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Effect of caffeine on shot-hole borer beetle (*Xyleborus fornicatus*) of tea (*Camellia sinensis*)

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Abstract

Caffeine was found to inhibit oviposition in shot-hole borer beetle (*Xyleborus fornicatus*) of tea (*Camellia sinensis*) in laboratory culture media. Caffeine also delayed the appearance of the different developmental stages in the life cycle, but apparently did not have a lethal effect on the beetle. The inhibitory effect of caffeine on the beetle was partially reduced in the presence of the polyphenol tannic acid.

Keywords: *Xyleborus fornicatus*; Shot-hole borer beetle; *Camellia sinensis*; *Monacrosporium ambrosium*; Fungus; Caffeine; Oviposition; Polyphenols; Tannic acid

1. Introduction

The purine alkaloid caffeine, the major alkaloid found in tea (*Camellia sinensis*), was found to inhibit the growth of the ambrosia fungus (*Monacrosporium ambrosium*), the fungal symbiote of the shot-hole borer beetle (*Xyleborus fornicatus*) (Kumar, Hewavitharanage, & Adikaram, 1995). We suggested that the accumulation of caffeine in tea stems after beetle attack, could be a plant defence strategy. This paper is a report of in vitro experiments carried out using laboratory culture media containing caffeine, to determine the effect, if any, of caffeine on the shot-hole borer (SHB).

Phenols and polyphenols are important constituents of tea plants. Phenolic compounds play a central role in plant–herbivore interactions and also influence the interaction of herbivores with microbial symbiotes. Caffeine has been reported to form stable complexes with polyphenols such as potassium chlorogenate (Gorter, 1907; Gorter, 1908) and complex formation was attributed to dipole-induced dipole interactions which may be of physiological significance (Booth, Boyland, & Orr, 1954). Complex formation between chlorogenic acid and caffeine reduced the toxicity of caffeine in rabbits (Gerhadt, 1939) and was also found to inhibit the action of caffeine on frog muscle (Straub & Domejoz, 1941). Thus interactions between caffeine and other types of naturally occurring molecules may well determine the relative susceptibility of cultivars to attack by insect pests and microbial pathogens. A preliminary study was carried out to determine whether the effect, if any, of caffeine on SHB could be altered in the presence of a polyphenolic compound such as tannic acid.

2. Results and discussion

The shot-hole borer beetle is found in symbiotic association with the ambrosia fungus (*M. ambrosium*) within galleries made by the beetle in tea stems (Gadd & Loos, 1947). The fungus is probably the sterol source necessary for the development of the larval stages (Wickremasinghe, Perera, & Perera, 1976). We have reported (Kumar et al., 1995) that caffeine inhibited the growth of the ambrosia fungus and that there was accumulation of caffeine after beetle attack. Previous studies have shown that caffeine has a toxic effect on insects at concentrations found in plants (Frischknecht, Ulmer-Dufek, & Baumann, 1986) and it has been suggested that caffeine may be an endogenous substance produced by plants to discourage insect feeding (Nathanson, 1984). Hence the effect of caffeine on SHB could be of interest.

Two separate experiments involving caffeine were carried out. In the first experiment, the beetle was cultured in the laboratory with varying amounts of caffeine in the

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culture medium. These experiments were carried out to obtain information regarding the concentrations of caffeine at which normal growth of the fungus was evident and the effect of caffeine on the development of the SHB. In the second study the number of days required to observe each stage of the life cycle was determined. During this study the effect of tannic acid in the culture medium, on appearance of different developmental stages in the life cycle of SHB, was also determined.

2.1. Effect of different concentrations of caffeine on SHB

Twenty-four diet tubes were prepared for each concentration of caffeine (50, 100, 200, 500, 1000, 2000 and 5000 ppm) and these consisted of six replicates of four types of diet media (Sivapalan, 1976). Thereafter, one female beetle per tube was added and the development of the fungus and larvae were monitored.

It was observed that galleries were made on all four types of diet tubes. The growth of *M. ambrosium* started from the second day of inoculation in the control tubes with diet types **1** and **2**. The presence of caffeine in the diet medium delayed the appearance of the fungus which was observed after 6, 10 and 19 days in media containing 50, 100 and 200 ppm respectively of caffeine. The fungus appeared only after 20–24 days in the tubes with 500 ppm of caffeine (Table 1). However the growth pattern of the fungus was normal in the presence of 50–500 ppm of caffeine and occurred to a similar extent in all these tubes. The inhibitory effect of caffeine was somewhat mitigated in the presence of the tea bark extract and the fungus was observed after 8 and 15 days when the medium contained tea bark extract together with 100 and 200 ppm of caffeine respectively. A similar effect was seen when tea bark extract was added to the medium containing 500, 1000 and 2000 ppm of caffeine respectively (Table 2). Only slight growth of the fungus was observed after 26 and 30 days, respectively, in the tubes with 1000 and 2000 ppm of caffeine. The fungus did not grow at all in tubes with 5000 ppm of caffeine (diet types **3** and **4**) confirming our earlier findings that caffeine has an inhibitory effect on the fungus at this concentration (Kumar et al., 1995).

The larval stages appeared after 18 days and the first female from the second generation emerged after 30 days from the two sets of control tubes **1** and **2**. Emerging females were not observed in any of the diet media **3** and **4**. The total number of emerging females was 77 in the control tubes **2** (with tea bark extract) while it was only 30 in control tubes **1** (without tea bark extract). Therefore tea bark extract apparently contains additional substances which are favorable to the development of the beetles. It is likely that secondary metabolites present in the tea bark either act as oviposition stimulants or signal the presence of a suitable host medium.

Although the fungus was absent in tubes containing 5000 ppm of caffeine, some of the introduced beetles were alive up to about 30 days. Hence caffeine does not have a lethal effect on SHB. However these beetles did not deposit eggs. In tubes with 1000 and 2000 ppm of caffeine, fungal growth was slight and some beetles were alive for

Table 1. Effect of caffeine on development of *Ambrosia fungus* and shot-hole borer

Composition of the medium	Diet type	Caffeine (ppm)	**A** galleries	**B** fungus	**C** larval stages	**D** adult females	**E** total females
Control medium (CM)	1	–	1	2	18	30	30
CM + extract[a]	2	–	1	2	18	30	77
CM + extract + caffeine	3	50	1	6	–	–	0
CM + caffeine	4	50	1	6	–	–	0
CM + extract + caffeine	3	100	1	8	–	–	0
CM + caffeine	4	100	1	10	–	–	0
CM + extract + caffeine	3	200	2	15	–	–	0
CM + caffeine	4	200	2	19	–	–	0
CM + extract + caffeine	3	500	2	20	–	–	0
CM + caffeine	4	500	2	24	–	–	0
CM + extract + caffeine	3	1000	3	26	–	–	0
CM + caffeine	4	1000	3	29	–	–	0
CM + extract + caffeine	3	2000	3	30	–	–	0
CM + caffeine	4	2000	3	32	–	–	0
CM + extract + caffeine	3	5000	3	none	–	–	0
CM + caffeine	4	5000	3	none	–	–	0

[a]Extract refers to tea bark extract.
A is the number of clays for appearance of galleries.
B is the number of days for appearance of fungus.
C is the number of days before observing larval stages.
D is the number of days before emergence of females.
E is the total number of emerging females.

about 30 days, but eggs were not deposited. Eggs were also not observed in tubes containing 500, 200, 100 and 50 ppm of caffeine, though fungal growth appeared normal. In the tubes with 50 ppm of caffeine some of the beetles remained alive up to 60 days. Eggs, larval stages and emerging females were not observed in all the tubes containing caffeine (diet media **3** and **4**). This experiment confirmed the inhibitory effect of caffeine on the fungus and also indicated that caffeine has an inhibitory effect on oviposition of the beetle.

According to our results, oviposition is inhibited even when the concentration of caffeine is 50–100 ppm. Nathanson (1984) reported that larvae of the tobacco hornworm *Manduca sexta* were killed in a nutrient medium containing 0.3% (3,000 ppm) caffeine and in *Callosobruchus chinensis* caffeine caused nearly 100% sterility at a caffeine concentration of 1.5% (Rizvi, Pandey, Mukerji, & Mathur, 1980). SHB infests and thrives in tea stems which contain 350–750 ppm of caffeine (dry weight) (Karunaratne & Kumar). Our experiments also showed that the highest number of beetles emerged from the control tubes **1** with tea bark extract in which the caffeine concentration is 1150–1320 ppm (Karunaratne & Kumar). The in vivo tolerance of SHB beetle to such high concentrations of caffeine may be due to the presence of other phytochemicals which are able to overcome the inhibitory effect of caffeine. It is also possible that caffeine may be in a biologically inactive or bound form in healthy tea stems and is released after attack by the beetle. For example, association between caffeine and naturally occurring molecules such as polyphenols in aqueous media could remove caffeine from the medium. Gorter (1907, 1908) reported that only a small fraction of caffeine can be extracted with chloroform from the crystalline complex formed between caffeine and potassium chlorogenate. It is also known that such complex formation results in aggregates which are precipitated from solution (McManus, Davis, Haslam, & Lilley, 1981). Therefore polyphenols, which are important constituents of tea could be of significance in reducing the inhibitory effect of caffeine on SHB in tea stems.

2.2. Effect of caffeine and tannic acid on shot-hole borer beetles

In these experiments the control medium **A** contained sucrose, yeast, casein, Wesson's salt, agar, cellulose and distilled water. The diet medium **B** contained the control medium with caffeine (100 ppm), while medium **C** was composed of control medium **A** + tannic acid + caffeine (100 ppm each). Medium **D** contained control medium **A** + tannic acid (100 ppm).

Observations were made on the time (number of days) taken before (i) formation of the first gallery, (ii) observation of the fungus, (iii) egg formation, (iv) appearance of larvae, (v) appearance of immature beetles (light brown in color) (vi) appearance of mature beetles (dark brown in color) and (vii) emergence of female beetles. The total number of females which emerged from each medium in each generation was also counted. Observations were made on six replicates of each of the four different culture media.

2.3. Observations made

1. Galleries were observed in 2–3 days in **A** (Fig. 1a) whereas they were observed after one day in both **C** and **D**. Therefore gallery formation takes place at a significantly faster rate in the media containing tannic acid. Galleries were observed after 3–4 days in the tube containing the medium **B** (only caffeine).
2. The fungus was observed in **A** as well as in **C** and **D** after 2 days, but in **B** this observation was made after 5 days (Fig. 1b). Therefore caffeine significantly slows down the development of the fungus while tannic acid did not affect fungal growth (**D**), and reduced the caffeine effect (**C**).
3. Eggs were observed first in **A** and **C** (four days) and only after 12 days in **B** (Fig. 1c). Observation of eggs was difficult because the eggs are laid at the bottom of the gallery and are sometimes covered by the beetle. In **D** (tannic acid) eggs were seen after 7 days, i.e. later than in **C** (4–5 days) containing both caffeine and tannic acid. Therefore the combination of tannic acid and caffeine was more favorable for the development of the beetle than the medium containing only tannic acid (**D**) or only caffeine (**B**).
4. Larvae were visible in **A** after about 10–11 days, whereas they were observed only after 16–18 days in **B**, **C** and **D** (Fig 1d). The larvae tended to move out of the galleries and into the space between the medium and the walls of the tube in search of food because the fungal mycelia were observed to grow well on the outer surface of the medium. Therefore the presence of either caffeine or tannic acid, as well as a combination of the two delayed the appearance of larvae.
5. The immature beetles are light brown in color and their movements are fast. The immature beetles were slowest to appear in **B** (28 days), while in **C** and **D** this observation was made after about 22 days (Fig. 1e). Immature beetles in **A** were observed after about 23–24 days.
6. The mature beetles were first observed in **A**, **C**, then in **D** (about 30 days) and later (about 35 days) in **B** (Fig. 1f). These beetles were dark brown in color and were very mobile. The mature beetles took a few days to move towards the cotton plugs and to emerge from the galleries. Both immature and mature beetles were slowest to appear in **B**, the medium containing only caffeine.
7. The total number of beetles which emerged from **B** was very small (4) while 50–55 beetles emerged from

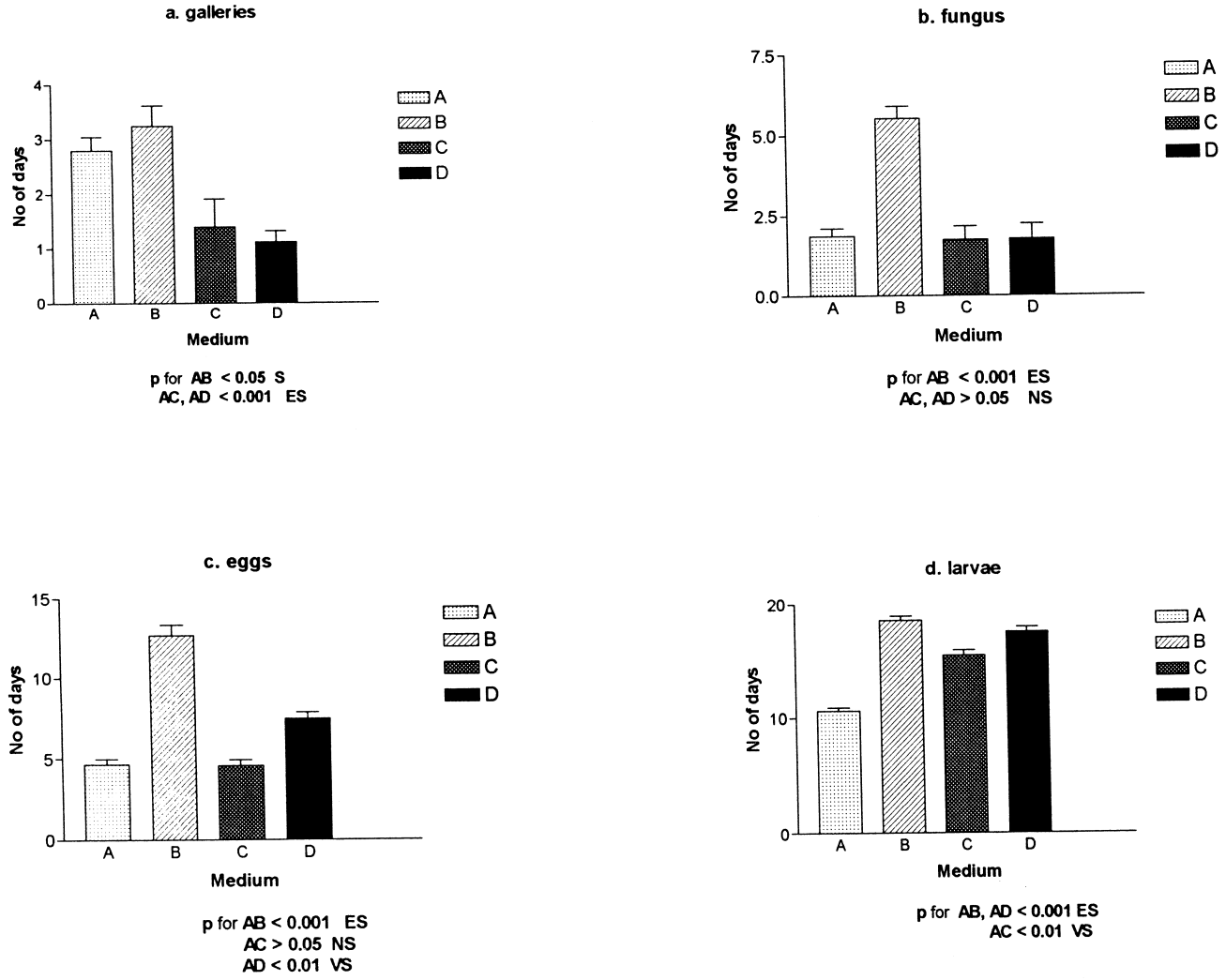

Fig. 1. Number of days for appearance of different developmental stages in the life cycle of Shot-hole borer (SHB).

A (Fig. 1h). The number which emerged from **C** (i.e. medium containing both caffeine and tannic acid) was larger (18) than from **D** (11), but much less than from **A**.

Some differences were noted in the results obtained from the two experiments. In the first experiment larval stages were observed only after 18 days in the control medium whereas in the second this observation was made in 10–11 days. The difference may be attributed to the fact that the diet medium was placed in glass tubes in the first experiment, whereas in the second the diet medium was placed as a thin layer between the inner wall of a boiling tube and the outer wall of a test tube placed in the center of the boiling tube. The latter set-up made observation of the developmental stages easier and more accurate.

The difference in the set-up of the medium may also account for the observation of larval stages in the medium **B** (containing caffeine) and the emergence of a larger number of beetles from the control medium during the second experiment. The thin layer of medium may well be more favorable for the construction of galleries and, the survival and development of the larval stages. This may also account for the emergence of a very small number of beetles from the caffeine containing medium **B** in the second experiment while none emerged from this medium during the first experiment.

Our results suggest that caffeine in laboratory culture media has an inhibitory effect on oviposition. Oviposition is the first stage in the insect–plant relationship at which the plant may show resistance (Stanley, 1965). Thus plant resistance to the onion maggot *Hylemya antiqua*, was found to be entirely due to resistance to oviposition (Perron & Jasmin, 1963). Plant secondary metabolites play an important role in ovipositional behavior. The tobacco hornworm *Protoparce sexta*, oviposits on the Solanaceae and eggs are deposited only in response to an alcohol soluble substance (Yamamoto & Fraenkel, 1960).

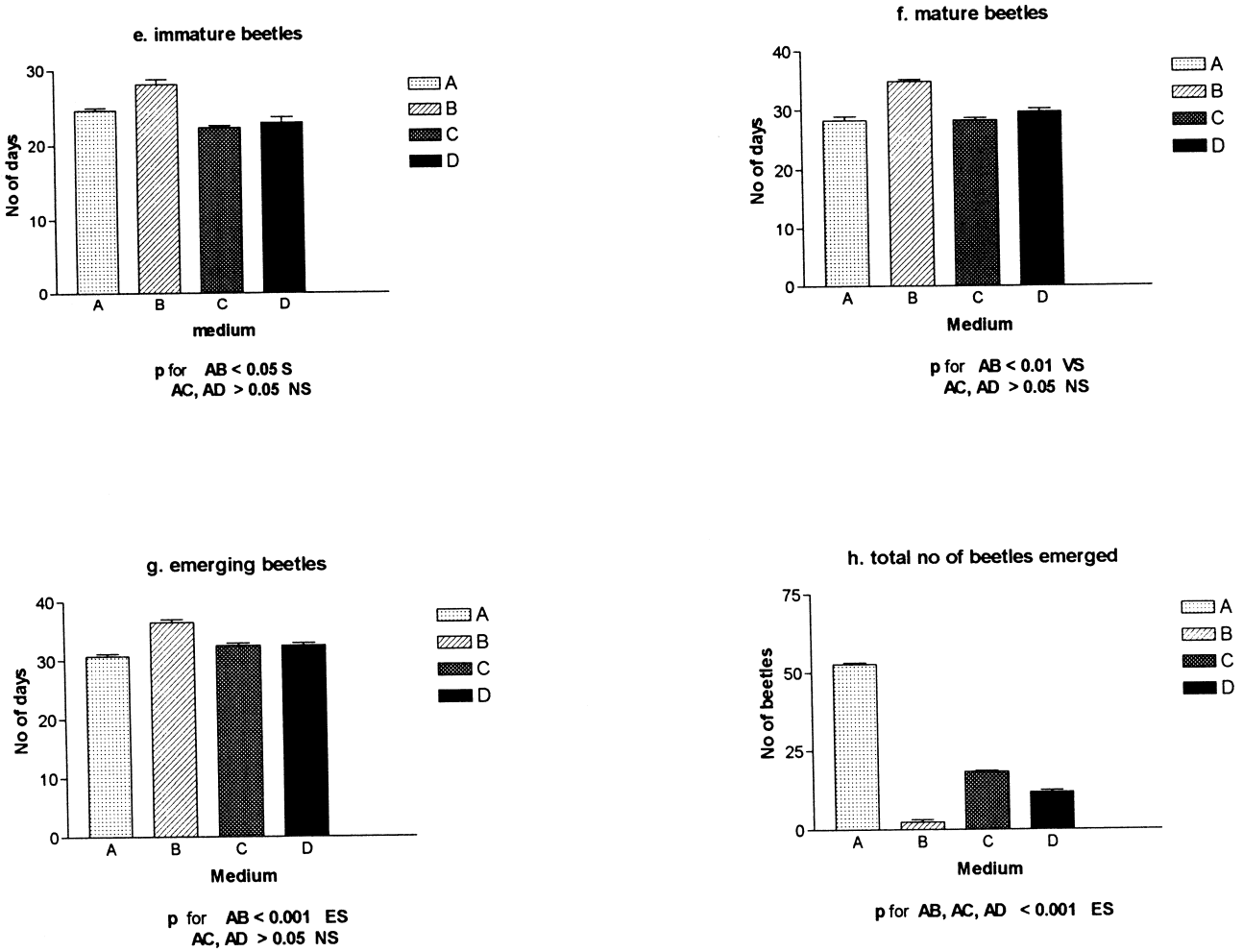

Fig. 1—*continued.*

Therefore the inhibition of oviposition in laboratory media containing caffeine may well be due to the absence of another host plant chemical which acts as an oviposition stimulant in tea stems.

According to our results caffeine significantly delayed the appearance of each developmental stage in the life cycle of the beetle. Thus eggs were observed after 12 days in medium **B**, whereas eggs were seen in 3–4 days in both the control medium **A** and the medium **C** containing both caffeine and tannic acid. The first instar larvae were observed after 10 days in **A**, whereas larvae were observed only after 18 days in **B**, the medium containing caffeine. The appearance of mature beetles and the number of days taken for the beetles to emerge were also delayed in medium **B**.

Observations on the media containing tannic acid suggest that an interaction, possibly complexation, between tannic acid and caffeine reduces the bioavailability of caffeine. We have observed that the phenolic content of the susceptible clone TRI 2025 is greater than the phenolic content of the less susceptible clone TRI 2023 (Abeysinghe, Kumar, & Ratnayake). It is likely that the content and the composition of phenolics in tea stems may have a bearing on differences in susceptibility of certain tea clones to infestation by SHB.

3. Experimental

3.1. Collection of beetles

The shot-hole borer beetle of tea is sensitive to both heat and light, and the activity of this beetle varies depending on the daily temperature. The beetle is most active from noon until about 3.30 p.m. at Peradeniya where the temperature is around $25 \pm 27^{\circ}C$ throughout the year. The beetles are active between 2.30 and 3.00 p.m at Talawakelle, where the temperature is lower and fluctuates between 16–20°C throughout the year. Therefore all observations were made at 2.30–3.00 p.m. each day when the beetles were presumed to be most active.

3.2. Laboratory culture of beetles

'Mother beetles' used to start the first generation of the laboratory culture were collected from beetle infested bushes of the tea clone TRI 2025 (tea clone reported to be the most susceptible to attack by SHB) at the TRI-substation in Hantana. Only healthy adult female beetles (*X. fornicatus*) which were about to emerge were collected. The beetles were surface sterilized with 2% NaOCl (2–3 min), washed with distilled water and introduced into tubes containing the culture medium prepared under sterile conditions. The composition of the culture media used are given below. Each medium was prepared by mixing all the constituents thoroughly in a mortar.

3.3. Effect of different caffeine concentration on shot-hole borer beetles

3.3.1. Preparation of culture media

Beetles were reared in the laboratory in a diet medium (250 ml) (Sivapalan, 1976) composed of sucrose (4.7 g), casein (3.1 g), yeast extract (3.1 g), Wesson's salt mixture (0.4 g), cellulose powder (50.0 g), fine agar (12.5 g) and H_2O (125 ml). Penicillin was added to the culture media to prevent bacterial contamination. Bacterial growth results in a medium which is sticky and the beetle is unable to move about freely under these conditions.

Dietary ingredients other than agar, were made into a paste with H_2O (25 ml). Agar was soaked in H_2O (100 ml), added to the paste and mixed well. Tea bark extract was made from freshly peeled bark (25 g) of healthy pencil thick tea stems, the preferential site of attack (Danthanarayana, 1973), from the clone TRI 2025, cut in to small pieces, blended with water (125 ml) and filtered. The tea bark extract (125 ml) was added to the agar paste and mixed well. Water alone (125 ml) was added to prepare the control diet medium.

Four types of diet media were prepared. (1) Control medium, (2) control medium + tea bark extract, (3) control medium + tea bark extract + caffeine and (4) control medium + caffeine.

Caffeine was dissolved in distilled H_2O (10 ml), mixed well with cellulose powder and incorporated in varying concentrations (5000, 2000, 1000, 500, 200, 100, 50 ppm) into the culture medium and the volume of H_2O used to dissolve caffeine was reduced from the volume of water used to soak the agar. Caffeine was incorporated into the diet medium before autoclaving.

The diet medium (15 ml) was placed in glass/boiling tubes (150×15 mm) which were then plugged with surgical cotton. The diet tubes were autoclaved and allowed to cool to room temperature. Penicillin (500 ml, 50 units/ml) was added and the tubes were kept at room temperature for 24 h. One freshly emerged young female of *X. fornicatus* was added into each tube under sterile conditions, the tubes were plugged immediately, and maintained at room temperature ($26 \pm 20^{\circ}$) in the dark. Observations regarding gallery construction, eggs, number of larvae, pupae and emerging female beetles were made daily for 72 days.

Twenty-four tubes were incubated for each caffeine concentration; six tubes (control medium with tea bark extract), six tubes (control medium), six tubes (control medium tea bark extract + caffeine) and six tubes (control medium with caffeine).

3.4. Effect of caffeine and tannic acid on shot-hole borer beetles

3.4.1. Preparation of culture tubes

An equal quantity of the medium was poured into each of six boiling tubes. A test tube was placed in the center of the boiling tube so that there was a thin layer of medium between the walls of the inner test tube and the outer boiling tube. The boiling tubes were sealed with cotton plugs and aluminium foil, and autoclaved. The culture tubes were kept for 3–4 days before use because excess moisture in the tubes or on the upper surface of the medium was found to restrict the movement of the beetles. The tubes were kept undisturbed in the dark (24 h) at $25 \pm 27^{\circ}$C (Sivapalan, 1976).

3.4.2. Composition of the media

The basal medium (= medium **A**) used to culture the beetles was composed (mg) of sucrose (1,409), yeast (929), casein (929), Wesson's salt (118), agar (3,749), cellulose (17,000) and distilled water (75 ml). Medium **A** served as control, and was supplemented with 100 ppm caffeine (medium **B**), 100 ppm caffeine and 100 ppm tannic acid (**C**), or with 100 ppm tannic acid (**D**).

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