Identification of a coffee berry borer-associated yeast: does it break down caffeine?

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Abstract

Two yeasts isolated from laboratory reared adult coffee berry borers [*Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae)] and from insects collected in the field in Colombia were identified as *Pichia burtonii* Boidin and *Candida fermentati* (Saito) Bai, based on sequencing of the nuclear large subunit 26S rDNA variable D1/D2 domain. Liquid culture experiments using *P. burtonii* in media containing different caffeine levels indicated that caffeine levels in a range found within coffee seeds can retard yeast growth. HPLC analysis shows that *P. burtonii* does not break down caffeine.

Introduction

The coffee berry borer, which is endemic to Central Africa and is now found in most coffee growing regions, is the most devastating pest of coffee throughout the world. Of more than 800 insects reported on coffee, the coffee berry borer is the only one that has developed an ability to exploit the coffee bean as a food source. This is remarkable due to the many reports of caffeine toxicity to insects (see below) and the caffeine content (as a percentage of dry weight) in the coffee seed, which averages 1.1–1.7% in *Coffea arabica* L. and 2–3% in *C. canephora* (robusta) Pierre ex Froehn er (Mösli Waldhauser & Baumann, 1996).

It has been proposed that the role of the alkaloid caffeine in plants might be as a defence against herbivores (Nathanson, 1984). This hypothesis is supported by reports of the negative effects of caffeine in diets fed to insects in various orders: Coleoptera (Rizvi et al., 1980; Nathanson, 1984; Akhtar & Mondal, 1994; Pöschko, 1995; Castellanos & Espinosa-García, 1997 Hewavitharanage et al., 1999); Diptera (Itoyama, 1990, 1993; Srinivasan & Kesavan, 1979; Mohamed & Nair, 1991, 2001; Itoyama & De Campos Bicudo, 1992; Itoyama et al., 1995, 1997, 1998); Lepidoptera (Muthukrishnan et al., 1979; Nathanson, 1984; Mathavan et al., 1985; Slansky & Wheeler, 1992; Mayilvaganan & Mathavan, 1994; Stamp et al., 1994; Laz et al., 1998); and Heteroptera (Nathanson, 1984). In addition, caffeine has been shown to act as a deterrent against several insects (Levinson, 1976; Usher et al., 1988; Glendinning & Slansky, 1994; Chen et al., 1996; Bernays et al., 2000; Liscia & Solari, 2000).

The ability of the coffee berry borer to successfully exploit a food source which is high in alkaloids (caffeine in the coffee seed) is likely due not only to biological traits predominant in members of the Scolytidae (e.g., burrowing into host plant; Wood, 1982) but also to detoxification mechanisms which are absent in species that cannot survive on the coffee seed. During routine examinations of coffee berry borers for microbial pathogens, we detected yeasts within the insect. This was interesting, because yeasts are known to serve as endosymbionts in insects, in some cases detoxifying plant toxins or producing enzymes that help in the digestion of food material (Shen & Dowd, 1991 and references therein). Therefore, we became interested in determining the possible role of yeasts in caffeine detoxification.

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Materials and methods

Insects

Coffee berry borers originating from insects collected in the field in Colombia were reared at the US Department

of Agriculture (USDA) quarantine facility in Stoneville, Mississippi, on an artificial diet containing ground dried green coffee beans, sugar, casein, powdered yeast, and preservatives (Portilla, 1999) and kept at 28 °C with a L12:D12 photoperiod. A separate batch of insects was obtained from a colony maintained at the Insect Biocontrol Laboratory (USDA) in Beltsville, Maryland, using Portilla's diet (1999) but with different suppliers for the ingredients used.

Yeast isolation from the insect

Batches of ten coffee berry borer adults were sterilized in a 70% ethanol solution containing Triton X-100 for 30 s while vortexing, followed by a wash in sterile distilled water while vortexing for another 30 s. Insects were then macerated in 0.5 ml of sterile water using a tissue homogenizer. The homogenate was transferred to a 2-ml Eppendorf tube and centrifuged for 5 min at 725 ***g***. Two µl of the suspension from around the pellet area were inoculated onto yeast malt (YM) extract agar (per 500 ml: 10 g agar, 5 g glucose, 2.5 g peptone, 1.5 g yeast extract, 1.5 g malt extract) with 0.5 ml antibiotics (0.02 g each streptomycin, penicillin, and tetracycline dissolved in 10 ml sterile water and filter sterilized); the same was done with the water used to clean the insects to assess whether there were yeasts on the cuticle of the insect (this was always negative). From the YM agar, the yeast was transferred to Wickerham's YM broth (3 g yeast extract, 3 g malt extract, 5 g peptone, and 10 g l^{-1} distilled water) for 24 h at 25 °C using a rotary shaker at 200 r.p.m. After centrifugation, the yeast was washed and lyophilized for subsequent DNA extraction using the method of Kurtzman & Robnett (1998a). The ca. 600 nucleotide 26S rDNA domain D1/D2 (Kurtzman & Robnett, 1998a) was amplified by polymerase chain reaction (PCR) and sequenced using an ABI TaqDyeDeoxy terminator Cycle sequencing kit and an ABI 377 DNA Sequencer (Applied Biosystems Inc., Foster City, CA).

Yeast growth in liquid culture media containing caffeine

To assess whether caffeine would affect yeast growth, *Pichia burtonii* from the coffee berry borer was selected for liquid culture experiments. This strain was chosen because it was the first to be isolated and identified. YM extract broth was prepared by completely dissolving 10 g glucose (Sigma Chemical Co., St. Louis, MO), 5 g tryptone peptone (Difco Laboratories, Detroit, MI), 3 g yeast extract (BBL Microbiology Systems, Cockeysville, MD), and 3 g malt extract (Sigma Chemical Co., St. Louis, MO) in 1000 ml distilled water, followed by the addition of 1 ml antibiotics (as previously described). The media (200 ml) was then dispensed in five beakers and caffeine (1,3,7-trimethylxanthine, anhydrous, Sigma Chemical Co., St. Louis, MO), was added as follows: 0 g (control), 0.2 g (0.1% caffeine), 1.0 g (0.5%), 2.0 g (1%), and 4.0 g (2%). A magnetic stirrer was used to completely dissolve the caffeine. The media was filter sterilized in a laminar flow hood using a 0.2 µm filter (Nalge Co., Rochester, NY) and 45 ml of each respective treatment was dispensed into each of three 125 ml sterile baffled flasks (three replicates per treatment). The media was inoculated using a 4-day-old coffee berry borer isolated *P. burtonii* culture (NRRL Y-27402) growing on YM broth. A dilution was prepared so as to make inoculations in flasks with 5 ml of 5×10^6 spores ml^{-1}, thus resulting in a final concentration of 5×10^5 spores ml^{-1} and a final caffeine concentration of 0.09, 0.45, 0.9, and 1.8%. Treatments were incubated in an INNOVA 4000 Digital Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, NJ) at 28 °C and 150 r.p.m., and spore yields were determined using a Bright-Line hemacytometer (Sigma Chemical Co., St. Louis, MO), counting at least four fields for each flask and three flasks per treatment, 24, 48, and 72 h post-inoculation. At each sampling point a 1-ml culture sample was placed in a sterile Eppendorf tube and stored at −80 °C until HPLC analysis could be performed. The experiment was repeated three times.

Spore counts were $\log_{10}$ transformed and analyzed as a three-factor general linear models using PROCMIXED (SAS Institute Inc., 1999) with concentration and hour as the fixed factors and block as a random factor. The correlation between cell counts over time was modeled using the compound symmetry heterogeneous covariance structure in PROCMIXED. The assumptions of the general linear model were tested. To correct for variance heterogeneity, the treatments were grouped into similar variance groups for the analysis.

HPLC analysis

Culture supernatants were analyzed for caffeine content by reverse-phase chromatography performed on a Hewlett-Packard 1100 HPLC (Hewlett-Packard GmbH, Waldbronn, Germany) equipped with a diode array detector. Supernatants were clarified by centrifugation at 10 000 ***g*** for 4 min. Samples of the clarified supernatants were then diluted with water (based on the initial caffeine concentration of the culture) such that the final concentration of the caffeine would be 100 p.p.m. Ten µl of each diluted sample was injected onto a Luna 250 × 3 mm C18(2) column (Phenomenex; Torrance, CA) and eluted isocratically with 20 mM sodium acetate buffer (pH 4.75) containing 25% methanol at a flow rate of 400 µl min^{-1}. The UV absorbance was monitored at 275 nm, where the caffeine exhibits an absorbance maximum. Ten µl of a standard containing 100 p.p.m. of anhydrous caffeine

dissolved in water was used to calibrate the system. Integration of the UV absorbance data was performed using HP CHEMSTATION software. Under the chromatographic conditions described, supernatants from cultures without caffeine exhibited no UV absorbing compounds that interfered with the caffeine analysis. Differences in caffeine concentration among hours post-inoculation were tested separately at each caffeine concentration using PROCMIXED (SAS Institute Inc., 1999) with hour specified as a fixed factor.

Results

Yeast identification

The nuclear large subunit 26S rDNA variable D1/D2 domain of four yeasts isolated from the coffee berry borer at different times was sequenced (Kurtzman & Robnett, 1998a). Two strains had a 600 nucleotide sequence which was identical to that of the type strain of *Pichia burtonii* Boidin (NRRL Y-1933, GenBank no. U45712); these have been accessioned in the USDA Agricultural Research Service Culture Collection (NRRL; National Center for Agricultural Utilization Research, Peoria, IL) as NRRL Y-27394 and NRRL Y-27402. The other two isolates have sequences which are identical to *Candida fermentati* (Saito) Bai (C.P. Kurtzman, unpubl.) and have been accessioned as NRRL Y-27401 (GenBank no. AY187283) and NRRL Y-27403. On the basis of phenotype, *C. fermentati* was believed to be a synonym of *Pichia guilliermondii* Wickerham (type strain, NRRL Y-2075, GenBank no. U45709). However, *P. guilliermondii* and *C. fermentati* differ by three nucleotides, and it appears that they may represent two separate but closely related species.

Pichia burtonii growth in caffeine-containing media

Results of *P. burtonii* spore counts in liquid culture revealed a statistically significant concentration by hour interaction (d.f. = 8; F = 103.4; P < 0.0001). Means were compared using Sidak adjusted P-values so that the experiment-wise error was 0.05. There was no significant difference in spore production 24, 48, or 72 h post-inoculation between the control and the lowest caffeine concentration, although at higher caffeine concentrations, spore production was significantly inhibited (Table 1).

Caffeine degradation by *P. burtonii*

Analysis of the HPLC data revealed no significant differences between initial caffeine concentrations in the media and those detected 24, 48, and 72 h later for each of the concentrations tested: 0.09% (P = 0.8369); 0.45% (P = 0.2662); 0.9% (P = 0.5111); and 1.8% (P = 0.9241).

Table 1 *Pichia burtonii* growth in liquid media containing different levels of caffeine after inoculation with 5.5×10^5 spores ml^{-1}. Means are for three experiments run on different dates, each with three replicates per treatment

	Cells $ml^{-1} \times 10^7$ (h post-inoculation)		
% Caffeine	24	48	72
0	40.7a[1]y[2]	74.3ax	100.3ax
0.09	33.8ay	66.6ax	90.7ax
0.45	18.9by	28.8bx	47.5bx
0.9	4.5cz	20.7cy	35.9bx
1.8	0.9dz	9.9dy	14.7cx

[1]Concentration means within hour with different a, b, c, d letters are statistically different at the 0.05 significance level.
[2]Hours means within concentration level with different x, y, z letters are statistically different at the 0.05 significance level.

Discussion

We have isolated two yeasts (*P. burtonii* and *C. fermentati*) from the coffee berry borer. Barnett et al. (2000) lists 33 different *Pichia* species associated with insects, out of 89 recognized *Pichia* species. *Pichia burtonii* had previously been isolated from an unspecified caterpillar (Barnett et al., 2000) while other *Pichia* species have been reported from insects, e.g., *P. guilliermondii* has been reported from the frass of *Synoxylon rufficorne* (Coleoptera: Bostrichidae), from fig wasps (Hymenoptera: Chalcidoidea), and from *Xestobium plumbeum* (Coleoptera: Anobiidae) (Barnett et al., 2000); *P. ramenticola* from the frass of wood-boring beetle larvae (Kurtzman & Robnett, 1998b; Kurtzman, 2000), and *P. pini* from galleries associated with various *Ips* and *Dendroctonus* species (Coleoptera: Scolytidae) (Holst, 1936; Phaff, 1956; Whitney, 1971). Various *Candida* species (*Pichia*'s asexual stage; Barnett et al., 2000) have been isolated from frass of unspecified wood boring beetle larvae and from the striped ambrosia beetle *Trypodendron lineatum* (Oliv.) (Coleoptera: Scolytidae) (Kurtzman & Robnett, 1998b; Kurtzman, 2000). *Candida* has also been isolated from two *Dendroctonus* species (Rumbold, 1941), from planthoppers (Homoptera: Delphacidae), where it is transmitted transovarially (Eya et al., 1989), and from a cerambycid, where it is associated with the midgut (Bismanis, 1976). *Candida fermentati* has been isolated from ants (Barnett et al., 2000).

If the role of *P. burtonii* in the coffee berry borer yeast is to detoxify caffeine, then its growth should not be impaired by caffeine concentrations present in the coffee berry, and therefore, consumed by the insect. To mimic caffeine concentrations in the coffee seed, we used various caffeine concentrations in liquid culture (0.09, 0.45, 0.9,

and 1.8%) that are well within the range found in coffee beans (Mösli Waldhauser & Baumann, 1996). *Pichia burtonii* tolerates low caffeine levels (0.09%), based on no significant differences in spore production with the control, but at the higher caffeine levels tested (0.45, 0.9, and 1.8%), spore production was impaired, indicating a detrimental effect of caffeine on the yeast. Our results suggest that caffeine levels in the coffee seed, which are much higher than those necessary to inhibit yeast growth in liquid culture, might act to inhibit yeast growth within the insect.

The HPLC results indicate that *P. burtonii* is not involved in the breakdown of caffeine in liquid media, as evidenced by no significant differences between initial caffeine levels and those observed after 24, 48, or 72 h in culture. In an additional experiment we assessed caffeine levels after 96 and 168 h (1 week) in culture with the yeast, and found that caffeine levels remained unchanged. Interestingly, yeast culture supernatants produced HPLC chromatograms which appeared little changed from fresh media; the HPLC system used to assay caffeine concentration revealed no peaks which could be ascribed to yeast metabolites. The yeast might be involved in the breakdown of metabolites of caffeine, but this possibility was not addressed in this study. Thus, even though the yeast might be acting as an endosymbiont, it is also possible that the presence of the yeast in the insect is mere happenstance.

In addition to detoxifying allelochemicals (Dowd, 1991, 1992), yeasts or yeast-like fungi play a nutritional role in insects, such as providing vitamins (Chararas & Pignal, 1981), and/or sterols (Pant & Fraenkel, 1954; Noda & Saito, 1979; Noda & Mittler, 1983). *Candida guilliermondii* (Castellani) Langueron and Guerra, and *Candida tenuis* Diddens and Lodder (*Candida* is the asexual form of *Pichia*) have been isolated from the alimentary canal of a cerambycid where they are believed to play an important nutritional role by synthesizing B vitamins and producing various enzymes (Chararas & Pignal, 1981). Lee & Hou (1987) demonstrated that yeasts provide proteins that are essential for the embryonic development of *Nilaparvata lugens* (Homoptera: Delphacidae). The ability of termites to degrade cellulose has been ascribed to the production of hemicellulolytic enzymes by yeasts present in the termite's gut (Schäfer et al., 1996). The detoxification role involves the production of enzymes that in some cases have the ability to detoxify plant allelochemicals, pesticides, and mycotoxins (Dowd, 1990). It is possible that *P. burtonii* is involved in providing nutritional factors for the coffee berry borer.

In conclusion, *P. burtonii* does not appear to be associated with caffeine breakdown in the coffee berry borer. Future studies will examine the exact location of the yeast within the insect, as well as the biology of yeast-free insects.

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References

Akhtar N & Mondal KAMSH (1994) Effect of caffeine and castor oil on fecundity and fertility of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae). Pakistan Journal of Zoology 26: 179–181.

Barnett JA, Payne RW & Yarrow D (2000) Yeasts: Characteristics and Identification, 3rd edn. Cambridge University Press, Cambridge, UK.

Bernays EA, Oppenheim S, Chapman RF, Kwon H & Gould F (2000) Taste sensitivity of insect herbivores to deterrents is greater in specialists than in generalists: a behavioral test of the hypothesis with two closely related caterpillars. Journal of Chemical Ecology 26: 547–563.

Bismanis JE (1976) Endosymbionts of *Sitodrepa panicea*. Canadian Journal of Microbiology 22: 1415–1424.

Castellanos I & Espinosa-García FJ (1997) Plant secondary metabolite diversity trait against insects: a test with *Sitophilus granarius* (Coleoptera: Curculionidae) and seed secondary metabolites. Biochemical Systematics and Ecology 25: 591–602.

Chararas C & Pignal M-C (1981) Étude du role de deux levures isolées dans le tube digestif de *Phoracantha semipunctata*, (Coléopterè, Cerambycidae), xylophage spécifique des eucalyptus. Comptes Rendus Academie des Sciences Paris 292: 109–112.

Chen H-CN, Xu X-F, Chen Z-M, Chen & Yu F-L (1996) On the resistance mechanisms of tea clones to pink tea rust mite. Acta Phytophylacica Sinica 23: 137–142.

Dowd PF (1990) Detoxification of plant substances by insects. CRC Handbook of Natural Pesticides, Vol. VI: Insect Attractants and Repellents (ed. by E D Morgan & N B Mandava), pp. 181–225. CRC Press, Boca Raton, Florida.

Dowd PF (1991) Symbiont-mediated detoxification in insect herbivores. Microbial Mediation of Plant–Herbivore Interactions (ed. by P Barbosa, V A Krischik & C G Jones), pp. 411–440. John Wiley & Sons, New York.

Dowd PF (1992) Insect fungal symbionts: a promising source of detoxifying enzymes. Journal of Industrial Microbiology 9: 149–161.

Eya BK, Kenny PTM, Tamura SY, Ohnishi M, Naya Y, Nakanishi K & Sugiura M (1989) Chemical association in symbiosis: sterol donors in planthoppers. Journal of Chemical Ecology 15: 373–380.

Glendinning JI & Slansky F Jr (1994) Interactions of allelochemicals with dietary constituents: effects of deterrency. Physiological Entomology 19: 173–186.

Hewavitharanage P, Karunaratne S & Savitri Kumar N (1999) Effect of caffeine on shot-hole borer beetle (*Xyleborus fornicatus*) of tea (*Camellia sinensis*). Phytochemistry 51: 35–41.

Holst EC (1936) *Zygosaccharomyces pini*, a new species of yeast associated with bark beetles. Journal of Agricultural Research 53: 513–518.

Itoyama MM (1990) Effect of caffeine on reproductive and developmental aspects in *Drosophila prosaltans*. Revista Brasileira de Genetica 13: 147–148.

Itoyama MM (1993) Effects of caffeine on reproduction and, in association with the stannous ion, on fecundity in *Drosophila prosaltans*. Revista Brasileira de Genetica 16: 882–883.

Itoyama MM & de Campos Bicudo HEM (1992) Effects of caffeine on fecundity, egg laying capacity, development time and longevity in *Drosophila prosaltans*. Revista Brasileira de Genetica 15: 303–321.

Itoyama MM, de Campos Bicudo HEM & Cordeiro JA (1997) Effects of caffeine on mitotic index in *Drosophila prosaltans* (Diptera). Revista Brasileira de Genetica 20: 655–657.

Itoyama MM, de Campos Bicudo HEM & Manzato AJ (1995) Effects of caffeine on mating frequency and pre-copulation and copulation durations in *Drosophila prosaltans*. Cytobios 83: 245–248.

Itoyama MM, de Campos Bicudo HEM & Manzato AJ (1998) The development of resistance to caffeine in *Drosophila prosaltans*: productivity and longevity after ten generations of treatment. Cytobios 96: 81–93.

Kurtzman CP (2000) Three new ascomycetous yeasts from insect-associated arboreal habitats. Canadian Journal of Microbiology 46: 50–58.

Kurtzman CP & Robnett CJ (1998a) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek 73: 331–371.

Kurtzman CP & Robnett CJ (1998b) Three new insect-associated species of the yeast genus *Candida*. Canadian Journal of Microbiology 44: 965–973.

Laz R, Salam MA & Islam MS (1998) Induction of dominant lethal mutations by ethyl merhanesulfonate and caffeine in the silkworm, *Bombyx mori* L. I. Effects on larval growth. Bangladesh Journal of Zoology 26: 95–102.

Lee YH & Hou RF (1987) Physiological roles of a yeast-like symbiote in reproduction and embryonic development of the brown planthopper, *Nilaparvata lugens* Stål. Journal of Insect Physiology 33: 851–860.

Levinson HZ (1976) The defensive role of alkaloids in insects and plants. Experientia 32: 408–411.

Liscia A & Solari P (2000) Bitter taste recognition in the blowfly: electrophysiological and behavioral evidence. Physiology and Behavior 70: 61–65.

Mathavan S, Premalatha Y & Christopher MSM (1985) Effects of caffeine and theophylline on the fecundity of four lepidopteran species. Experimental Biology 44: 133–138.

Mayilvaganan S & Mathavan S (1994) Induction of non-specific gene expression by secondary plant metabolite – caffeine. Phytophaga – Madras 6: 81–82.

Mohamed P & Nair VR (1991) Effect of caffeine on different developmental stages of *Drosophila melanogaster*. Proceedings of the Indian National Academy of Sciences, Part B, Biological Sciences 57: 141–145.

Mohamed P & Nair VR (2001) Effect of caffeine on wing morphogenesis. *Drosophila melanogaster*. Entomon 26: 87–90.

Mösli Waldhauser SS & Baumann TW (1996) Compartmentation of caffeine and related purine alkaloids depends exclusively on the physical chemistry of their vacuolar complex formation with chlorogenic acids. Phytochemistry 42: 985–996.

Muthukrishnan J, Mathavan S & Venkatasubbu K (1979) Effects of caffeine and theophylline on food utilisation and emergence in *Danaus chrysippus* L. (Lepidoptera: Danidae). Entomon 4: 307–312.

Nathanson JA (1984) Caffeine and related methylxanthines: possible naturally occurring pesticides. Science 226: 184–187.

Noda H & Mittler (1983) Sterol biosynthesis by symbiotes of aphids and leafhoppers. Metabolic Aspects of Lipid Nutrition in Insects (ed. by T E Mittler & R H Dadd), pp. 41–55. Westview Press Inc., Boulder, CO.

Noda H & Saito T (1979) The role of intracellular yeastlike symbiotes in the development of *Laodelphax striatellus* (Homoptera: Delphacidae). Applied Entomology and Zoology 14: 453–458.

Pant NC & Fraenkel G (1954) Studies on the symbiotic yeasts of two insect species, *Lasioderma serricorne* F. and *Stegobium paniceum*. Biological Bulletin 107: 420–432.

Phaff HJ (1956) A proposal for amendment of the diagnosis of the genus *Pichia* Hansen. Antonie Van Leeuwenhoek 22: 113–116.

Portilla M (1999) Mass rearing technique for *Cephalonomia stephanoderis* (Hymenoptera: Bethylidae) on *Hypothenemus hampei* (Coleoptera: Scolytidae) developed using Cenibroca artificial diet. Revista Colombiana de Entomología 25: 57–66.

Pöschko M (1995) Research into the Biology and Host-Specificity of *Teretriosoma nigrescens*, a Potential Natural Antagonist of *Prostephanus truncatus*. Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ). GmbH, Eschborn, Germany.

Rizvi SJH, Pandey SK, Mukerji D & Mathur N (1980) 1,3,7-trimethylxanthine, a new chemosterilant for stored grain pest, *Callosobrochus chinensis* (L.). Zeitschrift für Angewandte Entomologie 90: 378–381.

Rumbold CT (1941) A blue stain fungus, *Ceratostomella montium* n. sp. and some yeasts associated with two species of *Dendroctonus*. Journal of Agricultural Research 62: 589–601.

SAS Institute Inc. (1999) SAS/STAT User's Guide, Version 8. SAS Institute Inc., Cary, NC.

Schäfer A, Konrad R, Kuhnigk T, Kämpfer P, Hertel H & König H (1996) Hemicellulose-degrading bacteria and yeasts from the termite gut. Journal of Applied Bacteriology 80: 471–478.

Shen SK & Dowd PF (1991) Detoxification spectrum of the cigarette beetle symbionts *Symbiotaphrina kochii* in culture. Entomologia Experimentalis et Applicata 60: 51–59.

SlanskyF & Wheeler GS Jr (1992) Caterpillars' compensatory feeding response to diluted nutrients leads to toxic allelochemicals dose. Entomologia Experimentalis et Applicata 65: 171–186.

Srinivasan A & Kesavan PC (1979) Mechanisms of pupariation delay induced by caffeine in *Musca domestica*: effect of combination treatment of caffeine & ascorbic acid on pupariation. Indian Journal of Experimental Biology 17: 321–322.

Stamp NE, Temple MP, Traugott MS & Wilkens RT (1994) Temperature-allelochemical interactive effects on performance of *Manduca sexta* caterpillars. Entomologia Experimentalis et Applicata 73: 199–210.

Usher BF, Bernays EA & Barbehenn RV (1988) Anti-feedant tests with larvae of *Pseudaletia unipuncta*: variability of behavioral response. Entomologia Experimentalis et Applicata 48: 203–212.

Whitney HS (1971) Association of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) with blue stain fungi and yeasts during brood development in lodgepole pine. Canadian Entomologist 103: 1495–1503.

Wood SL (1982) The Bark and Ambrosia Beetles of North and Central America (Coleoptera: Scolytidae), a Taxonomic Monograph. Great Basin Naturalist Memoirs, no. 6. Brigham Young University, Provo, Utah, USA.