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In Vivo Safety Assessment of the Plant Growth-Promoting Rhizobacterium *Bacillus cereus* RS87 and Rhizo-product

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Authors' contributions

This work was carried out in collaboration between all authors. Author OL designed the study, wrote the protocol, data analysis and wrote the first draft of the manuscript. Author KJ prepared the bacterial spores. Author PP performed the statistical analysis. Author SW formulated and prepared the rhizo-product. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To assess the acute toxicity and skin irritation potential of a rhizobacterium *Bacillus cereus* RS87 and the rhizo-product in rats and rabbits.
Study Design: Adult Wistar rats were gavaged with a single dose of *B. cereus* RS87 in acute oral toxicity test and were applied with single doses of rhizo-product for 24 hours in acute dermal toxicity test. New Zealand albino rabbits were applied with 0.5g rhizo-product in acute dermal irritation test.
Place and Duration of Study: Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand, between November 2013 and March 2014.
Methodology: Animal toxicity studies were carried out by the methods described in the Organization for Economic Co-operation and Development (OECD) test guidelines. Mortality data of animals were used to determine the median lethal dose (LD_{50}) values after oral and dermal exposures to *B. cereus* RS87 and the rhizo-product along with distilled water as control. The skin irritation potential of the rhizo-product was evaluated in rabbits. Distilled water was used as a control. The average weight gains were calculated and gross examination at necropsy was performed.
Results: No mortality and no signs of toxicity were observed. The oral LD_{50} of *B. cereus* RS87 and dermal LD_{50} of rhizo-product in rats were greater than $9x10^8$ CFU kg^{-1} and 15,000 mg kg^{-1}(about $4.5x10^8$ CFU kg^{-1}), respectively. However, significant decrease in mean weight gain in the high-dose groups when compared to controls (21.40+/-1.47 *versus* 28.40 +/- 0.24 (male); 13.80+/-2.57 *versus* 20.20+/-0.58 (female)) were reported at day 8 after 24-hour dermal exposure to rhizo-product. No pathological changes in major organs were observed at necropsy.
Conclusion: *B. cereus* RS87 and the rhizo-product (about $3x10^7$ CFU/g) have low acute toxicity and very low skin irritation potential, which was considered safe for humans. However, adverse effect needed to be further explored in the field experiment or in practical use.

Keywords: Bacillus cereus; toxicity; safety; rhizobacterium; rhizo-product.

1. INTRODUCTION

Rhizobacteria is a group of rhizosphere bacteria capable in colonizing the root environment [1]. In the past few decades, several evidences demonstrated the benefits of rhizobacteria in promoting plant growth and controlling the plant pathogens [2]. Thus far, the use of rhizobacteria in agriculture continues to increase owing to sustainability and safe environment, crop productivity, and reduction of synthetic fertilizers [3]. Several rhizobacteria have been developed into commercial products as biofertilizers and biopesticides [4]. However, there is growing concern about safety of biofertilizers and the regulatory frameworks are being developed in several countries to assure the safety of the products prior to registration and commercialization [5].

Bacillus cereus RS87 is a saprophytic soil bacterium isolated from the rhizosphere of Green Kuang Futsoi (*Brassic chinensis* Jusl var. *parachinensis* (Bailey) Tsen & Lee) at Tumbol Bung-Phra, Phitsanulok province, Thailand. A study conducted by Jetiyanon et al. [6] showed that both fresh vegetative cell suspension and dormant spores of *B. cereus* RS87

had comparable efficacy in the enhancement of seedling emergence, root elongation and plant growth of cucumber and pepper seeds. *B. cereus* RS87 also promoted growth of Thai rice cultivars and showed the potential to replace 50% of the amount of synthetic fertilizer normally used [7,8]. The known mechanisms by which *B. cereus* RS87 promotes plant growth are production of indoleacetic acid, phosphate solubilization and production of siderophores [6-8].

Since the dormant spores of *B. cereus* RS87 have several advantages over the vegetative cells (e.g., resistance to environmental condition, rapid spore activation under favorable conditions), an end-use rhizo-product containing *B. cereus* RS87 spores has recently been formulated [9]. However, some strains of *B. cereus* are harmful to humans and toxicities of strain RS87 were still not elucidated *in vivo*. Therefore, this study aimed to assess the acute toxicity and skin irritation potential of *B. cereus* RS87 and the rhizo-product in experimental animals after oral and dermal exposures.

2. MATERIALS AND METHODS

2.1 Rhizobacteria *B. Cereus* RS87

The dormant spores of *B. cereus* RS87 were used for evaluating acute oral toxicity. The source of *B. cereus* RS87 and the preparation of bacterial spores were described earlier [6]. In brief, *B. cereus* RS87 isolated from the rhizosphere of Green Kuang Futsoi was identified by fatty acid analysis. The bacterium was maintained in tryptic soy broth (Becton Dickinson, Sparks, Maryland, USA) supplemented with 20% glycerol and stored at -80ºC. To prepare the spores, the bacterium was grown on tryptic soy agar for 24 hours and then transferred to a defined medium. After incubation at 30ºC for 4 days, spores were harvested by centrifugation at 10,000g for 10 min at 4ºC. The supernatant was discarded and the solid pellets containing spores of *B. cereus* RS87 were washed at least twice with sterile distilled water. The number of spores per milliliters were determined and the spores were then stored in the dark at 4ºC. In the acute oral toxicity test, the pellets were resuspended and diluted with distilled water at the time before dosing. The final concentrations were $5.4x10^4$, $5.4x10^6$ and $5.4x10^8$ $CFUmL^{-1}$.

2.2 Rhizo-product

The light brownish powder of rhizo-product used in acute dermal toxicity and acute dermal irritation tests was formulated and manufactured by PGPR laboratory, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand (Mfg. date 8 October 2013). Each gram of the product contained about $3x10^7$ CFU of *B. cereus* RS87 spores and other nontoxic, environment-friendly ingredients such as corn powder, natural clay, andcalcium carbonate [9]. For experimental purpose, small amount of distilled water was added to the known amounts of rhizo-product and mixed until it turned into paste before applying on the skin.

2.3 Acute Toxicity Testing

All toxicity tests in animals were conducted according to the OECD test guidelines no. 423 (acute oral toxicity), 402 (acute dermal toxicity), and 404 (acute dermal irritation/corrosion) [10-12]. The dose unit in acute oral toxicity test was modified to the number of rhizobacteria and the dose range was appropriately adjusted. All the tests were carried out between

November 2013 and March 2014 at Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand. The protocols were approved by TISTR animal ethics committee and were strictly followed.

Wistar rats were purchased from the National Laboratory Animal Center, Mahidol University (Salaya, Nakhon Pathom, Thailand). The New Zealand white rabbits were received from the Department of Animal Science, Kasetsart University (Bangkok, Thailand). Animals were acclimatized and quarantined for one week before experimentation.

2.3.1 Acute oral toxicity

Forty adult Wistar rats weighing 250-277g for male and 186-236 g for female were randomly divided into three treatment and one control groups (5 males/5 females per group). They were abstained from food for 16 hours before and 4 hours after receiving the bacterial spores. Water intake was *ad libitum*. On the day of experiment, the animals in each treatment group were orally gavaged with 0.5mL of different concentrations of *B. cereus* RS87 spores which were equivalent to $9x10^4$, $9x10^6$ and $9x10^8$ CFU kg^{-1}body weight. The rats in control group were dosed with distilled water at the same volume as the treatment groups.

2.3.2 Acute dermal toxicity

Thirty adult Wistar rats weighing 313-359g for male and 212-240g for female were randomly distributed to two treatment and one control groups (5 males/5 females per group). One day before experimentation, a 5x5cm area of skin on the dorso-lumbar region of each rat was clipped free of hair. The paste of rhizo-product equivalent to 2,000 or 15,000mg kg^{-1} body weight was applied onto a 3.5x3.5cm gauze patch. Distilled water on another patch was used as control. The gauze patch was applied to the selected skin site on each rat and secured by transpore adhesive tape, with an elastic bandage wrapping the entire trunk for 24 hours. After removing the patches, the treated skin was gently wiped by moistened cotton wool until the residual rhizo-product was completely removed.

For acute oral and dermal toxicity tests, the rats were observed at 30 min, 1, and 3 hours after administration and once daily thereafter for 14 days. The mortality, signs of toxicity, and the definite time at which toxicity appeared and recovered were recorded. The body weight of the rats was recorded on day 1, 8 and 15 or after death during the study period. Animals that survived after the 14-day observation period were terminated by CO_2 asphyxiation. All animals were then subjected to gross examination at necropsy and, if necessary, histopathologic examination.

2.3.3 Acute dermal irritation/corrosion

Three adult New Zealand white rabbits weighing 2-3kg were clipped free of hair on the dorso-lumbar region to get an area of 10x10 cm one day before experimentation. Two areas with the size of 2.5x2.5cm on the shaved skin were selected as treatment and control area. The paste of 0.5g rhizo-product or distilled water (as a control) was applied on a 2.5x2.5cm gauze patch. Both treatment and control patches were applied on each selected area and were fixed with transpore adhesive tape and elastic bandage wrapping the entire trunk for 4 hours. Then the patches were removed and the rhizo-product was completely removed from the skin by moistened cotton wool.

The severity degree of erythema/eschar and edema occurred on each treated area was independently assessed by two inspectors at 1, 24, 48 and 72 hours after removing the patches by using a rating scale from 0 to +4. Any lesions and other toxic effects were also recorded. If there were signs of irritation, further observation would be continued up to 14 days after application in order to establish the reversibility of the irritation signs. Other abnormalities, if any, were also recorded.

2.4 Statistical Analysis

For mortality data, the LD_{50} values were analyzed using probit analysis. If there was no mortality, the LD_{50} was reported to be greater than the highest dose in the experiment. The average weight changes of animals in each treatment group and each gender were calculated and expressed as mean ± standard error. A Student's t-test was used to establish the significant differences between the control and treatment groups. $P<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Acute Oral Toxicity

No mortality and no signs of toxicity were observed in all animals after a single oral administration of the bacterial spores at any doses during the 14-day study period. There were no statistically significant differences in weight gain and body weight between the treatment and control groups on days 8 and 15 (Table 1 and Fig. 1). At autopsy, no gross pathological changes were observed in any groups. Thus, the LD_{50} value of *B. cereus* RS87 after oral gavage to Wistar rats was greater than $9x10^8$ CFU kg^{-1} body weight.

Table 1. Mean weight gains of the rats after oral administration of single doses of *B. cereus* RS87 spores

Treatment	Weight gain[a] (g)			
	Male		Female	
	Day 8	Day 15	Day 8	Day 15
Control	24.40±2.56	55.80±4.82	14.60±2.63	44.20±11.69
B. cereus RS87 $9x10^4$CFU kg^{-1}b.w.[b]	23.20±2.10	54.60±5.14	11.60±0.68	21.00±2.23
B. cereus RS87 $9x10^6$CFU kg^{-1}b.w.	19.40±0.24	56.60±3.95	11.80±0.73	33.60±4.22
B. cereus RS87 $9x10^8$CFU kg^{-1}b.w.	20.20±0.37	57.80±1.20	14.20±2.22	30.40±5.85

*[a]Mean ± S.E.M = Mean values ± standard error of means of five animals (in each group); [b]b.w. = body weight; * B. cereus RS87: significant from control, P<0.05*

3.2 Acute Dermal Toxicity

No mortality and no signs of toxicity were observed in all animals after 24-hour dermal exposure to rhizo-product at any doses during the 14-day study period. No weight loss was observed in all animals and the total body weight was not affected by the rhizo-product (Fig. 2). However, the difference in body weight gain between animals in high-dose groups and controls became significant as soon as day 8 after dermal exposure in male and female

rats (Table 2). This is likely to be a temporary, acute effect that might be resulted from the stress induced by a 24-hour exposure to bacterial spores or the clay-like ingredients in the rhizo-product rather than their direct effects. Gross examination at autopsy did not show any pathologic changes in all animals. Hence, the dermal LD_{50} of *B. cereus* RS87 in Wistar rats was greater than 15,000mg kg^{-1} body weight (equivalent to $4.5x10^8$ CFU of *B. cereus* RS87kg^{-1} body weight).

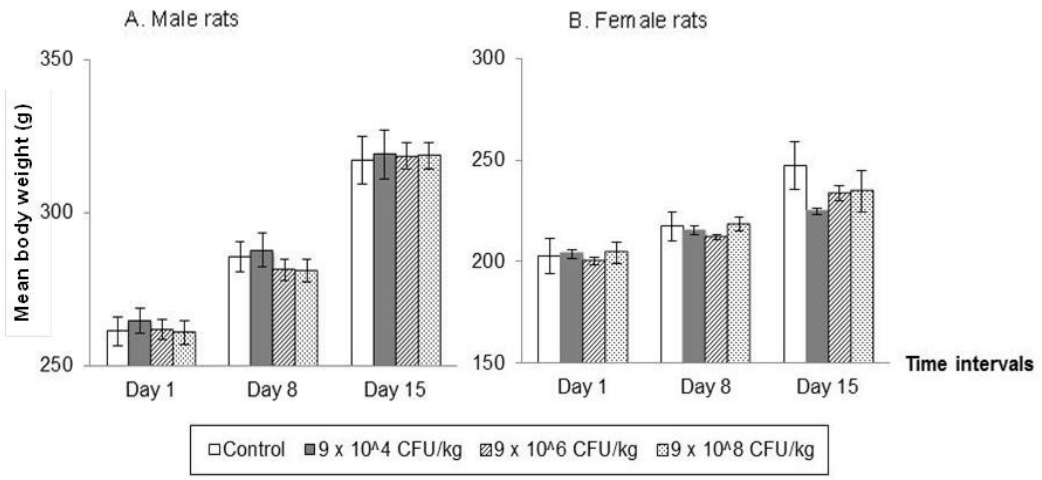

Fig. 1. Mean body weights of the rats after oral administration of single doses of *B. cereus* RS87 spores

Mean ± S.E.M = Mean values ± standard error of means of five animals (in each group)

Table 2. Mean weight gains of the rats after 24-hour dermal exposure to rhizo-product containing *B. cereus* RS87 spores

Treatment	Weight gain[a] (g)			
	Male		Female	
	Day 8	Day 15	Day 8	Day 15
Control	28.40±0.24	55.40±4.06	20.20±0.58	33.20±2.87
Rhizo-product 2,000 mg kg^{-1}	25.60±2.11	44.00±3.96	19.80±0.68	28.60±1.03
Rhizo-product 15,000 mg kg^{-1}	21.40±1.47*	41.00±4.92	13.80±2.57*	25.60±3.48

[a]*Mean ± S.E.M = Mean values ± Standard error of means of five animals (in each group);* Rhizo-product: significant from control, P<0.05*

3.3 Acute Dermal Irritation

No erythema/eschar and edema were reported in both treatment and control groups during the study after 4 hour exposure to rhizo-product.

The result from a series of toxicity tests indicates that rhizo-product containing *B. cereus* RS87 have low acute toxicity and very low skin irritation potential. In addition, the bacteria are unlikely to be harmful after activation since no mortality and clinical signs were observed in animals during 14 days of study period. In practical use, it is recommended that fifty grams

of rhizo-product will be mixed with 8 liters of water, which is equivalent to the concentration of $1.9x10^8$ CFU of *B. cereus* RS87 per liter. This estimated concentration of rhizo-product used in the field is much lower than the doses tested in animal experiments. Therefore, oral and dermal exposure to rhizo-product could be considered safe in humans.

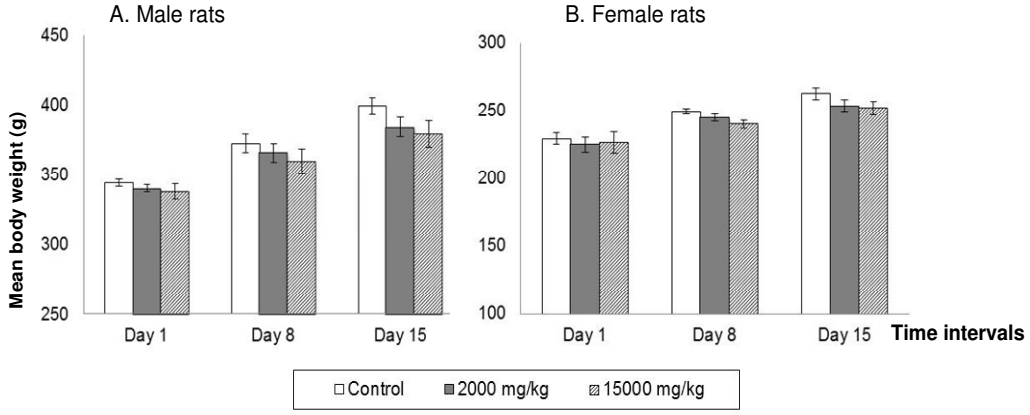

Fig. 2. Mean body weights of the rats after 24-hour exposure to rhizo-product containing *B. cereus* RS87 spores

Mean ± S.E.M = Mean values ± standard error of means of five animals (in each group)

4. CONCLUSION

Bacillus cereus RS87 and the rhizo-product have very low acute toxicity according to the results from a series of toxicity tests after oral and dermal exposure. The oral LD_{50} for *B. cereus* RS87 in rats is greater than $9x10^8$ CFU kg^{-1} body weight and the dermal LD_{50} for rhizo-product is greater than 15,000mg kg^{-1} (or $4.5x10^8$ CFU kg^{-1} body weight), respectively. In addition, the rhizo-product is not a skin irritant. *B. cereus* RS87 and the rhizo-product (about $3x10^7$ CFU/g) are considered nontoxic when given orally and dermally in standard animal testing and could be considered safe in humans. Nonetheless, adverse effects needed to be further explored in the field experiment or in practical use.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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