EFFECT OF THE EXTRACTS FROM LEAVES OF *ACANTHUS MONTANUS* (Nees) T. Anders. (ACANTHACEAE) ON GERMINATION AND OUTGROWTH OF SOME *BACILLUS* SPORES

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Summary

A study was done using the crude extract and ethyl acetate fraction from leaves of *Acanthus montanus* with the objective of examine their effect on germination and outgrowth of *Bacillus cereus* and *Bacillus stearothermophilus* spores.

After heat activation at 80°C during 10 min, spores were exposed to the crude extract and ethyl acetate fraction at the concentration of 1 mg/ml and 0,5 mg/ml respectively follow by water wash. To allow them to germinate and outgrow, they were spread by the use of bent glass rods onto plates containing trypticase soy agar. Furthermore, treated spores were exposed in trypticase soy broth. The number of CFU obtained from treated spores decreased significantly after 24 h of incubation. However, the optical density accompanying germination indicated that crude extract and ethyl acetate fraction delay this process. When 40 µg/ml and 20 µg/ml of ethyl acetate fraction were directly added to heated spores of *Bacillus cereus* and *Bacillus stearothermophilus* respectively in the germination medium, the germination and outgrowth were delayed in the same extent as were pre-treated spores. The active compound present in this fraction may act by preventing the action of germinant inside the spore or by inhibiting the RNA synthesis during outgrowth.

Keywords: spores, *Acanthus montanus*, *Bacillus*, germination.

Introduction

Clostridium and *Bacillus* bacteria are known to form spores that are highly resistant to adverse environmental conditions including dryness, heat and poor nutrient supply. They are also high resistant to chemical disinfectant, desiccation, extreme in pH, temperature, pressure, ultraviolet and ionizing radiation (1). To kill spores of these bacteria requires high temperature (121°C) under pressure for 15 min or strong chemical disinfectants such as formalin (2). It was

vital to detect germination of spore and to evaluate the effect of sporocidal and/or germination inhibitory agents. Spore germination is accompanied by the loss of refractivity, which can be observed visually using the phase contrast microscopy. Determination of the reduction in spore optical density has also been used to asses spore germination. The percent germination can be calculated from the absorbance decrease at 625 nm (3). In the case of spores of *B. cereus* and *B. stearothermophilus*, several studies have been carried out upon these strains (4; 5), none has been aimed at investigating the effect of some medicinal plant extracts on the germination of these spores. Crude extract and ethyl acetate fraction from leaves of *Acanthus montanus* are known for their bactericidal effects on vegetative *Bacillus stearothermophilus* and *Bacillus cereus* (6). The objective of this work is then to characterize germination and outgrowth of *Bacillus cereus* and *Bacillus stearothermophilus* spores after exposing them to crude extract and acetate ethyl fraction from leaves of *Acanthus montanus.*

Materials and methods

Plant material.The leaves of *A. montanus* were collected in Mbouda in the west province of Cameroon in January 2003 and identified by the National Herbarium in Yaoundé, where the voucher specimen was deposited under the reference number, 22730/SFR/CAM.

Extraction procedure. Fresh leaves of the plant were ground and macerated with methanol in the ratio 1:5 (w/v) at room temperature for 24 h. The concentrated methanol extract was decanted, filtered with Wathman number 1 filter paper and concentrated in vacuo bellow 40°C using a rotary evaporator to give the crude extract (3.14% w/w) used for the investigations . The methanol crude extract (M-L) was fractionated by successive extraction using hexane, ethyl acetate and acetone. The hexane (H-L), ethyl acetate (E-L), acetone (A-L) fractions and residue (R-L) were obtained respectively at: 7.33% w/w; 3.8% w/w; 1.37% w/w and 68.6% w/w. The crude extract and ethyl acetate fraction have significant antibacterial effects on vegetative *Bacillus stearothermophilus* and *Bacillus cereus* (6) and used in this work to examine their effect on germination and outgrowth of *Bacillus stearothermophilus* and *Bacillus cereus* spores.

Microorganisms. The test microorganism *Bacillus cereus* F3748 was obtained from the Microbiology Laboratory, Institute of Food Research Reading, UK and *Bacillus stearothermophilus* CNCH 5781 from the Institute Appert, France.

Production and purification of spores. Production of *Bacillus stearothermophilus* spores was adapted from the standard method described by (8). The bacterial strain was cultured for 12 – 14 h in the nutrient broth medium after which 2 ml of the culture was spread upon the surface of a sporulation agar. The incubation was made at the optimum growth of the strain. Daily observations were made using the phase contrast microscope (Olympus, Model BHT), and maximum spore concentration was obtained by the fifth day. Vegetative cells and spores were then harvested by scraping the agar surface with a sterile bent glass rod and rinsing of the spores with sterile distilled water. For *Bacillus cereus* the procedure for sporulation was as follows: abundant suspensions of the bacteria were obtained after growth at 37°C for 24 h in tryptone soya broth. Following this, several plates were surface-inoculated with 1 ml of the suspension. The inoculated plates were initially incubate in an upright position at 37°C for 24 h, then inverted for an additional 48 h at 37°C. The plates were afterwards held at 4°C for

24 h (8). Growth of each plate was harvested by scraping the surface with the bent glass rod which was then suspended in cold distilled water. Harvested spores were purified using a modified procedure of (9). Vegetative bacteria were removed by centrifugation at 4°C for 10 min at 3000 g in a refrigerated centrifuge (Sigma 202 MK, Bioblock Scientific). Microscopic observation was performed to evaluate final pellets containing more than 90 % refractile spores. The clean spores obtained were kept at 4°C until required.

Recovery of treated spores on agar plates. Spores (0.5 ml, 3 x 10^7) of *Bacillus cereus* and *B. stearothermophilus* after heat activation of 80°C for 10 min were suspended in distilled water containing M-L and E-L at: 1; 0.5; 0.25 and 0.125 mg/ml and 0.5; 0.25; 0.125 and 0.062 mg/ml for 20 min. Samples of these spores were serially diluted in sterile distilled water, and appropriate dilution was spread onto plates of agar media. Plates were incubated at appropriate temperature for 24 h and numbers of CFU were then counted. Survivals were expressed as the percentage of viable counts of untreated spores (10; 11; 2).

*Evaluation of germination.*A sample (0.5 ml of 10^7 /ml) of a stock spore suspension of each specie was heat activated at 80°C for 10 min in the water bath. Spores were then exposed to 1 and 0,5 mg of the M-L and E-L per ml respectively for 20 min. After exposure, the reaction was quenched by the addition of nine millilitres of cool sterile distilled water. The tubes were centrifuged at 5,000 x g for 15 min, the supernatant was discarded and the pellet was resuspended in the residual water remaining in the tubes after decantation of the supernatant. To allow the spores to germinate and outgrow five millilitres of trypticase soy broth was added to the spores and then incubated at the appropriate temperature. The optical density changes accompanying germination and outgrowth were recorded with spectrophotometer UV-120-01 at 560 nm. The optical density readings were expressed as percentage of the initial readings of untreated spores (11).

Determination of ethyl acetate fraction concentration effective in germination. A sample (0.5 ml of 10^7 /ml) of a stock spore suspension of each specie was centrifuged at 5,000 x g for 15 min and the pellet was resuspended in five millilitres of tryticase soy broth medium. Ethyl acetate fraction was immediately added at final concentration ranging from 0 to 80 µg/ml, and the pH of preparation was adjusted at 7.0. The spores were then incubated at the appropriate temperature. The optical density changes accompanying germination were recorded with spectrophotometer UV-120-01 at 560 nm.

Statistical analysis

Significant differences between the colony numbers from spores treated with M-L and E-L and untreated spores (control) were submitted to analysis using of variance (ANOVA) to determine the statistical difference ($p<0.05$).

Results

The recovery treated spores of *B. cereus* and *B. stearothermophilus* using M-L and E-L at concentration ranging from 0.125 to 1 mg/ml and 0.062 to 0.5 mg/ml in the trypticase soy agar medium is showed in figures 1 and 2.

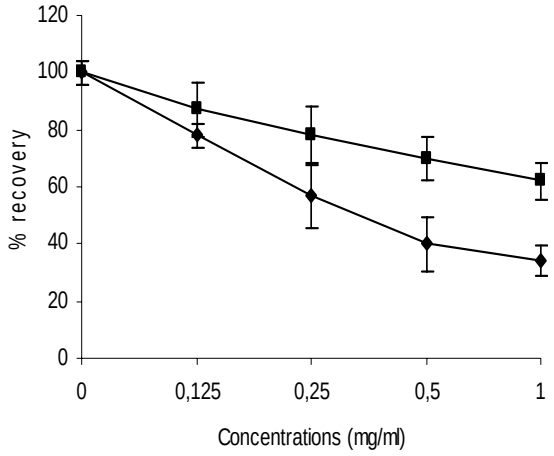

Figure 1: Percent recovery of crude extract treated *B. stearothermophilus* (♦) and *B. cereus* (■) spores on trypticase soy agar. The bar in the graph is the standard deviation.

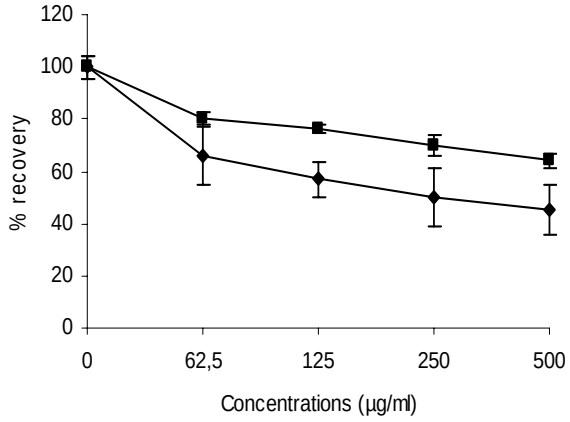

Figure 2: Percent recovery of ethyl acetate fraction treated *B. stearothermophilus* (♦) and *B. cereus* (■) spores on trypticase soy agar. The bar in the graph is the standard deviation

The germination and outgrowth patterns of samples of the same spores used in the previous experiment were followed by recording the changes in optical density of the medium. The untreated (control) spores appeared to germinate and outgrow normally as judged by the spectrophotometer readings (Fig 3 and 4). Germination process of untreated spores in this system ended after 30 min of incubation where as 45 and 35 min were required for *B. stearothermophilus* and *B. cereus* M-L treated spores respectively. Germination process of E-L treated spores ended after 45 min of incubation.

We assessed what amount of E-L added directly to the culture at 0 h would allow spores to germinate normally. *B. cereus* and *B. stearothermophilus* treated spores germs in 30 min after incubation when the final E-L concentrations were less or equal to 40 µg/ml and 20 µg/ml respectively. The germination process for each spore strains was delayed at E-L concentrations higher than those above. Since 80 and 40 µg of E-L per ml are the minimal effective concentrations in germination of *B. cereus* and *B. stearothermophilus* respectively.

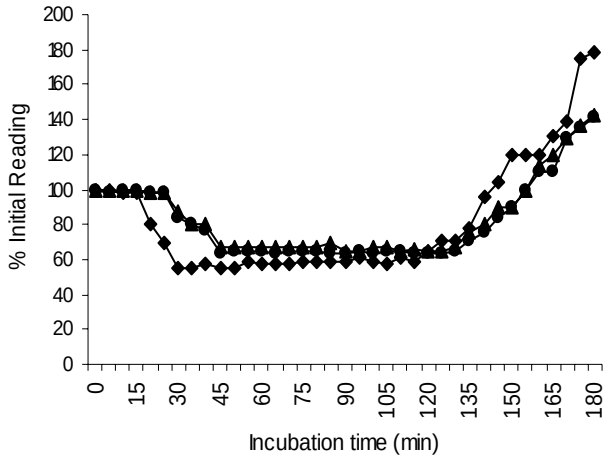

Figure 3: Germination and outgrowth expressed as percentage of initial reading as function of time, of *B. stearothermophilus* spores. Symbols: (♦), control (0 µg/ml); (●), ethyl acetate fraction (500 µg/ml); (▲), crude extract (1000 µg/ml)

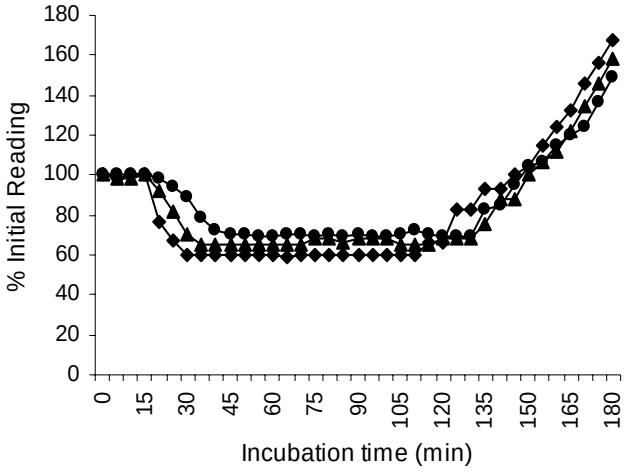

Figure 4: Germination and outgrowth expressed as percentage of initial reading as function of time, of *B. stearothermophilus* spores. Symbols: (♦), control (0 µg/ml); (●), ethyl acetate fraction (500 µg/ml); (▲), crude extract (1000 µg/ml)

Discussion and conclusion

The number of colony of each specie in trypticase soy agar using crude extract and ethyl acetate fraction decreased after 24 h of incubation by comparison with the untreated spores. This diminution was proportional to the crude extract and ethyl acetate fraction concentrations. Significant differences between the colony numbers from treated spores of *B. stearothermophilus* were obtained with all the doses used ($p<0.05$) in comparison with control. The ability to form visible colonies was the criterion used to measure survival in the above experiments. A liquid recovery system was used to follow the untreated, crude extract and ethyl acetate fraction treated spores through the various steps germination, outgrowth, emergence, elongation and symmetrical division of the spores to the many vegetative cells making up a colony. Dormant spores have a maximal absorbance, this absorbance decrease during germination. This event observed is the consequence of the refractivity diminution represented by the lag phases of the curves above. The results obtained indicate that crude extract and ethyl acetate fraction, delays germination process of *B. stearothermophilus* and *B. cereus*. The increase in turbidity of the medium during late outgrowth in all the cases, represent an increase in cellular mass due to the appearance and multiplication of vegetative cells. This was the consequence of the increase of optical density represented by the ascendant part of the curves (12; 5).

Spore suspensions were heat activated before their treatment. It was then possible that during outgrowth, active compound present in this extract prevented an aspect of spore recovery from heat. Since macromolecules synthesis operated during the outgrowth (11). We presumed that RNA and others macromolecules syntheses were the prerequisite for successful outgrowth into vegetative cells. If the germinated spores were unable to synthesis its RNA efficiently for any reason, it follows that fewer survivors would results. This effect can explain why fewer colonies were seen on agar plates of heated spores treated with these extracts.

Germination and outgrowth of *B. stearothermophilus* and *B. cereus* spores was found to be delayed by crude extract and ethyl acetate fraction from *A. montanus*. It is known that spores-forming bacteria are present in the soil, food and in the environment. These extracts may be useful to prevent the germination and outgrowth of the spore pathogens in the soil, in the food and in the environment.

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