JARONSKI, S. T.[1], C. FULLER[1,2], B. LARSON[3] and B. JACOBSEN[4], [1]USDA-ARS, Agricultural Research Service, Northern Plains Agricultural Research Laboratory, Sidney, MT 59270, [2(current address)]University of Cincinnati, Department of Molecular Genetics, Cincinnati, OH 45201, [3]Montanta State University Extension Service, Richland County Office, Sidney, MT 59270 and [4]Montana State University, Department of Plant Sciences and Plant Pathology, Bozeman, MT 59717. **Effect of three bacterial disease-control agents on the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*.**

ABSTRACT

USDA-ARS is developing a biocontrol strategy for the sugarbeet root maggot, *Tetanops myopaeformis*, using entomopathogenic fungi (EPF) such as *Beauveria bassiana* and *Metarhizium anisopliae*. These fungi have been under study as candidate microbial agents of the sugarbeet root maggot. Concurrently, Montana State University has been developing three bacteria to manage some of the sugarbeet pathogens: *Bacillus pumilis* LS201 (for use against *Pythium* species, *Rhizoctonia solani*, and *Aphanomyces cochlioides*); *B. mojavensis* MSU127 (against *Rhizoctonia solani* Root and Crown Rot); and *B. mycoides* BmJ (against *Cercospora beticola* Leaf Spot). The rhizosphere is the primary arena for EPF deployed against soil-dwelling pests as well as the site of action of many biological agents used against root pathogens. Moreover, the soil has the potential for contamination by microbial agents applied to the plant canopy through runoff and rain washoff. Interactions between EPF and soil microbes, especially biofungicidal agents, have rarely been studied, however. The micro-organisms may interfere with EPF by inhibiting conidial germination or mycelial growth in the rhizosphere. We therefore examined the in vitro effect of the three *Bacillus* spp. on three strains each of *B. bassiana* and *M. anisopliae*, on four different agar media, as a preliminary to in situ studies.

Because we recognized that fungi may exhibit within-species variability in their response to bacteria, we chose three strains of each fungus to obtain a small degree of comparative data (Table 1). *Metarhizium* Strain MA1200, originally isolated from soybean cyst nematode in Illinois, has been extensively studied by USDA for sugarbeet root maggot control, while strain F52, registered for other uses in the U.S., has recently emerged as the lead candidate in this regard. *Metarhizium* Strain TM109, originally isolated in Norway, is being studied there for the control of cabbage and turnip root maggots and is moderately efficacious against the root maggot. Strains TM28 and TM86 of *B. bassiana*, isolated from sugarbeet field soil in the Sidney MT area, were early candidates as microbial pest control agents of the sugarbeet root maggot; Strain GHA was originally registered against a wide variety of insects in the 1990s and is commercially available in the U.S., Mexico, Japan, and certain EU countries. All fungal cultures were derived from single conidium isolations. The fungal strains were obtained for our experiments from agar media slants stored at -80° C. (*B. bassiana*) or 3-4° C. (*M. anisopliae*). Upon recovery from storage, the fungi were routinely cultured on quarter-strength Sabouraud dextrose agar + 0.1% yeast extract (.25SDAY). The three bacteria were cultured on tryptic soy agar (TSA).

Two media favoring bacterial growth -- TSA and plate count agar (PCA) – and two media favoring fungal growth -- potato dextrose agar (PDA) and .25SDAY – were used. The three bacteria were grown at 27° C. for 18 h in shake flask cultures of tryptic soy broth, at 200 rpm, for use in our experiments. For additional tests we also cultured the bacteria for 60 hours to obtain plateau phase populations and ample opportunity for metabolite production. To test the effect of bacteria on conidial germination, we first created a monolayer of an aqueous suspension of fungal conidia on each of 4 replicate plates for each treatment. For challenge, as soon as the inocula had dried, we placed two 6-mm filter paper disks off-center on top of the fungus-inoculated agar surface. One disk was inoculated with 5 µL of bacterial culture and the other with sterile water, after which the plates were incubated in the dark, at 27° C., for 48 hours. The diameter of any zone of inhibition around the disk was then measured along two perpendicular axes across each disk using a digital vernier caliper. To test the effect of the bacteria on hyphal growth of the fungi, replicate fungus-inoculated plates were first incubated for 24 h at 27° C. to initiate conidial germination, after which bacteria were added as described. The diameter measurements were converted into a mean width of the inhibitory zone around each disk. The entire set of interactions was conducted twice. The experiment was repeated as described above, using 60-hr cultures of the bacteria to determine if plateau-phase populations registered different effects with two replications of the entire assay.

There were considerable differences between fungal species and within each species in sensitivity to the bacteria, as evidenced by the width of the inhibition zone around the bacterial disk (Figures 1A and 1B). In general, MSU127 showed the strongest, most consistent inhibition of conidial germination, with a mean width of the inhibition zone as great as 16 mm (for *B. bassiana* Strain GHA on PDA). In most cases the MSU127-induced zone of inhibition resisted fungal regrowth for at least several months. The medium affected the message, however. Depending upon the medium used and the strain of fungus, we observed very different inhibitory patterns, probably reflecting nutritional effects of the media on both fungi and bacteria. For example, the effect of BmJ on *M. anisopliae* F52 germination, as indicated by the zones of inhibition (Figure 1B), was greater on PCA than on TSA, and negligible on PDA and .25SDAY. For *M. anisopliae* MA1200, the effect of MSU127 on PCA was significantly less than on the other three media; but for the other two *M. anisopliae* strains the medium used showed little effect. In terms of bacterial effects on hyphal growth, only *B. bassiana* GHA was adversely affected, by BmJ and LS201 but not MSU127, and only on TSA, with large zones of inhibition (data not shown).

In a number of instances the inhibitory power of the bacteria harvested from older (60-hr) cultures was significantly different (t test, $p = .05$) from 18-hr cultures, but the differences were inconsistent in the direction of effect (Figure 2). The 60-hr cultures of MSU127 on all four media had significantly narrower zones of inhibition than their 18-hr counterparts when pitted against germinating conidia of *M. anisopliae* F52. The other *M. anisopliae* isolates were unaffected. The ability of MSU127 to inhibit MA1200 greatly decreased in the older cultures. Of the three *B. bassiana* strains only GHA showed a differential response to the 60-h cultures; neither TM28 nor TM86 showed a significantly different response to any 60-h bacterial culture. With BmJ, grown for 60 h, The zone of inhibition was significantly larger in 60-h than in 18-h BmJ cultures on TSA and PCA.

MSU127 on TSA exhibited the only other significant difference in response to culture age; the 60-h cultures registered a significantly smaller zone of inhibition with *B. bassiana* GHA.

Very few studies of the interactions between EPF and other soil microbes have been undertaken, fewer still between EPF and bacteria and fungi under development for biocontrol of plant pathogens. Our observations highlight the within-species variability of the sensitivity of two important EPF to three mycoantagonistic bacteria, the mediating effects of the test medium, and the effect of bacterial culture age. The real-world significance of our in vitro observations should be taken with circumspection, however. Antagonistic interactions between microorganisms may not translate from the in vitro environment to the natural soil and rhizosphere arena. Furthermore, in actual use BmJ would have little contact with the fungi; the bacterium would be applied to the sugarbeet foliage, while the fungi would be in the soil, the conidia having been applied either at planting or a month before the first use of the bacterium. MSU127 would be applied as a crown spray, also somewhat limiting exposure. Only LS201 has the potential for close contact with the fungal conidia in normal use; it would be applied as a seed coat with subsequent rhizosphere colonization, while the fungi would be applied in furrow as conidial sprays or as granules.

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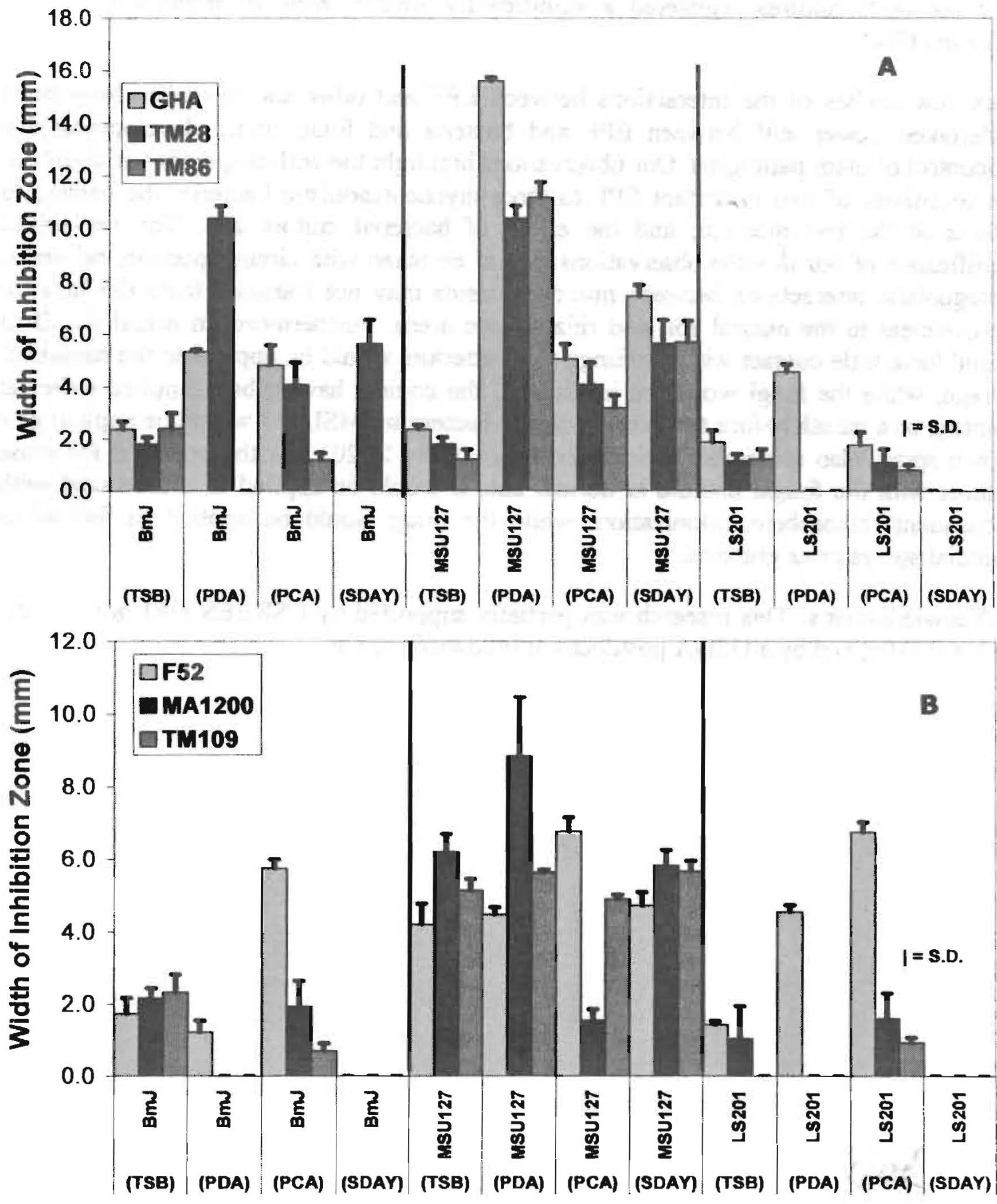

Figure 1. Effect of three mycoantagonistic bacteria, *Bacillus pumilis* LS201, *B. mojavensis* MSU127, and *B. mycoides* BmJ, on conidial germination of (A) three *Beauveria bassiana* strains (GHA, TM28, and TM86) and (B) three *Metarhizium anisopliae* strains (F2, MA1200, TM109). The interactions were examined on four different media: Tryptic soy (TSA), potato dextrose (PDA), plate count agar (PCA), and quarter-strength Sabouraud dextrose yeast extract agar (SDAY). Error bars represent the standard deviation for each interaction .

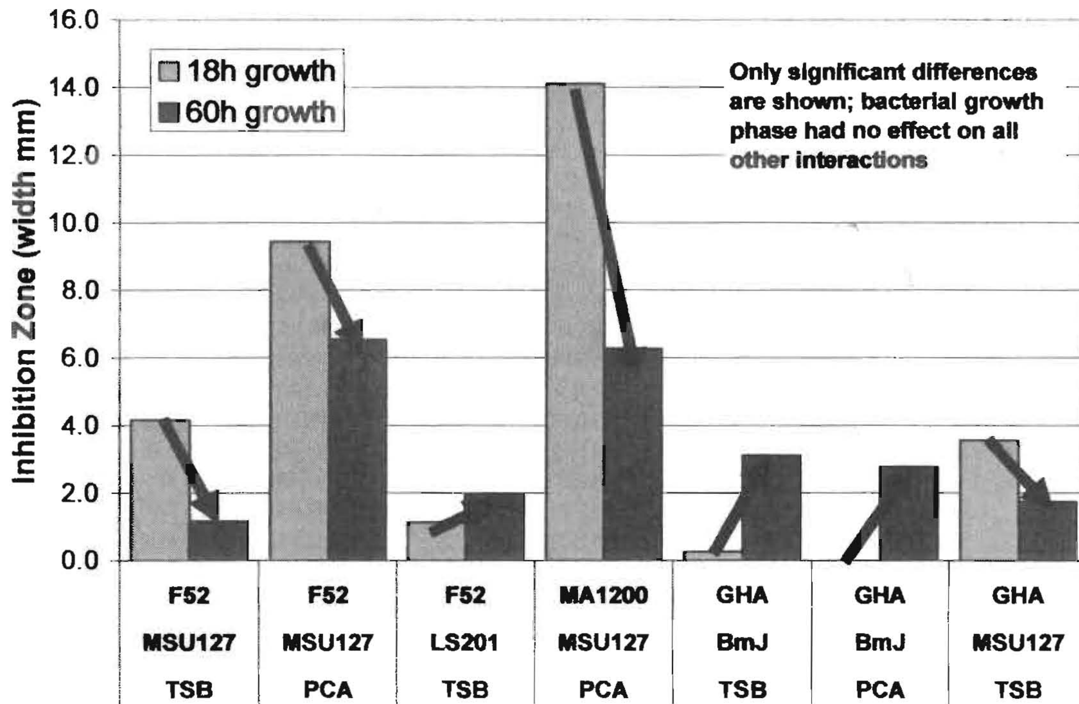

Figure 2. Effect of bacterial growth phase on fungal germination. Only the statistically significant interactions (t-test, p = 0.5) are shown. Column labels are fungal strain, bacterium, and medium, resp. See Figure 1 for explanation of abbreviations.

Table 1. Test microorganisms

Species	Strain	Source
Entomopathogenic fungi		
Beauveria bassiana	GHA	Laverlam International, Butte MT
B. bassiana	TM28	USDA ARS Sidney MT (deposited as ARSEF* 6937)
B. bassiana	TM86	USDA ARS Sidney MT (deposited as ARSEF 6948)
Metarhizium anisopliae	MA1200	ATCC92061, reisolated from sugarbeet root maggot
M. anisopliae	F52	Novozymes Biologicals, Salem VA
M. anisopliae	TM109	ARSEF 5520
Biological control bacteria		
Bacillus pumilis	LS201	Montana State University
Bacillus mojavensis	MSU127	Montana State University
Bacillus mycoides	BmJ	Montana State University

* ARS Entomopathogenic Fungus Collection, USDA ARS, Ithaca NY.