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The Effects of a Naturally Produced Benzoquinone on Microbes Common to Flour

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Abstract Many species of insects are known to produce and secrete benzoquinone derivatives. These compounds are usually defined as "defense chemicals." However, in many cases, it has not been determined what the chemicals are meant to defend against. Tribolium beetles produce up to three benzoquinones, but their specific function is not known. In this experiment, one of the derivatives, methyl-1,4-benzoquinone (MBQ) was tested for its effectiveness for one of its purported functions as an antimicrobial. Methyl-1,4-benzoquinone was added in three concentrations (0.3, 30, and 150 μg/ml) to the liquid media of three species of *Bacillus* bacteria and eight species from four genera of yeasts, and the effect on their growth was monitored. The presence of MBQ altered growth in all species. The bacteria responded more negatively than the yeasts. All bacteria species showed reduced growth at all levels of MBQ. Yeasts were more tolerant to the presence of the chemical, and two species, *Saccharomyces microellipsoides* and *Pichia burtonii*, actually showed increased growth at the lowest level of MBQ.

Keywords Benzoquinone · *Bacillus* · Yeast · *Pichia* · *Cryptococcus* · *Rhodoturula* · *Saccharomyces* · *Tribolium*

Introduction

Over 100 species of arthropods synthesize benzoquinone derivatives (Blum 1981). Tribolium flour beetles produce up to three benzoquinone derivatives: methyl-1, 4-benzoquinone (MBQ), ethyl-1,4-benzoquinone, and methoxybenzoquinone (Weatherston 1967; Blum 1981). Although often termed "defense chemicals," it is not clear what these compounds actually defend against. Based on potential organismal interactions in their

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stored grain environment, one theory has suggested that the compounds prevent rodents from becoming potential predators on flour beetles (El-Mofty et al. 1992; Dettner 1993; Bouchard et al. 1997). Other hypotheses suggest that the compounds act as antimicrobials (Geiger 1946; Engelhardt et al. 1965; Prendeville and Stevens 2002; Yezerski et al. 2005).

Williams (1966) established a list of criteria for a trait to be considered adaptive. These included widespread examples across species, complexity of the trait, cost of the trait to the organisms, and a well-defined genetic basis. The production of benzoquinones by Tribolium beetles fits these criteria. Benzoquinone derivatives are found in many genera of insects (Blum 1981). There is great complexity in the creation and storage of the compounds, as well as metabolic costs to the organisms. In flour beetles, the compounds are derived from an ill defined, but apparently intricate, mechanism in specialized prothoracic and postabdominal glands (Happ 1968). In addition to the metabolic costs, the substances are known to be harmful to the beetles themselves and thus pose a survival cost (Chapman 1926; Roth and Howland 1941). Further supporting the fact that benzoquinone production is an adaptation, the storage of the benzoquinone derivatives has been shown to have a strong and quantitative genetic background (Yezerski et al. 2000, 2004). However, the exact functions of these chemicals for these beetles has not been clearly defined.

In this research, we explored the hypothesis that the benzoquinone trait is an adaptation in the stored grain pest, *Tribolium confusum*, that controls microbial growth in their flour environment. To this end, we obtained type species of bacteria and yeasts known to be associated with stored flours, and we subjected them to an environment containing varying levels of the benzoquinone derivative, MBQ. The effect of the compound on the growth of the microbial species was monitored via turbidity.

Methods and Materials

Source of Microbial Specimens The three species of bacteria are known to inhabit flour and other stored grain products (Norris et al. 1981). These species were provided in both type strain and flour isolate forms by L. K. Nakamura of the USDA in lyophilized preparations. The species included *Bacillus circulans* [NRRL B-380 (type) and B-393 (isolate)], *Bacillus subtilis* [NRRL NRS-744 (type) and B-571 (isolate)], and *Bacillus licheniformis* [NRRL NRS-1264 (type) and NRS-766 (isolate)]. Specimens were grown and maintained in TGY media containing tryptone, glucose, and yeast extract.

Yeasts were provided in lyophilized form by C. P. Kurtzman of the USDA and included the following species known to inhabit wheat flour (Kurtzman et al. 1970): *Pichia fermentans* (NRRL Y-7138), *Pichia anomala* (NRRL Y-7081), *Pichia burtonii* (NRRL Y-7082), *Pichia farinosa* (NRRL Y-7084), *Saccharomyces microellipsoides* (NRRL Y-6524), *Cryptococcus albidus* (NRRL Y-7079), *Cryptococcus laurentii* (NRRL Y-7139), and *Rhodotorula glutinis* (NRRL Y-7140). Specimens were grown and maintained in YM-2 media containing yeast extract, malt extract, peptone, and glucose.

Inoculation Procedure Measurement of the growth of each species began with inoculation of 1 ml of the microbe that had reached maximum turbidity for the species into 100 ml of the appropriate media in a sidearm flask containing one of three concentrations of MBQ: 0.3, 30, or 150 μg/ml. These values were based on the average amount of MBQ found to be on the external surface of a typical beetle and equated to 1, 100, and 500 beetles, respectively (Yezerski et al. 2000, 2004). A control was simultaneously inoculated similarly into a flask containing no MBQ. All measurements were taken in triplicate. Prior testing by plating

found no inherent microbial growth in the purchased MBQ. Methyl-1, 4-benzoquinone has a strong orange to red color in microbial media, and therefore, "color blanks" were needed that contained the same concentrations as the experimental flasks. However, the presence of the microbes themselves also altered the color of the media as growth occurred and, thus, interfered with the accuracy of the readings. Therefore, blanks were created identical to the control, but cells were centrifuged to the bottom of the tube, removing turbidity, before being used to zero the machine. These blank cultures were then resuspended until the next measurement was taken to allow growth to continue as seen in the controls.

Growth Measurements Turbidity of the inoculations was determined by using a manostat colorimeter. Readings began 6–8 hr after inoculation depending on the previously determined lag time for each species. After the colorimeter was zeroed by using the blanks, readings were taken on the experimental cultures to obtain turbidity in Klett units. This procedure was repeated for each inoculation every hour until the control flasks approached a stationary phase. Notable events between and within species and concentrations were compared via a repeated-measures analysis that used the JMP™ Statistical Program (Sall et al. 2006).

Results

Growth curves for the bacteria species are shown in Fig. 1 and growth curves for the yeast species in Figs. 2 and 3. A summary of a statistical comparison of growth in each condition vs. the control flask is shown in Table 1. The control flasks for all species indicated successful and consistent growth under these conditions. The yeasts tended to reach higher turbidities (around 300 Klett units) than the bacteria, which tended to plateau at levels around 200 Klett units. However, the bacteria reached their stationary phases much sooner than the yeasts under the same conditions.

Compared to the controls, the MBQ-treated flasks showed significantly reduced growth rates in most species (Table 1). The equivalent of the presence of 500 beetles, 150 μg/ml MBQ, appears to have eliminated growth altogether in all of the bacterial species and in three of the eight yeast species. However, considering that 30 μg/ml MBQ was not tolerated by any bacterial species, it is notable that the majority of yeast species was somewhat resistant to the effects of the presence of MBQ even at this highest level. *Pichia anomali* demonstrated little reduction in growth with any level of MBQ, while *B. circulans*' growth was completely eliminated by even 0.3 μg/ml MBQ.

Several of the yeasts appeared unaffected by the compound at the two lowest levels, but their growth was strongly suppressed at the highest level. This is not unexpected because the dilutions were serial and not parallel in nature. However, there is a surprising result for three yeasts, *Rhodotorula glutinus*, *P. burtonii*, and *S. microellipsoides*, where growth increased at the lowest level of 0.3 μg/ml MBQ (Figs. 2 and 3). *Saccharomyces microellipsoides* and *P. burtonii* have significantly higher turbidity than the controls in the second half of their growth curves (Table 1; indicated by Xs rather than asterisks).

In general, the microbial growth was consistently decreased during the measurement time, but bacterial species were more greatly affected than yeast species. There is some indication that low levels of MBQ may actually encourage growth in certain species of yeast.

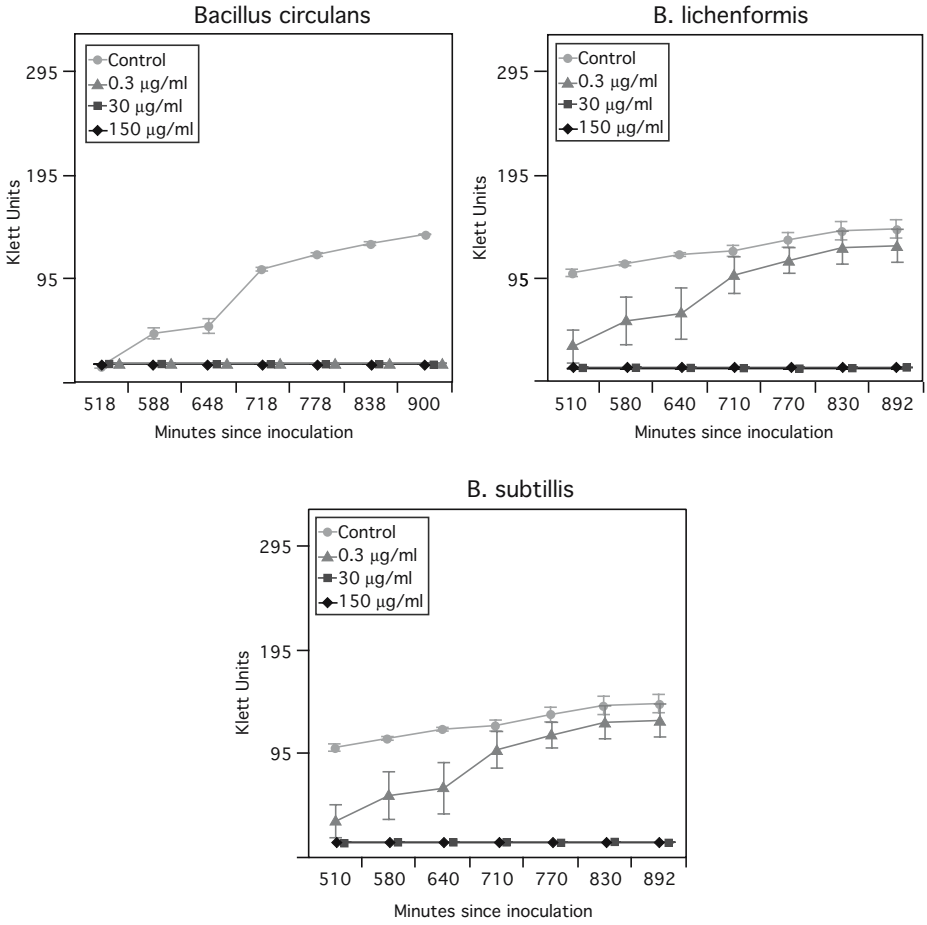

Fig. 1 Growth of three Bacillus species in the presence of MBQ in Klett units + SEM. As indicated in the *legends*, *symbols* represent growth in media without MBQ (*circles*) or with 0.3 µg/ml (*triangles*), 30 µg/ml (*squares*), or 150 µg/ml of MBQ (*diamonds*) measured at the indicated minutes after initial inoculation

Discussion

Our results indicate that MBQ has an effect on microbial growth. However, the compound is more of a deterrent to bacteria than yeast. From an evolutionary standpoint, these results are expected. Bacteria species, especially members of the *Bacillus* genus, can be harmful to insect species by causing numerous diseases and developmental difficulties (Faust 1974; Kreig 1981; Kumari and Neelgund 1985; Abdel-Razek et al. 1999; Abdel-Razek 2002).

Microbes might also compete with beetles for nutritional resources. However, it has been shown that yeast species associated with flour obtain distinct nutritional benefits for the beetles. The beetles do poorly in flour that does not contain sufficient levels of yeast (Offhaus 1952; Charbonneau and Lemonde 1962; Via and Conner 1995). In fact, a veritable symbiosis has been noted (Dunkel 1988). Therefore, the complete elimination of yeast species from the environment would be detrimental to beetle survival.

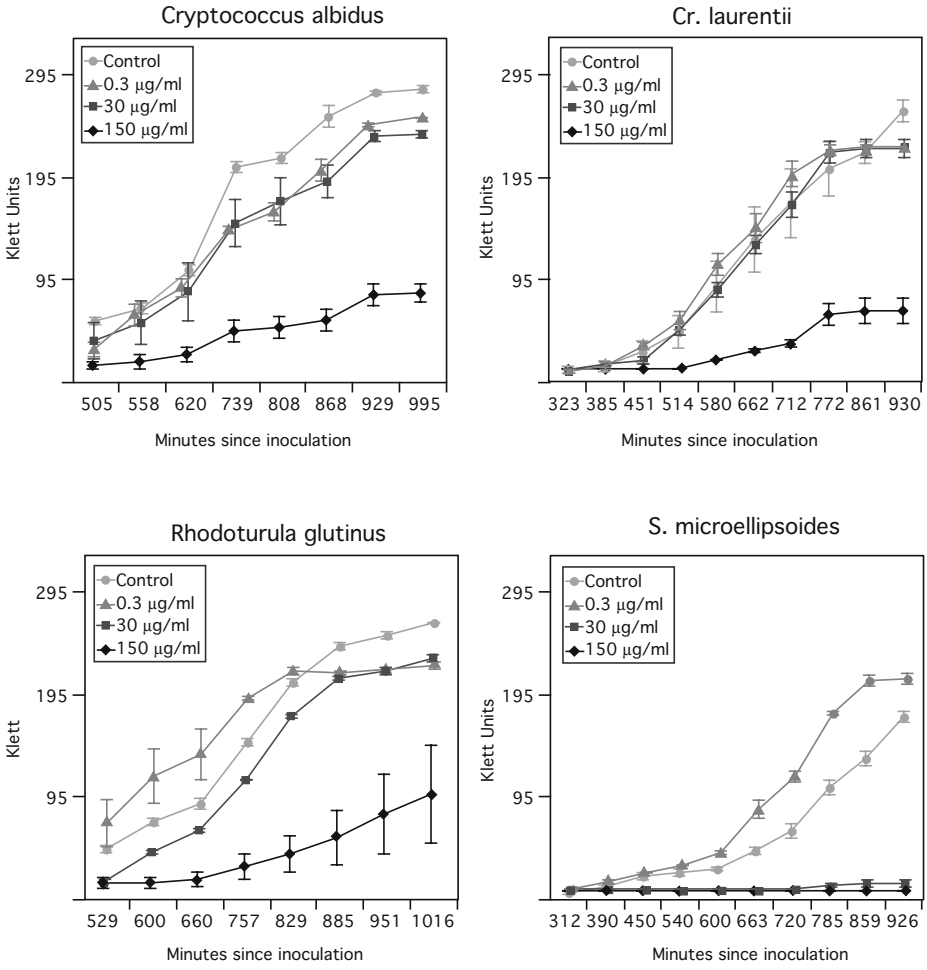

Fig. 2 Growth of four yeast species in the presence of MBQ in Klett units + SEM. As indicated in the *legends*, *symbols* represent growth in media without MBQ (*circles*) or with 0.3 µg/ml (*triangles*), 30 µg/ml (*squares*), or 150 µg/ml of MBQ (*diamonds*) measured at the indicated minutes after initial inoculation

The choice of the bacterial species for this study was based on data for species commonly found in flour according to USDA experts. Species were selected based on previous work specifically with flour isolates. Other sources, however, indicate that, whereas the presence of *Bacillus* species is quite usual, other genera such as *Salmonella* and *Escherichia* should also be considered for future investigation (Farmiloe et al. 1954; Rogers 1978; Okagbue 1990; Richter et al. 1993; Sorokulova et al. 2003). Additionally, our selection of species did not include those that might have been already associated with the beetles themselves because the flour isolates were not from stores containing insects. Recent studies have suggested that insect flour pests introduce members of the Enterococcidae and Enterobacter families into their flour environment, but Tribolium species that produce high levels of benzoquinones generally lack these bacteria species in their stocks (Yezerski et al. 2005). Therefore, although these bacterial species may be

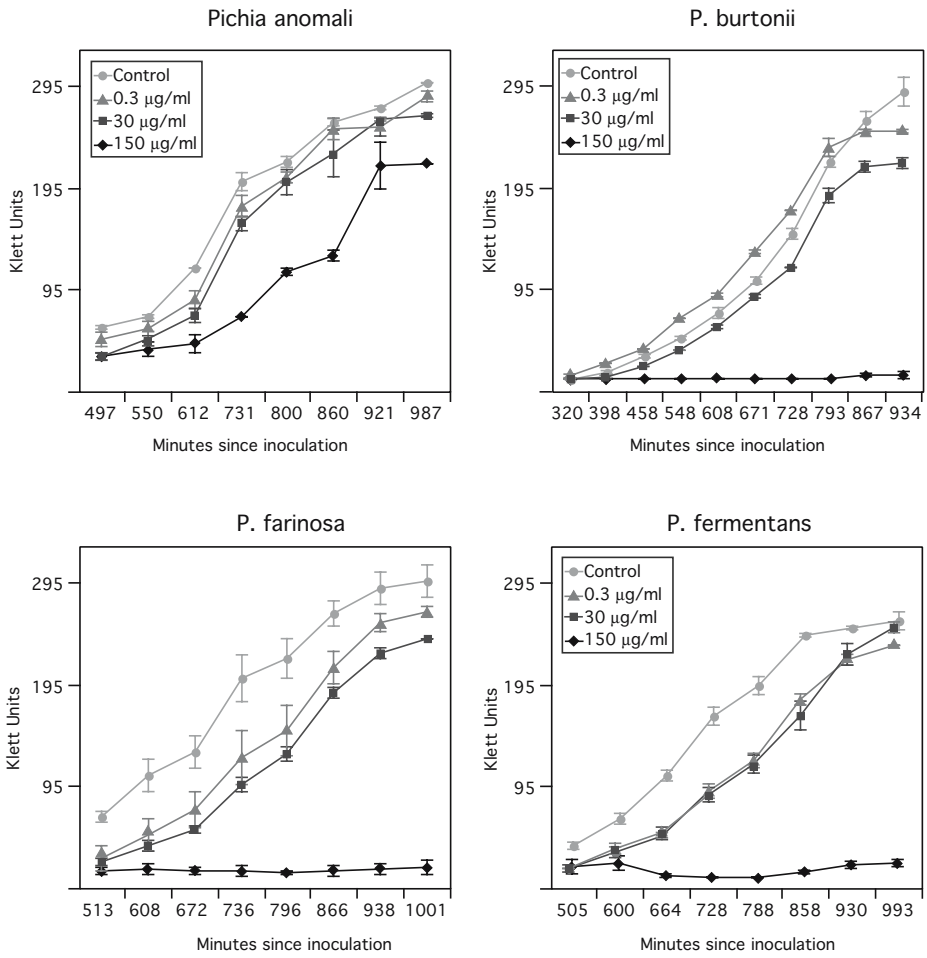

Fig. 3 Growth of four yeast species from the *Pichia* genus in the presence of MBQ in Klett units + SEM. As indicated in the *legends*, *symbols* represent growth in media without MBQ (*circles*) or with 0.3 µg/ml (*triangles*), 30 µg/ml (*squares*), or 150 µg/ml of MBQ (*diamonds*) measured at the indicated minutes after initial inoculation

present in the guts of flour pests, previous work, along with these present data, suggests that the beetles are curtailing their presence.

It is well known that yeasts are associated with flour, but the species varies. The species that we used based on the USDA's flour isolates were not in complete congruence with those suggested by the literature (Kurtzman et al. 1970; Okagbue 1990). However, the species used represent common species. Regardless, the conclusion that yeast species generally grow better in the presence of MBQ than bacterial species is supported.

The levels of MBQ chosen for this study were based on previously determined levels on the external surface of a *T. confusum* beetle (about 0.3 µg per beetle at any given time) (Yezerski et al. 2000, 2004). However, amounts of MBQ on the beetles themselves may differ from amounts present in their flour environment. Other work has shown that beetles secrete approximately 7 µg MBQ over 72 hr, but that much of this degrades into methyl-1,

Table 1 Summary of the statistical comparison of turbidity between control and experimental cultures over time using a repeated measures ANOVA

Species	[MBQ] (μg/ml)	Time Period[a]									
		1	2	3	4	5	6	7	8	9	10
B. circulans	0.3	**	***	***	***	***	***	***			
	30	**	***	***	***	***	***	***			
	150	**	***	***	***	***	***	***			
Bacillus lichenformis	0.3	**	**	**	ND	ND	ND	ND			
	30	***	***	***	***	***	***	***			
	150	***	***	***	***	***	***	***			
Bacillus subtillis	0.3	**	***	***	ND	ND	ND	ND			
	30	***	***	***	***	***	***	***			
	150	***	***	***	***	***	***	***			
C. laurentii	0.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	150	ND	ND	ND	ND	***	***	***	***	***	***
C. albidus	0.3	ND	ND	ND	ND	ND	ND	ND	ND		
	30	ND	ND	ND	ND	ND	ND	ND	ND		
	150	ND	ND	**	***	***	***	***	***		
R. glutinis	0.3	ND	ND	ND	ND	ND	ND	ND	ND		
	30	ND	ND	ND	ND	ND	ND	ND	ND		
	150	ND	ND	ND	**	***	***	***	***		
S. microellipsoides	0.3	ND	ND	ND	ND	xxx	xxx	xxx	xxx	xxx	***
	30	ND	ND	ND	**	***	***	***	***	***	***
	150	ND	ND	ND	**	***	***	***	***	***	***
P. anomali	0.3	ND	ND	ND	ND	ND	ND	ND	ND		
	30	ND	ND	ND	ND	ND	ND	ND	ND		
	150	ND	ND	ND	***	***	***	***	***		
P. burtonii	0.3	ND	ND	ND	xx	xxx	xxx	xxx	ND	***	
	30	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	150	ND	ND	ND	ND	***	***	***	***	***	
P. farinosa	0.3	ND	ND	ND	**	*	ND	ND	ND		
	30	ND	*	**	***	***	**	**	*		
	150	*	**	***	***	***	***	***	***		
P. fermentans	0.3	ND	ND	***	***	***	***	**	ND		
	30	ND	***	***	***	***	***	ND	ND		
	150	**	***	***	***	***	***	***	***		

Blank cells indicate no measurement was taken in this time interval.

ND=no difference

[a] Measurements were taken in 1-hr intervals. The first measurement time from inoculation (1) varied for each species. Actual time in minutes from inoculation can be seen in detail in the accompanying figures. Statistical significance of the comparison of the turbidity in Klett units between indicated culture and respective control. Asterisks represent Klett units significantly lower than control (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$). Xs represent Klett units significantly higher than control (x $P<0.05$, xx $P<0.01$, and xxx $P<0.001$).

4-hydroquinone (MHQ) (Yezerski, unpublished data). It can be assumed that the highly reactive MBQ naturally degrades into MHQ in the flour environment. Therefore, by introducing a reasonable amount of MBQ into the environment and allowing it to degrade normally, one realistically replicates a natural situation. The reactivity of MBQ may be part of the effectiveness of the antimicrobial activity, but this has not been tested directly here.

In this study, we also did not test the chemically related ethyl-1,4-benzoquinone that is also produced by many insect species (Weatherston 1967; Blum 1981). This compound is actually the more abundant compound in the beetle excretion, being produced at a ratio of almost 2:1 in *T. confusum* (Pappas and Morrison 1995; Pappas and Wardrop 1996; Yezerski et al. 2000, 2004). This compound is not commercially available and is also extremely susceptible to light and temperature, making work with it exceedingly difficult (Yamada and Hosaka 1977). The slight structural difference in this substance vs. MBQ perhaps suggests a similar result. The purpose of producing twice as much may be related to ease of natural synthesis.

Although benzoquinone derivatives are a common part of many organisms' physiology, Tribolium beetles and several other insect species incur a metabolic cost that produces a substance that may be harmful to their own survival and development. The results of this experiment indicate that one of these compounds, MBQ, is an effective antimicrobial that is selective to species that are potentially harmful or beneficial. It effectively reduces the growth of bacterial species known to cause harm while controlling and even encouraging the growth of yeast species that are an important food source. Another suggested function may be an antipredatory defense against rats that cohabitate grain storage with flour beetles. We are currently conducting research to determine the efficacy of this alternative hypothesis.

References

ABDEL-RAZEK, A. S. 2002. Comparative study on the effect of two Bacillus thuringiensis strains of the same serotype on three coleopteran pests of stored wheat. *J. Egypt. Soc. Parasitol.* 32:415–424.

ABDEL-RAZEK, A. S., SALAMA, H. S., WHITE, N. D. G., and MORRIS, O. N. 1999. Effect of Bacillus thuringiensis on feeding and energy use by Plodia interpunctella (Lepidoptera: Pyralidae) and Tribolium castaneum (Coleoptera: Tenebrionidae). *Can. Entomol.* 131:433–440.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

BOUCHARD, P., HSIUNG, C. C., and YAYLAYAN, V. A. 1997. Chemical analysis of defense secretions of Siploidea sipylus and their potential use as repellents against rats. *J. Chem. Ecol.* 23:2049–2057.

CHAPMAN, R. N. 1926. Inhibiting the process of metamorphosis in the confused flour beetle (Tribolium confusum Duval). *J. Exp. Zool.* 45:293–299.

CHARBONNEAU, R., and LEMONDE, A. 1962. Indeterminate growth factors in brewer's yeast. IV. Relation between these factors and the nitrogen levels in the diet of Tribolium confusum Duval larvae. *Arch. Int. Physiol. Biochim.* 70:379–392.

DETTNER, K. 1993. Dabbing and shooting of benzo- and napthoquinone secretions: defensive strategies of bark-inhabiting Aleocharine (Col.: Staphylinidae) and Tenebrionid (Col.: Tenebrionidae) beetle larvae. *J. Chem. Ecol.* 19:1337–1354.

DUNKEL, F. V. 1988. The relationship of insects to the deterioration of stored grain by fungi. *Int. J. Food Microbiol.* 7:227–244.

EL-MOFTY, M. M., KHUDOLEY, V. V., SAKR, S. A., and FATHALA, N. G. 1992. Flour infested with Tribolium castaneum, biscuits made of this flour, and 1,4-benzoquinone induce neoplastic lesions in Swiss Albino mice. *Nutr. Cancer* 17:97–104.

ENGELHARDT, M., RAPOPORT, H., and SOKOLOFF, A. 1965. Odorous secretion of normal and mutant Tribolium confusum. *Science* 150:632–633.

FARMILOE, F. J., CONCORD, S. J., COPPOCK, J. B. M., and INGRAM, M. 1954. The survival of Bacillus subtilis spores in the making of bread. *J. Sci. Food Agric.* 5:292–304.

FAUST, R. M. 1974. Bacterial diseases, pp. 87–183, in G. E. Cantwell (ed.). Insect Diseases. Marcel Dekker, New York.

GEIGER, W. B. 1946. The mechanism of the antibacterial action of quinones and hydroquinones. *Arch. Biochem.* 11:23–32.

HAPP, G. M. 1968. Quinone and hydrocarbon production in the defensive glands of Eleodes longicollis and Tribolium castaneum (Coleoptera, Tenebriondae). *J. Insect Physiol.* 14:1821–1837.

KREIG, A. 1981. The genus Bacillus: insect pathogens, in M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (eds.). The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Springer-Verlag, Berlin.

KUMARI, S. M., and NEELGUND, Y. F. 1985. Preliminary infectivity tests using six bacterial formulations against the red flour beetle, Tribolium castaneum. *J. Invertebr. Pathol.* 46:198–1999.

KURTZMAN, C. P., WICKERHAM, L. J., and HESSELTINE, C. W. 1970. Yeasts from wheat and flour. *Mycologia* 62:542–547.

NORRIS, J. R., BERKELEY, R. C. W., LOGAN, N. A., and O'DONNELL, A. G. 1981. The genera Bacillus and Sporolactobacillus, pp. 1709–1742, in M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (eds.). The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Springer-Verlag, Berlin.

OFFHAUS, K. 1952. Vitamin requirements of the confused flour beetle (Tribolium confusum Duval). I. The water-insoluble yeast fraction required by Tribolium confusum. *Z. Vitam. Horm. Fermentforsch.* 4:555–563.

OKAGBUE, R. N. 1990. Identification of yeasts and aerobic spore forming bacteria from cassava flour. *Food Microbiol.* 7:27–32.

PAPPAS, P. W., and MORRISON, S. E. 1995. Benzoquinones of the beetles, Tribolium castaneum and Tribolium confusum. *Prep. Biochem.* 25:155–168.

PAPPAS, P. W., and WARDROP, S. M. 1996. Quantification of benzoquinones in the flour beetles, Tribolium castaneum and Tribolium confusum. *Prep. Biochem. Biotechnol.* 26:53–66.

PRENDEVILLE, H. R., and STEVENS, L. 2002. Microbe inhibition by Tribolium flour beetles varies with beetle species, strain, sex, and microbe group. *J. Chem. Ecol.* 28:1183–1190.

RICHTER, K. S., DORNEANU, E., ESKRIDGE, K. M., and RAO, C. S. 1993. Microbiological quality of flours. *Cereal Foods World* 38:367–369.

ROGERS, R. F. 1978. Bacillus isolates from refrigerated doughs, wheat flour, and wheat. *Cereal Chem.* 55:671–674.

ROTH, L. M., and HOWLAND, R. 1941. Studies on the gaseous secretion of Tribolium confusum Duval. I. Abnormalities produced in Tribolium confusum Duval by exposure to a secretion given off by the adults. *Ann. Entomol. Soc. Am.* 34:151–171.

SALL, J., CREIGHTON, L., and LEHMAN, A. 2006. JMP Start Statistics. Thomson Learning, Cary

SOROKULOVA, I. B., REVA, O. N., SMIRNOV, V. V., PINCHUK, I. V., LAPA, S. V., and URDACI, M. C. 2003. Genetic diversity and involvement in bread spoilage of Bacillus strains isolated from flour and ropy bread. *Lett. Appl. Microbiol.* 37:169–173.

VIA, S., and CONNER, J. 1995. Evolution in heterogeneous environments: genetic variability within and across different grains in Tribolium castaneum. *Heredity* 74:80–90.

WEATHERSTON, J. 1967. The chemistry of arthropod defense substances. *Q. Rev. Chem. Soc. Lond.* 21:287–313.

WILLIAMS, G. C. 1966. Adaptation and Natural Selection. Princeton University Press, Princeton.

YAMADA, Y., and HOSAKA, K. 1977. A convenient synthesis of alkyl-substituted p-benzoquinones from p-alkylphenols. *Synthesis* 143:53–54.

YEZERSKI, A., GILMOR, T. P., and STEVENS, L. 2000. Variation in the production and distribution of substituted benzoquinone compounds among genetic strains of the confused flour beetle, Tribolium confusum. *Physiol. Biochem. Zool.* 73:192–199.

YEZERSKI, A., GILMOR, T. P., and STEVENS, L. 2004. Genetic analysis of benzoquinone production in Tribolium confusum. *J. Chem. Ecol.* 30:1034–1044.

YEZERSKI, A., CUSSATT, G., GLICK, D. L., and EVANCHO, M. 2005. The effects of the presence of stored product pests on the microfauna of a flour community. *J. Appl. Microbiol.* 98:507–515.