تأثير التربية المخبرية للفطر *Beauveria bassiana* في قدرته الإمراضية لحشرة السّونة

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الملخص

تعد حشرة السونة من آفات القمح الرئيسة في دول الشرق الأوسط والأدنى وشرق أوربا. يرتبط استخدام الممرضات للحدّ من انتشار الآفات بقدرة أجزائها التكاثرية، كالأبواغ الكونيدية، في إحداث العدوى بعد التطبيق الحقلي. من أهم مصاعب تطوير الفطور الممرضة للحشرات كالفطر *Beauveria bassiana*، انخفاض شراستها جراء التربية المخبرية. تمت تربية ثلاث عزلات من الفطر ممرض *B. bassiana* لثلاثين مرة على بيئة $SDAY_{1/4}$ ثم مررت بعد ذلك ضمن حشرات السونة. أظهرت النتائج زيادة شراسة العزلات المدروسة بعد التمرير الأول ضمن العائل مقارنة بتربيتها المستدامة 30 مرة على البيئة الغذائية. انخفضت قيمة الجرعة القاتلة النصفية بعد تمريرها ضمن الحشرة وبشكل واضح. وبشكل مشابه، انخفضت قيمة الزمن القاتل النصفي للعزلات المدروسة بعد تمريرها ضمن حشرة السونة مقارنة بتربيتها المتتالية على المستنبتات الغذائية. أظهرت النتائج أن شراسة العزلات وثباتها تتغير تبعاً للعزلة المدروسة، وبالتالي يجب مراقبة أي تغير في الشراسة عند التجهيز التجاري لعزلات الفطور الممرضة للحشرات.

كلمات مفتاحية: حشرة السونة، *Beauveria bassiana*، التربية المخبرية، الشراسة.

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Effect of in Vitro Culturing on the Virulence of *Beauveria bassiana* against Sunn Pest

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Abstract:

Sunn Pest, *Eurygaster integriceps* Puton, is a major pest of wheat in Eastern Europe and the Near and Middle East. The success of any mycoinsecticide depends on the virulence of the isolates that are sprayed. One of the hurdles in the development of entomopathogenic fungi such as *Beauveria bassiana* is the loss of virulence when successively maintained in vitro. Three Isolates of *B. bassiana* were sub-cultured 30 times on $SDAY_{1/4}$, and then passed through the Sunn pest insect. The three isolates tested became significantly more pathogenic to the pest after the first passage through the insects. Lowering of the LC_{50} values was obtained when the isolates were passed through the Sunn Pest. Similarly, the LT_{50} value decreased after passage of the sub-cultured isolates through the insect host. Results show that the virulence and stability of isolates can vary according to the fungal isolates; therefore, they should be monitored in a commercial production setting.

Keywords: Sunn Pest, *B. bassiana*, sub-culturing, virulence.

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1- Introduction:

Sunn Pest, *Eurygaster integriceps* Puton, is a major pest of wheat in Eastern Europe and the Near and Middle East [1]. The insect injects enzymes while feeding on the grains and the baking quality of bread made from the flour is poor and unfit for human consumption [2]. Control of Sunn Pest mostly relies on the use of chemical insecticides [3]. The continuous use of chemical insecticides has resulted in serious management problems such as resistance to the insecticides, pest resurgence, elimination of beneficial insects and toxicity to humans and wildlife [4]. Entomopathogenic fungi that parasitize insects are valuable weapons for biocontrol and play an important role in promoting IPM [5]. Fungal pathogenicity to a target insect pest varies largely among species and isolates [6, 7]. The success of any mycoinsecticide depends on the virulence of the infective conidia that are sprayed in the field and overwintering site. Stability in continuous in vitro cultivation is desirable for the purpose of large-scale production of fungal bio-pesticides. The maintenance of virulence is critical, as strains of entomopathogenic fungi including *Beauveria bassiana* (Balsamo) Vuillemin, tend to reduce the sporulation and virulence during the successive sub-culturing on artificial media [8, 9, 10]. Until now, the sole possibility of overcoming this phenomenon is with a periodical passage through the target host [11, 12]. Information about the reasons of why fungus becomes attenuated is little; however, it is apparent that strains differ in their stability when cultured on artificial media [13]. Stable strains conserve virulence for several generations, whereas unstable strains usually become attenuated after a few subcultures [9, 10]. Thus, the selection of a strain that is stable throughout the production period is axiomatic to the successful development of fungi as biopesticides. The extent of the effect of repeated in vitro sub-culturing on viability and morphological, biochemical, and molecular characteristics, on the virulence and host specificity, varies considerably within entomopathogenic fungus strain. The aim of this study was to investigate the change of the virulence of some *B. bassiana* isolates reared on artificial media after passing them through their host.

2- Methodology

2-1- Organism:

Four Isolates of *B. bassiana*, SPSR2, SP22, SPT566 and GHA

(the active fungal ingredient of commercially available BotaniGard, as compared treatment), were obtained from the ICARDA fungal culture collection maintained at plant protection research lab. Fungal isolates were sub-cultured 30 times on $SDAY_{1/4}$ (neopeptone 2.5 g/L, dextrose 10 g/L, agar 15 g/liter, and yeast 2.5 g/L; all Difco, Becton-Dickinson). For in vivo passage in the insect host, conidial suspension $1x10^7$ conidia ml^{-1} of the sub-culture *B. bassiana* isolates were prepared. Sunn pest adults were sprayed with conidial suspension, and were kept in clean plots containing wheat leaflet, and then incubated at 22 ± 2°C and 65 ± 5% rH. The conidia from cadavers were harvested and single-spore isolates were prepared from Sunn pest cadavers killed in the initial bioassay.

For each of the treatments, five culture plates were prepared by spreading 100 µl of 1×10^7 conidia ml^{-1} onto $SDAY_{1/4}$. Plates were held for 14 days at 22 ± 2°C and 65 ± 5% rH, to maximize spore production. Conidia were then harvested by flooding each plate with 10 ml^{-1} of sterile distilled water (SDW) containing 0.01% (v/v) Tween 80 (Sigma) and dislodging the conidia into suspension by stirring with a glass rod. All samples were vortexed 3 min to break up the conidial chains or clumps. Conidia were separated from hyphae and substrate materials by filtration of the suspension through two layers of cheese-cloth. The concentrations of fungal conidia in suspensions were determined using a haemocytometer.

2-2- Bioassay:

Sunn pest adults were collected from wheat, one day before treatment, and stored in plastic ventilated containers in a refrigerator at 5°C until being used.

Sunn Pest adults were treated with *B. bassiana* isolates at $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ conidia ml^{-1}, for the two treatments (30^{th} sub-culturing and after passing through the Sunn pest). Concentrations were prepared in SDW containing Tween 80 (0.01% v/v). Ten adults (5 ♂ and 5 ♀) per isolate and concentration were used. To secure the insects for topical application, individuals were placed on a strip of scotch tape with dorsal side down and 5µl of conidial suspension was applied/insect to the mesosternum. Control insects were treated with SDW containing Tween. After the application was dry (20 min), insects were transferred to wheat growing in pots (7 cm diameter and 8.5 cm height) surrounded by clear plastic cages and incubated at 22 ± 2°C and 65 ± 5% rH for 22 days. A split-plot design

with five replicates was used. Mortality was counted 4, 6, 8, 10, 12, until 22 days post application. Dead insects from each treatment were surface sterilized and kept separately in petri dishes containing sterile paper toweling moistened with 0.10% streptomycin sulfate and 0.02% penicillin G. Dishes were then incubated at 22 ± 2°C and 65 ± 5% rH for 2 weeks to observe fungal outgrowth [14].

2-3- Conidia germination assays

The germination of conidia obtained from the 30th subcultures and after passing through the Sunn Pest was assessed by inoculating 50 µl of conidial suspension (1×10^5 conidia ml^{-1}) on $SDAY_{1/4}$ plates. Plates were incubated for 10 h in the dark at 22 ± 2°C for each treatment. Germination was observed in three separate fields of view at ×40 magnification. One hundred conidia were observed randomly in each field of view and were considered to have been germinated when their germ tubes were equal to or greater than their width.

2-4- Statistical analysis

Cumulative mortality was corrected for natural mortality using Abbott's formula [15], and analyzed statistically using ANOVA. Means were separated using Fisher's Unprotected LSD at P= 0.05. The computations were done using GenStat Ed:12 [16]. Probit analysis was used to estimate LC_{50} of the isolates with 95% confidence limits (CL) and LT_{50} values using R program [17].

3- Results and Discussion

No significant differences (P > 0.05) were observed in conidia germination rates on $SDAY_{1/4}$ plates for 30th subcultures and after passing through Sunn Pest. The highest germination rates were from Isolates SPSR2 after passing through Sunn Pest and reached 98%, but with no significant differences with other isolates used in this study.

Corrected mortalities caused by the tested isolates are shown in table 1. The three isolates tested became significantly more pathogenic to the pest species from the first passage through the insects (F=2.62, P =0.001), causing 68–81% mortalities compared with 23-78% before passing through the host when 1×10^8 conidia ml^{-1} were used.

Under the same concentrated spray, the three isolates derived from the first passage through the insect killed more Sunn pest. However, significant mortality changes were not found among the SPSR2 isolate, which reflects the stability of these isolates under sub-culturing conditions, while significant mortality was observed when SPT566 isolate was sub-cultured for 30 times.

Table 1: Changes in corrected Sunn Pest mortality during the passages of three *Beaueria bassiana* isolates through the host insect species.

Day 18				
Treatment	Concentration			
	1×10^5	1×10^6	1×10^7	1×10^8
SPSR-2.B	13.22±1.83	14±4.08	44.26±7.45	78±6.63
SPSR.2.A	23.74±4.27	22.96±4.33	51.65±9.45	81.48±7.58
SPT-22.B	10±4.47	10±3.16	50.87±5.44	62.43±4.85
SPT-22.A	12.26±2.86	32.26±10.98	55.65±10.10	72.96±7.65
SPT-566.B	8±3.74	18.43±5.50	32.96±3.74	48.61±3.03
SPT-566.A	14±5.10	23.57±8.07	57.74±6.86	67.48±7.98
GHA	22±8.60	28.96±5.09	36.96±3.75	45.3±5.62
	d.f.	f	F pr.	l.s.d.
Treat	6	3.97	0.001	8.78
Concentration	3	93.35	<.001	6.64
Treat.Concen	18	2.62	0.001	17.56
Total	139			

B. 30th sub-culturing isolates. A after passing throughout Sunn pest.

The passage of *B. bassiana* through insect's host resulted in lowering the LC_{50} values from 1.76×10^7 to 6.04×10^6 conidia ml^{-1} when SPSR2 isolate was used. The highest differences were recorded when the SPT566 was sub-cultured or passed through Sunn Pest, and the LC_{50} was decreased from 7.27×10^7 to 1.12×10^7, which reflected the decrease in the isolate's virulence when it was sub-cultured for 30 times in the artificial media.

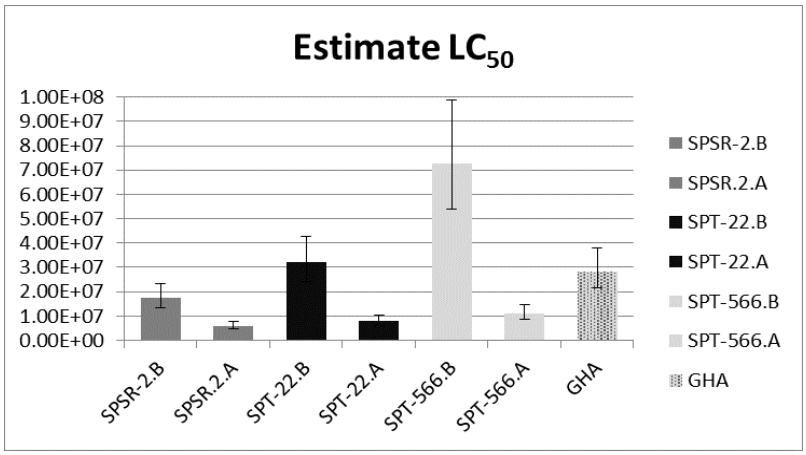

Figure 1: Changes in median lethal concentration (LC_{50}) of three *Beaueria bassiana*. B, 30th sub-cultured isolates. A, after passing throughout Sunn Pest.

Similarly, the LT_{50} value was decreased after passage of the sub-cultured isolates through the insect host. Decrease of the LT_{50} value varied according to fungal isolates. The lowest LT_{50} value was

observed from isolate SPSR2, while the highest LT_{50} value was from sub-cultured isolates SPT566. The most virulent conidia were from isolate SPSR2, which had LT_{50} values ranging from 10.52 to 8.85 days at the concentration of 1×10^{8} conidia ml^{-1}

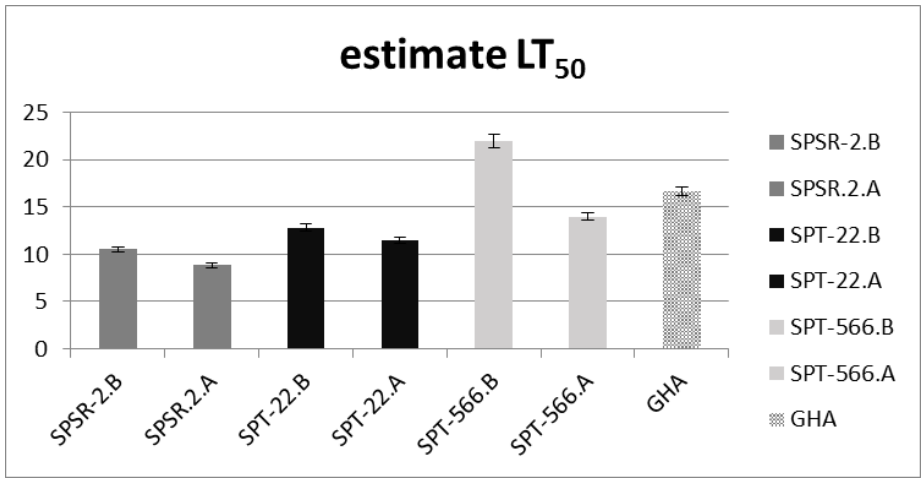

Figure 2: Changes in median lethal time (LT_{50}) of three *Beaueria bassiana* isolates. B, 30th sub-cultured isolates. A, after passing throughout Sunn Pest.

Discussion

The present study demonstrates that successive subcultures of *B. bassiana* on solid media differ significantly in their virulence. The pattern of change was strain dependent. In this study, *B.bassiana* isolates were declined in virulence after 30 successive in vitro subcultures. Safavi [18] demonstrated that three isolates of *B. bassiana* tested for their stability after 15 subcultures showed attenuation of virulence. Similarly, 20-40 subcultures caused *M. anisopliae* to decline in virulence against *Helicoverpa armigera* Hubner (Lepidoptera; Noctuidae) [19]. A decline in virulence after nine subcultures was accompanied by rapid changes in the surface properties of conidia of *M. anisopliae*-infecting *T. molitor* [20]. In contrast, other researchers did not observe a decline in virulence afte 15 and 30 successive subcultures on nutrient-rich media for *Beauveria bassiana* (Balsamo), *Isaria farinosa* Holmskiold (=*Paecilomyces farinosus*) and *Isaria fumosoroseus* Wize (=Paecilomyces fumosoroseus) [21, 22; 23, 24]. The LT_{50} values were in accordance with mortality data, as they gradually increased among subcultures of isolates, which was supported by Safavi's [18] results. Butt et al. [9] stated that isolates of entomopathogenic fungi differ in their stability when

maintained on artificial media with some isolates clearly degenerating more rapidly than others, which was supported by our results.

It is still unclear exactly which characteristics of the fungi are changed when a decline in virulence occurs and a passing through a host can often restore virulence. Mechanisms behind this phenomenon are still unclear; Butt et al. [9] suggested inheritance of altered characteristics involving several genetic mechanisms such as DNA methylation and transposon activity.

Finally, our results show that the virulence and stability of isolates can vary according to the fungal isolates; therefore, they should be monitored in a commercial production setting. Stable isolates retain virulence for several generations, whereas unstable ones usually become attenuated after a few subcultures [9]. However, determination of isolate stability virulence through successive sub-culturing may provide an alternative for the selection of best strain for commercial purposes.

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