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Evaluation of *Beauveria Bassiana* Spore Compatibility with Surfactants

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***Abstract*—**The spores of entomopathogenic fungi, *Beauveria bassiana* was evaluated for their compatibility with four surfactants; SDS (sodium dodyl sulphate) and CABS-65 (calcium alkyl benzene sulphonate), Tween 20 (polyethylene sorbitan monolaureate) and Tween 80 (polyoxyethylene sorbitan monoleate) at six different concentrations (0.1%, 0.5%, 1%, 2.5%, 5% and 10%). Incubated spores showed decrease in concentrations due to conversion of spores to hyphae. The maximum germination recorded in 72 h incubated spores varied with surfactant concentration at 49-68% (SDS), 39-53% (CABS), 78-92% (Tween 80) and 80-92% (Tween 20), while the optimal surfactant concentration for spore germination was found to be 2.5-5%. The surfactant effect on spores was more pronounced with SDS and CABS-65, where significant deterioration and loss in viability of the incubated spores was observed. The effect of Tween 20 and Tween 80 were comparatively less inhibiting. The results of the study would help in surfactant selection for *B. bassiana* emulsion preparation.

Keywords*—Beauveria bassiana*, spore, surfactant, compatibility, germination.

I. Introduction

ADVERSE effect of chemical insecticides and rising incidence of insecticide resistance among insects by traditional control through chemical insecticides has awakened the interest in entomopathogenic fungi as insect control agents [1, 2]. *Beauveria bassiana* is an entomopathogen which has extremely broad host range and is used as commercial biopesticide for the control of insects of agricultural, veterinary and medical significance [3-5]. Bioactivity of *B. bassiana* has been established against several pests at the lab level, while efforts are underway to simulate these results in practical scenario and in field condition. However, successful implementation of entomopathogenic activity shown at lab level to field scale necessitates the development of a suitable formulation.

Entomopathogenic fungi are usually applied in the form of spores, which need a stabilizing agent to facilitate application, stability and enhancement of activity [6]. Oil based emulsion formulations are superior spray carrier with increased probability of direct contact between fungal spores and host insects [7]. Surfactants used in the emulsion have been shown to enhance the production of enzymes such as cellulase, α-amylase and lignase [8]. Also, Surfactants have been shown to be activators of fungi [9, 8]. Further, the hydrophobicity of fungal spores renders the use of surfactants indispensable for laboratory bioassays and field trials [10]. However, inherent properties of some surfactants to induce amino acid leakage through the cell membrane are shown to have inhibiting effect on spore germination, which may ultimately leads to death of entomopathogenic fungi.

The selection of proper surfactant, therefore, is prerequisite before proceeding to emulsion preparation. In view of the above, present study has evaluated the compatibility of four surfactants; two anionic, SDS (sodium dodyl sulphate) and CABS 65 (calcium alkyl benzene sulphonate) and two non-ionic, Tween 20 (polyethylene sorbitan monolaureate) and Tween 80 (polyoxyethylene sorbitan monoleate) for the growth, viability, and germination kinetic of *B. bassiana* spores.

II. Materials and Methods

A. Materials

Surfactants; Tween 20 (polyethylene sorbitan monolaureate), Tween 80 (polyoxyethylene sorbitan monoleate), and SDS (sodium dodyl sulphate) and were purchased from Hi-Media India Ltd. Calcium alkyl benzene sulponate (CABS 65) was obtained from Standard Surfactant Ltd. (Kanpur, India).

B. Fungal Culture

Beauveria bassiana HQ917687 used in the present study was isolated in our laboratory from soil samples collected from Northern part of Uttar Pradesh, India. The fungal isolate was grown on Potato Dextrose Agar (PDA) slants and maintained at 4ºC.

C. Preparation of Spore Suspensions

For preparation of spore suspensions, spores were harvested from fresh slants cultured for 5 days, by adding 10 ml of 0.1% sterile Tween 80 to the culture slant. The slant was vortexed for 5 min and the resulting suspension was filtered through a sterilized 8 µm membrane filter disk. The filtered sporel suspension was enumerated for spore concentration and viability using an Automatic Cell Counter (Cellometer® Vision HSL, Nexcelom Bioscience). For determination of sporel viability, spores were stained with Trypan blue.

D. Culture Condition for Surfactant Evaluation

Two non-ionic surfactants Tween 20 and Tween 80, and

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two anionic surfactants, SDS and CABS-65 were chosen for evaluation of *B. bassiana* spore compatibility. Different concentrations (0.1%, 0.5%, 1%, 2.5%, 5% and 10%) of surfactants were taken in 50 ml of liquid Composite Media (CM) [containing (in g/l) Glucose 20, Peptone 3, MgSO4 0.3, KH2PO4 0.3 and NaCl 0.3] in 100 ml of Erlenmeyer flasks. A controlled medium containing no surfactant was also incorporated. All the flasks were inoculated with 1 ml of spore suspension (10^8 spore/ml) and incubated at 28 °C in a shaking incubator at 180 rpm. At a regular interval of 4, 8, 16, 24, 48 and 72 h, 3 ml of samples (inoculums) were taken aseptically from each flask and stored at 4 °C, until further use. Spore concentration of the samples was determined using Automatic Cell Counter (Cellometer® Vision HSL, Nexcelom Bioscience).

E. Evaluation of Spore Germination

Spore germination of *B. bassiana* spores from different surfactant treatments was studied to evaluate spore viability and germination kinetics. The inoculums (20 µl, 10^5 spore/ml) from stored samples were placed on 2 ml of Potato Dextrose Agar Yeast (PDAY) medium on glass slides (75 mm × 25 mm). Glass slides were observed microscopically (Leica microscope, DM2500) after 48 h at 40 X magnification to determine percentage of conidia germinated. The inoculums from 48 h inoculated samples, placed on glass slide, were observed periodically at regular interval of 4, 8, 16, 24 and 48 h, to resolve germination kinetics. Only those spores with germ tube longer than the spore diameter were considered germinated. A total of 100 spores were scored for each treatment in each of the trials. For each surfactant treatment, three replications were incorporated. The data on total numbers of germinated conidia in different treatments were analyzed by two-way analysis of variance (ANOVA) using StatPlus [11].

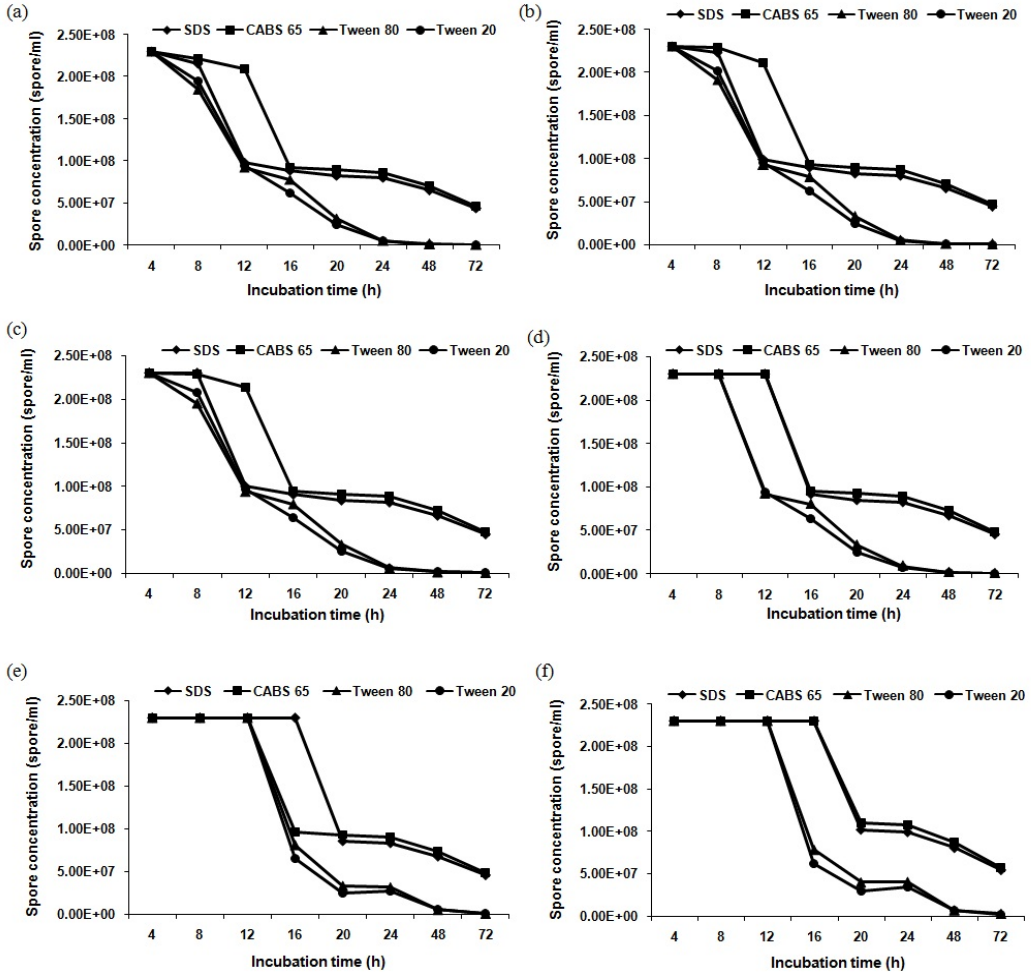

Fig. 1 Effect of incubation period on *B. bassiana* spore concentration at different surfactant concentrations, (a) 0.1%, (b) 0.5%, (c) 1%, (d) 2.5%, (e) 5%, and (f) 10%

III. Results

A. Spore Concentrations

B. bassiana spore concentrations in different surfactant treatments showed variable decrease with increase in incubation period (Fig. 1). The decrease in spore concentration was significant between different incubation periods ($p<0.001$), for all the surfactant concentration. The spore concentration reduction was most prominent in treatments containing Tween 80 and Tween 20. In comparison, decrease in spore concentration in CABS-65 and SDS containing systems were relatively less. The decline in spore concentration could be attributed to conversion of spores into hyphae, supported by media components. However, in case of SDS, its property to accumulate around spores could have prevented their germination into hyphae. Similarly, foam formation observed with CABS, could be attributed to have caused a stress on spores to reduce their germination into hyphae, resulting in final high spore concentration.

B. Spore Germination

B. bassiana spores incubated with different surfactants showed variable germination with incubation period and surfactant concentrations. Maximum percentage germination was recorded at 48h, beyond which monitoring became difficult due to the growth of fungus mycelia. Spores incubated with SDS showed significant variation with incubation period ($F=185.9$; $df=7, 35$; $p<0.001$); however, variation in spore germination at different concentration of SDS was non-significant ($p>0.05$). CABS incubated spores showed significant variation in germination with incubation period ($F=240.8$; $df=7, 35$; $p<0.001$) and surfactant concentration ($F=2.3$; $df=5, 35$; $p<0.05$). Significant variation in spore germination was observed with incubation time in Tween 80 ($F=45.1$; $df=7, 35$; $p<0.001$) and Tween 20 ($F=28.5$; $df=7, 35$; $p<0.001$). Also, spore germination showed significant variability with different concentration of Tween 80 ($F=4.9$; $df=5, 35$; $p<0.05$) and Tween 20 ($F=4.8$; $df=5, 35$; $p<0.05$). The maximum germination recorded in 72 h incubated spores varied with surfactant concentration at 49-68% (SDS), 39-53% (CABS 65), 78-92% (Tween 80) and 80-92% (Tween 20) of (Fig. 2). In the present study, *B. bassiana* spores inoculated with surfactants showed maximum germination when incubation period was less. This highlights the adverse effect of surfactants on spores with increase in time period of contact between spores and surfactants.

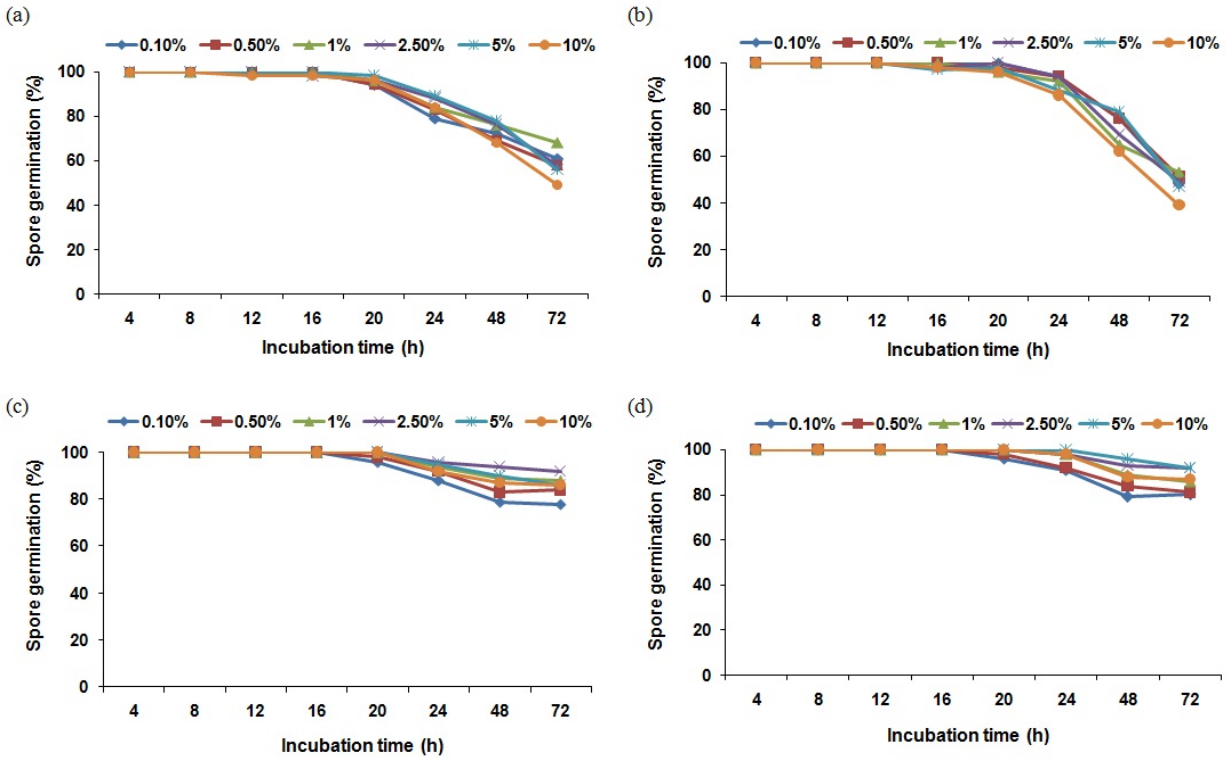

Fig. 2 Effect of incubation period on *B. bassiana* spore germination with different concentrations of surfactants, (a) SDS, (b) CABS 65, (c) Tween 80, (d) Tween 20

C. Spore Germination Kinetics

Germination kinetics of 48 h incubated *B. bassiana* spores was studied microscopically to deduce the pattern of spore germination. Germination kinetics was studied till 48 h, beyond which growth became substantial for germ tube count. Control showed 100% spore germination in 48h. Significant variation in spore germination was observed with variation in germination time with SDS ($F=337.4$; $df=4, 20$; $p<0.001$), while with surfactant concentration variation was non-significant ($p>0.05$). Spore germination with CABS 65 varied with its concentration ($F=5.8$; $df=5, 20$; $p<0.05$) and germination time ($F=624.7$; $df=4, 20$; $p<0.001$). Significant results were recorded for spore germination with variation in concentration of Tween 80 ($F=31.6$; $df=5, 20$; $p<0.001$) and Tween 20 ($F=20.6$; $df=5, 20$; $p<0.001$). Also, significant variability was observed in spore germination at different time of observations, with Tween 80 ($F=716.0$; $df=4, 20$; $p<0.001$) and Tween 20 ($F=394.5$; $df=4, 20$; $p<0.001$). The optimal surfactant concentration with respect to *B. bassiana* spore germination was found to be 5% for SDS, CABS and Tween 20; for Tween 80, it was observed to be at 2.5% of surfactant concentration.

IV. Discussion

The assessment of spores compatibility with surfactants is primary requirement in successful development of surfactant based formulation, viz. emulsion. Particularly, fungi with hydrophobic conidia render the use of surfactants indispensable for laboratory bioassays and field trials [10].The present study indicated slight to significantly negative effect of surfactants on *B. bassiana* spores viability. Further, negative effect on spores' germination was also observed. The effects were more pronounced with SDS and CABS-65, where significant deterioration and loss in viability of the incubated spores was observed. In comparison, Tween-20 and Tween-80 were shown to be better performing, with high number of spores showing germination.

The negative impact of surfactants to microorganism was probably due to increased cell permeability and amino acids leakage through inner membrane [12]. The above findings were supported by the study of Luz and Batagin [7] which reported relatively less toxic effect of non-ionic surfactants

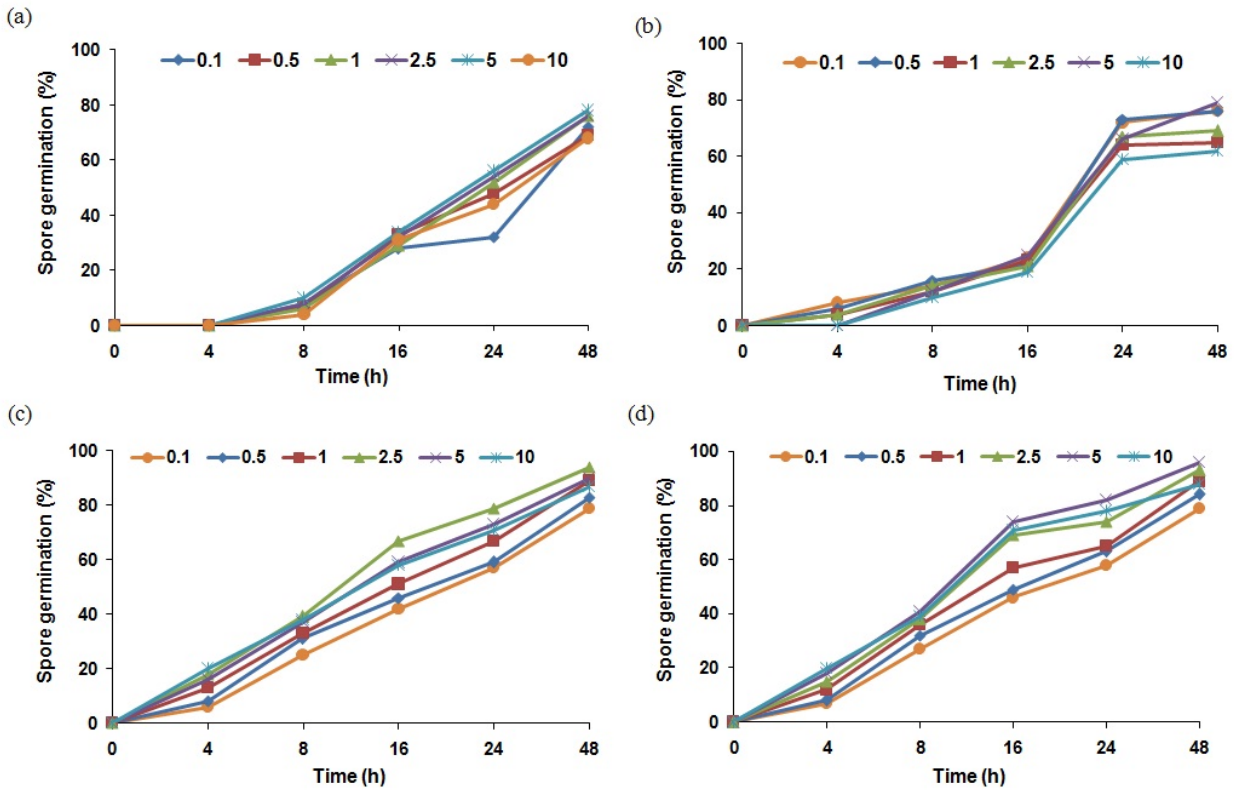

Fig. 3 Germination kinetics of *B. bassiana* spore with different concentrations of surfactants, (a) SDS, (b) CABS 65, (c) Tween 80, (d) Tween 20

(Tween-20 and Tween-80) than the ionic surfactants on the spores of *B. bassiana*. Similarly, Kollmann et al. [13] reported stimulation of spore production and germination with non-ionic surfactant in *Fusarium oxysporum*. Tween 20 is reported to enhance inoculum development by shortening the lag period during culture growth [8]. Also, the addition of Tween

20 and Tween 80 in the media has been shown to stimulate lipase production [14, 15]. However, in contrast to the present study, significant reduction in mycelia growth with surfactants, Tween 20 and Tween 80, was reported by Prasad [16] for phytopathogenic fungus *Chondrostereum purpureum*. Likewise, Silva et al. [15] reported SDS, alongwith Tween 80 to be the best surfactants for growth and germination of *M. anisopliae* spores. Tanuja et al. [12] reported complete inhibition of spore germination with SDS, while spore germination got delayed when inoculated with Tween 20. Detrimental effect of surfactants on the speed of germination was reported for *M. anisopliae* spores [15]. Polar [17] observed mild initial inhibitory effect of surfactants on spores' germination; however, the adverse effects were lessened with progression in time.

The above discussion indicates the variation in response of different fungal strains to various surfactants. The variation in spore viability and germination kinetics was suggested to be dependent upon surfactant toxicity along with nature of host cell membrane [18]. Overall this highlights the importance of surfactants selection to minimize their negative impact on spore germination and mycelial growth of particular fungal strain. The results of this study would help in selection of surfactant for emulsion formulation using *B. bassiana*. Also, the optimization of surfactant concentration results could be utilized for formulation design.

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