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Inhibiting the Growth of *Bacillus cereus* in Raw Sprouts and Cooked Rice using Red Clover Seeds

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The growth of enterotoxigenic *Bacillus cereus* in perishable foods is a well-known cause of food poisoning. In this study, we evaluated sprouting seeds (broccoli, green peas, lentil, mung bean, mustard, radish, red clover, soybean, and triticale) for their antimicrobial activity toward *B. cereus*. The filter-sterilized seed extracts of red clover, lentil, and mung bean yielded inhibition zones of 11.7, 9.2, 8.3 mm, respectively. However, no significant inhibition (≤ 6 mm) was observed with other seed extracts. Naturally occurring *B. cereus* multiplied from 1.1 to near 5.0 log CFU/g during broccoli and radish sprouting. In contrast, *B. cereus* growth was suppressed when brassica and red clover seeds were grown together. A mixture of seeds containing 10% red clover reduced *B. cereus* counts to ≤ 1 log CFU/g at the end of sprouting. Artificially inoculated *B. cereus* (1.1 log CFU/g) grew to 5.9 and 5.6 log CFU/g in white and black rice, respectively, during storage for 24 h at 24°C. Adding a small amount of red clover seed extract (2.5 ml per 25 g of cooked rice) reduced the respective growth by 3.2 and 2.3 log CFU/g. *B. cereus* counts on the treated white and black rice were about 4.9 and 3.7 log CFU/g, respectively, after 48 h of storage at 24°C. In conclusion, the antimicrobial activity of some legume seeds has potential to be utilized to inhibit *B. cereus* in food systems.

Some legume seeds and plants (such as soybean and red clover) contain high levels of isoflavonoid compounds that have been shown to exert beneficial effects on many human disorders, including cancer, menopausal symptoms, bone loss, and cardiovascular diseases (Beck *et al.* 2005; Campbell *et al.* 2004; Lam *et al.*; Powles 2004). In addition, studies have revealed that some phytochemicals derived from these seeds work as natural antimicrobial agents against the growth of foodbrone pathogens including *B. cereus* (Emmert *et al.* 2005; Rajkowski 2004; Verdrengh *et al.* 2004), a widely distributed spore-forming bacterium in the agricultural environment and food crops (Granum 2001; Schraft *et al.* 1996).

Although *Salmonella* spp. and pathogenic *Escherichia coli* were responsible for most reported outbreaks involving contaminated sprouts, the potential risk of *Bacillus cereus* contamination in sprout production should not be ignored (Pao *et al.* 2005). Previous seed surveys showed a high prevalence of enterotoxigenic *Bacillus* spp. in seeds sold for home-sprouting use (Pao *et al.* 2005; Harmon *et al.* 1987). Portnoy *et al.* (1976) suggested that *B. cereus* was the causative agent in a food poisoning outbreak linked to contaminated vegetable sprouts. Furthermore, enterotoxigenic *B. cereus* was isolated from raw soybean sprouts by Kim *et al.* (2004).

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A recent study by Pao *et al.* (2005) found that naturally occurring enterotoxingenic *Bacillus* spp. on radish seeds could multiply during glass-jar sprouting to > 5 log CFU/g, a population level high enough to cause food poisoning (Harmon *et al.* 1987). However, some legume seeds may release antimicrobial substances to prevent *Bacillus* growth during sprouting (Pao *et al.* 2005).

Outbreaks of *B. cereus* food poisoning have been frequently linked to the consumption of contaminated rice (Holmes *et al.* 1981; Raevuori et al. 1976). For instance, fried rice served at two Virginia child day care centers was linked to an outbreak of *B. cereus* food poisoning in 1993 (Khodr *et al.* 1994). In 1998 a non-typical *B. cereus* outbreak occurred in Texas at a church day school; sufferers had handled hydrated, orange-colored rice before consuming a meal, *B. cereus* organisms were found in the rice at a level exceeding 5 log CFU/g (Briley *et al.* 2001).

The purpose of this study was to screen sprouting seeds for their antimicrobial activity toward *B. cereus*. In addition, the potential usage of a legume seed (red clover) for controlling the growth of *B. cereus* in fresh sprout production and cooked rice storage was explored.

MATERIALS AND METHODS

Inoculum preparation. Three strains of *Bacillus cereus* (ATCC11778, ATCC13061 and ATCC14579) were cultured in nutrient broth (NB; Unless otherwise noted, all media were purchased from Biotrace, Bothell, WA) and individually streaked onto tryptone soy agar (TSA). After overnight incubation at 35°C, colonies were suspended in sterile peptone water (0.1%) and vortexed thoroughly. The bacterial suspension was adjusted to match the 0.5 McFarland turbidity standard for the agar disk assay as previously described (NCCLS 2003). The suspension was diluted to ~2 log CFU/ml (as later confirmed by plating) for inoculating cooked rice.

Seed extract. Nine types of sprouting seeds (including broccoli and red clover from Frontier Natural Products, Norway, IA; green peas, soybean, and triticale from Emergency Essential, Orem, UT; lentil, mung bean, and mustard from The Sprout House, Saugerties, NY; and radish from Handy Pantry, Apache Junction, AZ) were ordered through the Internet. Each type of seed (100 g) was soaked in sterile de-ionized water (300 ml) for 24 h at 24°C. The water was then sterilized through a syringe filter (0.20 µm, Fisher Scientific, Pittsburg, PA) and used for the agar disk assay and rice studies.

Agar disk assay. A sterile cotton swab was wetted with the *B. cereus* suspension (ATCC11778 or ATCC14579) and then streaked 3 times over Mueller-Hinton agar (MH) as previously described (NCCLS 2003). Blank paper disks (6 mm diameter; Bection Dickinson and Company, Sparks, MD) for antimicrobial evaluation were placed individually on MH using sterilized forceps and gently pressed down onto the agar. Freshly prepared seed soaking waters were individually dispensed onto the disks (0.05 ml/disk) before the agar plates were inverted for incubation at 30°C for 16-18 h. The diameter of each inhibition zone (including the disk) developed on the agar plates was measured.

Seed Sprouting. Before sprouting, broccoli seeds or radish seeds were mixed with 1, 5 or 10 % of red clover seeds. Approximately 50 g of the mixed sprouting seeds were placed in a sterilized glass jar (1-liter) and soaked with 150 ml of sterilized tap water (Matoca, VA) for 8 h at 24°C. After draining, the glass jar was placed horizontally for seed germination at 24°C for 6 days. The glass jar was loosely capped to allow air exchange and covered with a plastic sheet to reduce light exposure (*Pao et al.* 2005). Once per day, the germinating seeds in the jars were gently rinsed with 250 ml of sterilized tap water for about 30 seconds. The sprouting samples (10 g) were taken consecutively from the jars at hour 8 (immediately after soaking) and day 2, 4, and 6 before rinsing for microbial testing. The study was replicated three times.

Rice preparation. Two hundred grams of rice (enriched long-grain white rice, Mahatma, Riviana Foods, Houston, TX or natural short-grain black rice, Havista, Superlucky food, Shanghai, China) were boiled in a rice cooker (Black & Decker, Miramar, FL) with sterile de-ionized water (400 ml) for approximately 30 min. The heated rice was cooled to 24°C and then distributed onto petridishes (100 × 15 mm; 25 g rice/dish). A 0.1-ml of inoculum containing mixed *B. cereus* strains (ATCC11778, ATCC13061 and ATCC14579) was inoculated into each dish of cooked rice and mixed manually with sterile gloves for 1 min. The inoculated samples were then mixed with 2.5 ml of red clover seed extract or sterilized de-ionized water followed by incubation at 24°C for 8, 16, 24, or 48 h before microbial testing. Without inoculation, *B. cereus* was not found (<1 log CFU/g) on the cooked rice at either 0 or 48 h. The study was replicated for three times.

Microbial enumeration. The sprout or rice samples were blended with an equal amount of peptone water (0.1%) in a laboratory blender (Massticator Silver, IUL Instruments, Barcelona, Spain) for 120 s. Appropriate dilutions of each macerated sample were analyzed for *B. cereus* counts as previous described (USFDA 2001). Diluted samples were surface plated on mannitol-egg-yolk-polymyxin agar (MYP; Difco, Becton Dickinson, Sparks, MD) and incubated for 24 h at 30°C for presumptive *B. cereus* counts. To differentiate *B. cereus* from culturally similar species, representative colonies found on the MYP plates were isolated using nutrient agar and incubated at 30°C. Microscopic examination was performed at day 1 for positive cell motility and Gram-staining. The absence of bipyramidal-shape crystal formation at day 4 with basic fuchsin (Fisher Scientific, Fair Lawn, NJ) staining was further used to characterize the cells (Bennett and Belay 2001). Diluted isolates were inoculated to phenol red glucose broth, nitrate broth, modified VP medium, tyrosine agar, and lysozyme broth for biochemical assays. Cultures showing positive reactions with all assays were confirmed as *B. cereus*. To determine the enterotoxin producing capability of each isolate, a loopful of each was enriched with 10 ml BHI + 0.1% glucose for 16-18 h at 36°C before performing a *Bacillus* diarrhea enterotoxin visual immunoassay (Tecra, Frenchs Forest, Australia) according to the manufacturer's instruction (Tecra 2001).

Statistical analysis. One-way ANOVA was performed on data with all paired multiple comparison procedures (Holm-Sidak method) using SigmaStat (Version 3.0, SPSS Inc., Chicago, IL) software. Significance of difference was defined at $P \leq 0.05$.

RESULTS AND DISCUSSION

Agar disk assay. Components of many seeds and plants are known to have antimicrobial activity. In this study we found that seed soaking water (seed extracts) of red clover, lentil, and mung bean seeds can produce significant inhibition zones on MH plates streaked with *Bacillus cereus* (Table 1). Red clover seed extract showed the greatest inhibition zone diameter (≥ 11.0 mm). No measurable inhibition was observed with extracts from broccoli, radish,

Table 1. Inhibitory effects of seed extracts on the growth of *B. cereus* in the agar disk assay

***B. cereus* strain**	Inhibition zone diameter (mm)			
	Red Clover	Lentil	Mung bean	Other seeds
ATCC11778	12.3 ± 0.6a[1]	10.8 ± 0.4ab	9.3 ± 1.0b	NMI[2]
ATCC14579	11.0 ± 0.5a	7.6 ± 0.2b	7.3 ± 0.7b	NMI

[1]Data represent means ± standard errors of 6 replications. Other seeds refer to broccoli, radish, mustard, triticale, green peas, and soybean. In the same row, means not followed by at least one identical letter are significantly different ($P < 0.05$).
[2]NMI (Not Measurable Inhibition) indicates a zone diameter ≤ 6.0 mm.

mustard, triticale, green peas, or soybean seeds.

The presence of antimicrobial substances in legume seeds was not unexpected. Our previous study had suggested that lentil and mung bean seeds may release antimicrobial substances during sprouting to inhibit the growth of enterotoxigenic *Bacillus* spp. (Pao *et al.* 2005). Furthermore, red clover and many other legume seeds contain canavanine, a nonprotein amino acid, that has inhibitory activity toward *B. cereus* (Rajkowski 2004; Emmert *et al.* 1998). The presence of these naturally occurring compounds in selected seeds offers a possible explanation for the observed differences in inhibition zone development on MH plates. Since the greatest inhibition zone was found with red clover seed extract, it was used in subsequent sprout and rice studies.

Sprout study. Sprouting seeds used in this study were naturally contaminated with an average of 1.1 log CFU/g of *B. cereus*. Figure 1 shows that the naturally occurring *B. cereus* organisms were able to multiple to near 5.0 log CFU/g level during broccoli and radish sprouting (the control group, without red clover seeds) in glass jars at 24°C. Other reported findings with various vegetable sprouts agree with our results (Kim *et al.* 2004; Harmon *et al.* 1987).

Figure 1 also shows that the growth of the naturally occurring *B. cereus* was inhibited by the presence of red clover seeds as demonstrated by sprouting with seed mixtures containing 1, 5 or 10% of red clover. Red clover seeds showed a dose-related inhibition of *B. cereus* in both broccoli and radish sprouts. The levels of *B. cereus* were effectively control at ≤ 1 log CFU/g by the end of broccoli and radish sprouting (day 6) with the presence of 10% of red clover seeds. The study showed that red clover seeds could be incorporated in the sprouting process to prevent *B. cereus* poisoning, although it did not address sprout contamination issues associated with infectious bacteria such as *Salmonella* and pathogenic *E. coli*.

Rice study. Figure 2 shows that artificially inoculated *B. cereus* can grow from an initially low level (1.1 log CFU/g) to dangerous levels (5.9 and 5.6 log CFU/g in white and black rice, respectively) when stored at 24°C for 24 h. The observed rapid growth of *B. cereus* on cooked rice at ambient temperature is expected as contaminated starchy foods including leftover rice, potato, pasta, and macaroni dishes have caused numerous outbreaks of *B. cereus* food (Dierck *et al.* 2005; Khodr *et al.* 1994; Holmes *et al.* 1981).

However, Figure 2 indicates that adding a small amount of red clover extract (2.5 ml per 25 g of cooked rice) reduced *B. cereus* growth in day 1 by 3.2 and 2.3 log CFU/g in white and black rice, respectively. No beneficial effect was observed by adding water (the control group) to the inoculated rice samples. The seed extract showed antimicrobal activity toward *B. cereus* on both long-grain (white) and short-grain (black) rice. *B. cereus* counts of 4.9 and 3.7 log CFU/g were noted on the extract-treated white and black rice, respectively, following an additional 24 h of storage at 24°C. This finding suggests that the extract of red clover seeds has potential to be utilized along with other conventional food safety measures against the growth of *B. cereus* in cooked rice.

Although proper temperature control (hot holding or refrigeration) is the standard practice relied upon for preventing the growth of *B. cereus*, alternative approaches are highly valuable for safeguarding perishable foods that might be exposed to a broad range of temperatures during production, transportation, or storage (Pao *et al.* 2005; Ultee *et al.* 2000). Since red clover seed extract suppressed *B. cereus* on cooked rice, it would be interesting to test the usage of seed extracts for controlling *B. cereus* on mashed potatoes, pasta, and other food items that are vulnerable to growth of enterotoxigenic *B. cereus*.

CONCLUSIONS

Legume seeds and sprouts have long been noticed for their nutritive value and potential health benefits to human. The current study further demonstrated that 1) mixing broccoli or radish seeds with a small amount of red clover seeds could prevent the proliferation of *B. cereus* during sprouting and 2) the extract of red clover seeds has a potential to be used for controlling B. cereus growth in cooked rice. We conclude that the antimicrobial activity of legume seeds (such as red clover) may be utilized in food production or storage systems for preventing *B. cereus* growth. Further research in isolating and identifying the inhibitory compounds from legume seeds and exploring their safe use in food protection applications is recommended.

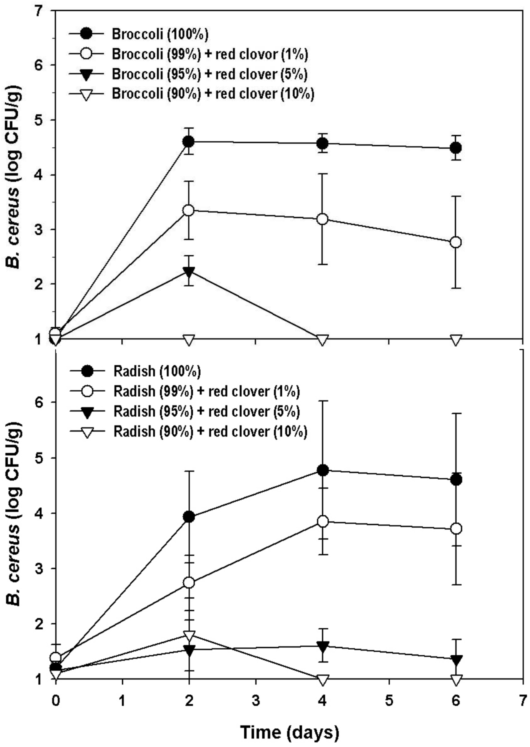

Figure 1. Effect of brassica and red clove seed ratio on the growth of naturally occurring *B. cereus* during seed sprouting.

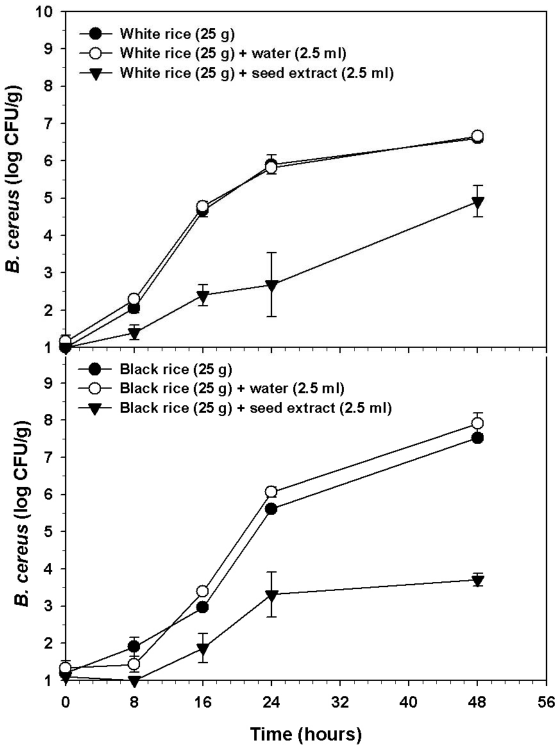

Figure 2. Reducing the growth of *B. cereus* in cooked rice stored at 24°C by adding red clover seed extract.

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