

NOTE

Effects of *in Vitro* Passage of *Beauveria bassiana* on Virulence to *Bemisia argentifolii*

Effects of repeated *in vitro* subculture on the virulence and morphological characteristics of entomopathogenic fungi appear to vary considerably among isolates and species. Some reports indicate a loss of pathogenicity in isolates of *Beauveria bassiana*, *Metarhizium anisopliae*, *Nomuraea rileyi*, *Paecilomyces farinosus*, and *Verticillium lecanii* (Kawakami, 1960; Schaerffenberg, 1964; Nagaich, 1973; Fargues and Robert, 1983; Morrow *et al.*, 1989), whereas other isolates apparently retain their virulence after repeated transfer (Ferron *et al.*, 1972; Hall, 1980; Hayden *et al.*, 1992).

Potential loss of virulence is highly relevant to the maintenance of strains in general and in the production of strains for comparative bioassays. It is particularly critical for commercialized strains that undergo several routine conidium-to-conidium cycles prior to large-scale fermentation. The potential for virulence loss has obvious implications for the quality of product resulting from a particular fermentation batch; maintenance of virulence throughout the mass-production process is essential to ensure the consistent quality of the marketed material. The objective of the current study, therefore, was to assess effects of repeated *in vitro* passage on the virulence of the *B. bassiana* GHA strain, the active ingredient in the commercial products BotaniGard and Mycotrol (Mycotech Corp., Butte, MT).

Vidal *et al.* (1997) and Fargues *et al.* (1997) initially passed fungal isolates through insect hosts in an attempt to ensure that full insecticidal activity was restored to each of the test strains prior to bioassay. In this way, differences detected in their relative pathogenicity for the target pests could be attributed to real interstrain variation rather than a function of prior culture conditions. The *B. bassiana* GHA strain was originally isolated from a chrysomelid beetle and deposited as ARSEF201; it was subsequently passed through grasshoppers in 1990 and later (1995) through *Bemisia* nymphs (S.T. Jaronski, unpublished data). The preliminary culture used in the current study was derived from a single spore isolate of the *Bemisia*-passaged GHA strain (S.T. Jaronski, unpublished data); to ensure the virulence of the initial culture used in the trials, the fungus was first passed through

larval *Galleria mellonella* L. with conidia isolated from a sporulating cadaver. Thereafter, the fungus was sequentially subcultured by mixed conidial transfer to full-strength Sabouraud dextrose agar supplemented with 0.1% yeast extract (SDY). Each passage was incubated in the dark at 28°C for 2 weeks before the next subculture. Conidia were harvested from the 1st, 5th, 10th, and 15th subcultures by physically scraping the spores from the surface of 21-day-old cultures. Conidia were dried at 25–28°C over moisture-indicating calcium sulfate (Drierite; W.A. Hammond, Drierite Co., Ltd., Xenia, OH) for 4–5 days. The moisture content of the resulting powders was between 5 and 9% and they were stored at –20°C until assay. Viability was assessed immediately after drying and prior to each assay by microscopic examination of conidia on SDY agar after 16 h incubation at 28°C. A conidium was considered germinated if a hyphal peg was visible. Observed viabilities were >95%. There appeared to be no qualitative differences in the growth morphology or conidiation characteristics of the different passages. Spore yield following each passage was approximately the same at the time of harvest.

The fungi were assayed against neonate and 2-day-old (pre-molt) *Bemisia argentifolii* (“whitefly”) nymphs infesting bean leaves (var. Royal Burgundy). To prepare leaves for assay, terminal leaves were excised from 3- to 4-week-old bean plants and placed in Oasis rooting cubes (Smithers-Oasis U.S.A., Kent, OH) to maintain leaf turgor and delay senescence for the duration of the assay. Three to five days later the leaves, which had developed roots, were infested with 12 pairs of adult whiteflies. These were removed after 24 h to promote homogeneity in the age structure of the developing nymphal population. Test populations ranged from 13 to 166 (average 71) nymphs per leaf. Two dose rates, 4.0×10^7 and 2.5×10^6 viable conidia/ml of diluent (hereafter called “high” and “low,” respectively) were used in the assays. Two concentrations were used to enhance the possibility of detecting small changes in virulence that could be missed if only a single dose was used. Conidial treatments were prepared in 0.02% (v/v) Silwet L-77 (Loveland Industries, Inc., Greeley, CO) and 2 ml of suspension applied to each leaf, individually, via a Potter spray tower; control leaves were

TABLE 1

Mean Percentages of Mortality (\pm SE) 6, 10, and 14 Days Posttreatment for Whitefly Nymphs Exposed to *Beauveria bassiana* as Neonates or Just Prior to Their First Molt (Means Calculated from Three Replicate Experiments)

Treatment ^a	Spore dose ^b	Mean % mortality (\pm SE)					
		Day 6		Day 10		Day 14	
		Neonate	Premolt	Neonate	Premolt	Neonate	Premolt
Control	No spores	2.0 (0.7)	5.4 (0.9)	4.4 (0.6)	7.9 (1.2)	5.8 (1.6)	13.6 (2.5)
Passage 01	L	4.3 (1.3)	14.2 (1.5)	12.4 (1.1)	24.3 (1.7)	17.5 (2.2)	35.2 (2.8)
	H	9.1 (2.1)	41.2 (6.1)	41.5 (4.2)	71.7 (4.6)	61.8 (4.6)	91.2 (2.4)
Passage 05	L	5.6 (1.0)	14.6 (2.1)	15.6 (2.1)	29.1 (2.1)	21.6 (3.0)	45.7 (2.8)
	H	10.8 (1.8)	41.8 (5.5)	45.5 (3.6)	74.7 (5.3)	66.6 (5.5)	92.5 (2.5)
Passage 10	L	3.4 (0.6)	10.7 (1.1)	15.0 (2.2)	23.8 (2.4)	18.7 (2.2)	36.4 (3.0)
	H	20.7 (2.7)	37.3 (6.4)	52.2 (5.0)	69.8 (5.0)	76.4 (2.9)	87.6 (2.6)
Passage 15	L	5.4 (1.0)	11.3 (2.4)	15.3 (2.4)	25.3 (3.6)	26.4 (1.7)	41.9 (5.2)
	H	11.9 (1.6)	34.2 (6.3)	54.7 (4.6)	74.6 (4.1)	70.5 (6.4)	90.5 (3.0)

^a *B. bassiana* was cultured *in vitro* and passages 1, 5, 10, and 15 were assayed.

^b Spore concentrations of L = 2.5×10^6 and H = 4.0×10^7 viable spores/ml of 0.02% Silwet were used.

sprayed with 2 ml of 0.02% Silwet only. This spray volume ensured that the leaves were evenly coated with fine droplets but there was no runoff. Leaves were allowed to air dry and then transferred to vented plastic deli containers (two 2.5-cm-diameter mesh-covered holes in the side of 16-oz containers), one leaf per container, which had an internal relative humidity of approximately 85%. The assay containers were held at $25 \pm 2^\circ\text{C}$ under a 16:8 L:D regimen. The total number of dead whiteflies (nymphs desiccated and/or discolored and infected individuals) and infected whiteflies (as defined by symptomatic larvae—pink/red coloration, fungal outgrowth or conidiation on the cadavers) were concurrently recorded 6, 10, and 14 days after spraying. Each experimental assay was replicated three times, and in each assay four replicate leaves were sprayed with the respective fungal or control treatment.

For statistical analysis, data were corrected for control mortality for the corresponding assay and then subjected to arcsine transformation (Zar, 1974) to improve the homogeneity of variances. The data were analyzed as a randomized complete block design using the general linear model of analysis of variance (SAS, 1996). The significance ($\alpha = 0.05$) of treatment effects and interactions was initially evaluated with type III sums of squares and the mean square error. However, the persistent occurrence of a significant interaction between fungal concentration and the age of the whitefly nymphs at the time of assay, i.e., neonate or 2 days old, for both mortality and infection prompted a reevaluation of each assay separately as it suggested differences in the relative susceptibility of the two test populations. The error terms used in the second analysis were the interaction of the variable being evaluated

with the replication of the experiment. The analysis was weighted for the total number of insects in a replication.

No significant differences were detected between mortality and infection rates for fungi passaged 1, 5, 10, or 15 times at either test concentration (Tables 1 and 2). Infection and mortality rates were significantly greater at the higher test concentration on all observation days. With one exception, percentages of mortality and infection were not significantly influenced by interactions between the concentration and the number of passages. The exception ($P = 0.02$) occurred with the percentage of infection of 2-day-old nymphs on day 14 and was not of obvious consequence. Interestingly, 2-day-old nymphs appeared to be significantly more susceptible than neonates and this probably contributed to the significant interaction between assay and concentration. Reasons for this observation cannot be defined from the current series of assays. Wraight *et al.* (1998) had similar findings but Fransen *et al.* (1987) showed that neonate *Trialeurodes vaporariorum* were more sensitive to *Aschersonia aleyrodis* than older nymphs. Differences between the test fungi and whitefly species used may account for this disparity. The higher mortality among older nymphs may have been due to a variety of factors, including the fact that the insects may have just molted to the second instar at the time of treatment or simply that the older larvae provided a larger target for the spray droplets, thereby increasing the likelihood of their acquiring a lethal dose of conidia.

Our observations of no loss in virulence contrast with those of Aizawa (1971) and Morrow *et al.* (1989), where a decline in pathogen virulence was detected after passaging *N. rileyi* and *B. bassiana* 10 and 16 times,

TABLE 2

Mean Percentages of Infection (\pm SE) 6, 10, and 14 Days Posttreatment in Whitefly Nymphs Exposed to *B. bassiana* as Neonates or Just Prior to Their First Molt (Means Calculated from Three Replicate Experiments)

Treatment ^a	Spore dose ^b	Mean % infection (\pm SE)					
		Day 6		Day 10		Day 14	
		Neonate	Premolt	Neonate	Premolt	Neonate	Premolt
Control	No spores	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Passage 01	L	1.5 (0.5)	4.5 (1.5)	6.0 (1.4)	12.7 (2.1)	9.8 (2.3)	24.0 (3.1)
	H	14.4 (2.7)	32.3 (5.8)	26.3 (4.5)	68.8 (4.6)	39.9 (6.3)	87.6 (3.6)
Passage 05	L	1.6 (0.5)	6.7 (2.0)	8.3 (1.4)	15.9 (2.6)	13.1 (3.2)	33.9 (3.1)
	H	4.6 (1.0)	33.6 (6.3)	27.9 (4.8)	71.2 (6.5)	45.3 (7.4)	89.8 (3.3)
Passage 10	L	0.9 (0.5)	2.6 (0.8)	7.1 (1.6)	11.6 (2.4)	9.7 (1.8)	19.3 (2.8)
	H	14.4 (2.7)	29.1 (7.8)	38.9 (6.5)	58.8 (7.4)	64.0 (4.6)	74.3 (4.8)
Passage 15	L	1.8 (0.6)	5.8 (2.5)	8.5 (2.0)	17.9 (4.3)	12.8 (2.7)	31.5 (6.2)
	H	5.9 (0.9)	25.9 (6.9)	33.6 (4.8)	62.2 (7.0)	56.3 (7.6)	76.2 (6.8)

Note. Only nymphs showing clear symptoms of infection were used to calculate these values as opposed to total percentages of mortality, i.e., death from all causes, which are presented in Table 1.

^a *B. bassiana* was cultured *in vitro* and passages 1, 5, 10, and 15 were assayed.

^b Spore concentrations of L = 2.5×10^6 and H = 4.0×10^7 viable spores/ml of 0.02% Silwet were used.

respectively, on artificial growth media. Other workers, however, have reported no loss of virulence for isolates of *V. lecanii*, *P. farinosus*, *N. rileyi*, and *B. tenella* (Ferron *et al.*, 1972; Hall, 1980; Ignoffo *et al.*, 1982; Hayden *et al.*, 1992). These apparent contradictions serve to highlight the considerable inter- and intraspecies variation that exists in the effects of repeated subculturing on virulence and the genetic stability of this trait. The variability in published reports may be due to the different methodologies used in the studies, e.g., single spore vs multispore transfers, use of enriched culture media, etc., especially because events leading to attenuation may be random (mutation) or due to culture conditions.

Further quantitative studies would be useful to document effects of additional *in vitro* passages beyond 15 of GHA as well as other isolates of *B. bassiana*. The effects of repeated culture on artificial media and the influence of culture conditions (medium, temperature, etc.) on growth, conidiation, and spore viability are also poorly defined for most entomopathogenic fungi and need to be addressed. The results of the current study strongly indicate that the genetic factors controlling pathogenicity in the *B. bassiana* GHA strain are stable enough to facilitate limited repeated transfer and culture on artificial media, such as during the mass-production process, without affecting the virulence of the isolate. This stability is another favorable characteristic of strain GHA, in addition to its ability to grow and sporulate profusely on solid substrate, which has enhanced its commercial development.

Key Words: *Beauveria bassiana* GHA strain; *Bemisia argentifolii*; *in vitro* passaging, virulence.

REFERENCES

- Aizawa, K. 1971. Strain improvement and preservation of virulence. In "Microbial Control of Insects and Mites" (H. D. Burges and N. W. Hussey, Eds.), pp. 666-668. Academic Press, New York.
- Fargues, J. F., and Robert, P. H. 1983. Effects of passaging through scarabaeid hosts on virulence and host specificity of two strains of the entomopathogenic hyphomycete *Metarhizium anisopliae*. *Can. J. Microbiol.* **29**, 576-583.
- Fargues, J. F., Ouedraogo, A., Goettel, M. S., and Lomer, C. J. 1997. Effects of temperature, humidity and inoculation method on susceptibility of *Schistocerca gregaria* to *Metarhizium flavoviride*. *Biocontrol Sci. Technol.* **7**, 345-356.
- Ferron, P., Deotte, A., and Marshal, M. 1972. Stabilité de la virulence d'une souche de *Beauveria tenella* (Decacr.) Siemaszko [Fungi Imperfecti] pour les larves du coleoptere, *Melolontha melolontha*. *L. C. R. Acad. Sci. Paris* **275**, 2977-2979.
- Fransen, J. J., Winkelmen, K., and van Leteren, J. C. 1987. The differential mortality at various life stages of the greenhouse whitefly, *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae), by infection with the fungus *Aschersonia aleyrodis* (Deuteromycotina: Coelomycetes). *J. Invertebr. Pathol.* **50**, 158-165.
- Hall, R. A. 1980. Effect of repeated subculturing on agar and passaging through an insect host on pathogenicity, morphology, and growth rate of *Verticillium lecanii*. *J. Invertebr. Pathol.* **36**, 216-222.
- Hayden, T. P., Bidochka, M. J., and Khachatourians, G. C. 1992. Entomopathogenicity of several fungi toward the English grain aphid (Homoptera: Aphididae) and enhancement of virulence with host passage of *Paecilomyces farinosus*. *J. Econ. Entomol.* **85**, 58-64.
- Ignoffo, C. M., McIntosh, A. H., Garcia, C., Kroha, M., and Johnson, J. M. 1982. Effects of successive *in vitro* and *in vivo* passages on the virulence of the entomopathogenic fungus, *Nomuraea rileyi*. *Entomophaga* **27**, 371-378.

- Kawakami, K. 1960. On the changes of characteristics of the silk-worm muscardines through successive cultures. *Bull. Sericul. Exp. Stn. Jpn.* **16**, 83–99.
- Morrow, B. J., Boucias, D. G., and Heath, M. A. 1989. Loss of virulence in an isolate of an entomopathogenic fungus, *Nomuraea rileyi*, after serial *in vitro* passage. *J. Econ. Entomol.* **82**, 404–407.
- Nagaich, B. B. 1973. *Verticillium* species pathogenic on aphids. *Indian J. Phytopathol.* **26**, 163–165.
- SAS Software, Release 6.12. 1996. SAS Institute Inc., Cary, NC.
- Schaerffenberg, B. 1964. Biological and environmental conditions for the development of mycoses caused by *Beauveria* and *Metarhizium*. *J. Insect Pathol.* **6**, 8–20.
- Vidal, C., Lacey, L. A., and Fargues, J. 1997. Pathogenicity of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay method. *J. Econ. Entomol.* **90**, 765–772.
- Wraight, S. P., Carruthers, R. I., Bradley, C. A., Jaronski, S. T., Lacey, L. A., Wood, P., and Galaini-Wraight, S. 1998. Pathogenicity of the entomopathogenic fungi *Paecilomyces* spp. and *Beauveria bassiana* against the Silverleaf whitefly, *Bemisia argentifolii*. *J. Invertebr. Pathol.* **71**, 217–226.
- Zar, J. H. 1974. "Biostatistical Analysis." Prentice Hall, Englewood Cliffs, NJ.

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