* 194 $[M]^+$ (78%).

Caffeine content of extracts. Quantification of caffeine present in samples of tea stems (50 g, dry wt) was made using HPLC. A Bondapak C_{18} column (3.9 mm × 30 cm, Waters) was used to separate the components in the CH_2Cl_2 extracts of healthy and infected samples of tea stems in the same age group. MeOH–HOAc–H_2O (20:1:79) at a flow rate of 1.5 ml min^{-1} at 2000 psi was used as the mobile phase.

Testing for antifungal activity. Each CH_2Cl_2 extract (2 mg) obtained from healthy and infected stems of the two clones, 2023 and 2025, was spotted onto a TLC plate prepd using silica gel (60pf 254 + 366) and the plates developed with 50% CH_2Cl_2 in hexane. After air-drying overnight, plates were sprayed with a suspension of conidia scraped from a 7-day-old culture of *Cladosporium cladosporioides* and *M. ambrosium* in Czapek Dox nutrient soln. Plates were incubated in a moisture chamber at 26 ± 2° for 48 hr and areas of inhibition were observed as a white circular patches against a dark green background. To visualize inhibition areas, *M. ambrosium* sprayed plates were exposed to I_2 vapour. Areas of inhibition were seen as white circular patches against a brown background. Activity of extracts was compared by measuring the diameter of the white zones.

Effect of caffeine on mycelial growth. Effects of caffeine on mycelial growth were determined in Czapek Dox agar and liquid media into which different concentrations of caffeine were incorporated. Preparation of media with 500, 1000, 2000 and 5000 ppm of caffeine was done by dissolving different amounts of caffeine in sterilized dist. H_2O (10 ml). Caffeine was incorporated into agar and liquid media after autoclaving and cooled to 45°. To observe mycelial growth in a solid medium, mycelial discs cut from the edge of a well grown colony of *M. ambrosium*, using an 8 mm diameter cork borer, were placed on agar plates containing 500, 1000, 2000 and 5000 ppm of caffeine. Growth of colonies was observed for 7 days.

To determine mycelial growth in a liquid medium, 100 ml portions of Czapek Dox medium containing 500, 1000, 2000 and 5000 ppm of caffeine were placed in 250 ml conical flasks. A spore suspension (1 ml) of conidia of *M. ambrosium* was added to each flask under sterile conditions and mixed well. Flasks were then incubated on an orbital shaker (100 rpm) for 3 days. Mycelia were separated by filtering (Whatman No. 1) and dry wts obtained by freeze-drying (−60° under 0.2 mbar). Experiments were repeated using the fungicide, Benlate, for comparison.

Effect of caffeine on sporulation. The agar medium was prepared in four separate flasks, autoclaved and cooled to 45°. Caffeine (500, 1000, 2000 and 5000 ppm) was dissolved separately in sterilized dist. H_2O (10 ml) in bottles, added into the flasks containing agar medium at 45° and agar plates prepared. Mycelial discs cut out from the edges of a well grown culture of *M. ambrosium* were placed in the centre of the caffeine-containing agar plates and the plates incubated for 10 days. Two lines were drawn across the diameter of the colony on the lower surface of each petri dish and 15 mycelial discs separated

from each plate using a 6 mm cork borer. Mycelial discs were placed in dist. H_2O (5 ml) and crushed with the blunt end of a glass rod, shaken well and filtered through glass wool. The number of conidia (cm^{-1}) in each sample was counted using a haemocytometer.

Effect of caffeine on germination of conidia. A series of solns containing 1000, 2500, 5000, 7500 and 10000 ppm of caffeine were prepared. One drop of spore suspension was placed on a clean slide and one drop of caffeine soln was mixed with it to make the final concn. Slides were then incubated in a moisture chamber for 12 hr and germination stopped by adding a drop of lactophenol. The number of germinated conidia in 15 microscopic fields was counted. This procedure was repeated for all concns of caffeine. Percentage germination in each sample was calculated. The expts were repeated using the fungicide, Benlate.

All liquid media used were sterilized by autoclaving for 15 min at 120° under 120 bars.

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