Pathogenicity of two new isolates of *Metarhizium anisopliae* from Canadian soil to *Melanoplus bivittatus* (Orthoptera: Acrididae) and *Tenebrio molitor* (Coleoptera: Tenebrionidae)

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Abstract—Worldwide biological-control research has shown that the fungal entomopathogen *Metarhizium anisopliae* (Metschnikoff) is an alternative to chemical insecticides for controlling grasshoppers and locusts. The pathogenicity of two recently discovered isolates of *M. anisopliae* var. *anisopliae* Driver and Milner from Canadian soil to the key grasshopper pest *Melanoplus bivittatus* (Say) and the yellow mealworm, *Tenebrio molitor* L., was determined by means of laboratory bioassays. Insects were fed a single dose of 10^5 conidia suspended in sunflower oil on food (a standard-size lettuce wafer). Subsequent feeding activity, movement, and mortality were assessed daily. The isolates were equally pathogenic, and similar in pathogenicity to the industry standard, Green Guard (*M. anisopliae* var. *acridum* Driver and Milner). Treatment with the three isolates resulted in 50% grasshopper mortality in 5–6 days and 90% mortality in 6–7 days.

Résumé—La recherche en lutte biologique à l'échelle globale a démontré que le champignon entomopathogène *Metarhizium anisopliae* (Metschnikoff) représente une solution de rechange aux insecticides chimiques pour le contrôle des criquets et des locustes. La pathogénicité de deux isolats de *M. anisopliae* var. *anisopliae* Driver et Milner découverts récemment dans du sol canadien a été déterminée dans des essais de laboratoire sur l'important criquet ravageur *Melanoplus bivittatus* (Say) et l'adulte du ver de farine jaune *Tenebrio molitor* L. Les insectes ont reçu une seule dose de 10^5 conidies suspendues dans de l'huile de tournesol sur de la nourriture (une pastille de laitue de taille standard). Les jours suivants, leur activité alimentaire, leurs déplacements et leur mortalité ont été notés. Les deux isolats sont également pathogènes et leur pathogénicité est semblable à celle de la norme industrielle de Green Guard (*M. anisopliae* var. *acridum* Driver and Milner). Un traitement avec les trois isolats cause une mortalité des criquets de 50 % en 5–6 jours et de 90 % en 6–7 jours.

[Traduit par la Rédaction]

Introduction

Management of insect pests, such as grasshoppers (Orthoptera: Acrididae), that present risk to crops in Canada continues to rely on chemical insecticides, primarily pyrethroids, carbamates, and organophosphates. Growers and managers who prefer integrated control, with less dependence on chemical insecticides, need alternatives. In addition, crop protection in organic agriculture systems and control of pests in protected areas (*e.g.*, wildlife conservation projects) may depend on nonchemical methods of control. Given that grasshopper outbreaks may be exacerbated by warm, dry spring and summer weather, future outbreaks are likely to be longer or more frequent if the recent warming trend continues (Sauchyn and Kulshreshtha 2007).

Research on biological control of grasshoppers is focused on natural enemies; the current best candidates appear to be fungal

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entomopathogens (Lomer *et al.* 2001), and successes include the development of isolates of *Metarhizium anisopliae* (Metschnikoff). Green Muscle (Lomer *et al.* 2001) and Green Guard (Long and Hunter 2005) (strains of *M. anisopliae* var. *acridum* Driver and Milner) are used to control locusts in Africa, Australia, and China. Species of *Metarhizium* are generally considered safe for the environment, human health, and nontarget species (Zimmermann 2007), including birds (Smits *et al.* 1999; Johnson *et al.* 2002), reptiles (Peveling and Demba 2003), aquatic organisms (Milner *et al.* 2002), and arthropods not targeted for control (Peveling and Demba 1997; Danfa and van der Valk 1999; Peveling *et al.* 1999; Arthurs *et al.* 2003). Other potential biocontrol agents such as *Verticillium lecanii* (Zimmerman) and *Beauveria bassiana* (Balsamo) Vuillemin are also environmentally safe, but in Canadian field tests did not significantly reduce populations of grasshoppers (Johnson *et al*. 1988; Inglis *et al.* 1997). Currently, no biological agents are registered in Canada for grasshopper control; however, at least one other indigenous isolate of *M. anisopliae* var. *anisopliae* Driver and Milner recently discovered in Canadian Prairie soil (Entz *et al.* 2008) is a potential candidate for use in integrated pest management systems.

This study reports the pathogenicity of two previously untested native Canadian isolates (Alberta 11S-1 and Alberta 6W-2) of *M. anisopliae* var. *anisopliae* against the two-striped grasshopper, *Melanoplus bivittatus* (Say) (Orthoptera: Acrididae), and the yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). We chose *T. molitor* as a nontarget model because tenebrionid habitat in Canadian grassland includes areas likely to be sprayed with *Metarhizium anisopliae* during grasshopper outbreaks. Generally omnivorous, tenebrionid beetles are significant decomposers in arid and semi-arid ecosystems and, in some cases, act as natural enemies by preying on grasshopper egg pods (Danfa and van der Valk 1999). Therefore, the effects of *M. anisopliae* on *T. molitor* may offer an indication of potential environmental impacts.

Materials and methods

Insect collection and maintenance

We collected adult *M. bivittatus* from grass borders of unsprayed agricultural pastureland in southern Alberta, Canada, and maintained them in oviposition cages. The resulting egg pods were allowed to mature and then stored at 5 °C for at least 60 days to allow diapause. F_1 hatchlings were allowed to develop to the 4th instar in the cages and then used in the laboratory bioassays. Prior to bioassay use, the nymphs were maintained on a lettuce diet in cages with a 16L:8D photoperiod. *Tenebrio molitor* adults and larvae (derived from a colony maintained at the Lethbridge Research Centre (Agriculture and Agri-Food Canada) by D.J. from 1990 to 2004) were reared in groups of 400–800 on a wheat bran diet in eight 4 L plastic containers. Larvae were used in the laboratory bioassays when they were nearly mature (approximately the 9th instar).

Spore formulations

Spores (conidia) of each *M. anisopliae* isolate (Alberta 11S-1, Alberta 6W-2, Green Guard) were obtained from colonies grown on selective medium (35 g oatmeal agar, 5 g agar, 0.9 g Cyprex (dodeine), 5 g cystal violet, 0.4 g penicillin, and 1.0 g streptomycin in 1.0 L of deionized water; Chase *et al.* 1986). Spores were resuspended in sunflower oil and diluted 1000-fold. To estimate the concentration of the diluted suspension, the conidia were counted using a microscope and a hemocytometer. The original suspension was further diluted to achieve a concentration of 5×10^7 conidia mL^{-1} sunflower oil.

Bioassays

The bioassays consisted of two separate experiments (randomized complete block design). Each ''block'' consisted of challenges with Alberta 11S-1, Alberta 6W-2, Green Guard, and a no-treatment control to three classes of insect subjects: *M. bivittatus*, *T. molitor* larvae, and *T. molitor* adults. Each treatment and control group started with 10 insects per block. *Melanoplus bivittatus* nymphs, *T. molitor* adults, and *T. molitor* larvae were selected, weighed, and placed in

Fig. 1. Total cumulative mortalities, from two bioassays ($n = 20$), of *Melanoplus bivittatus* challenged with one dose of 10^5 conidia from each of three isolates of *Metarhizium anisopliae*.

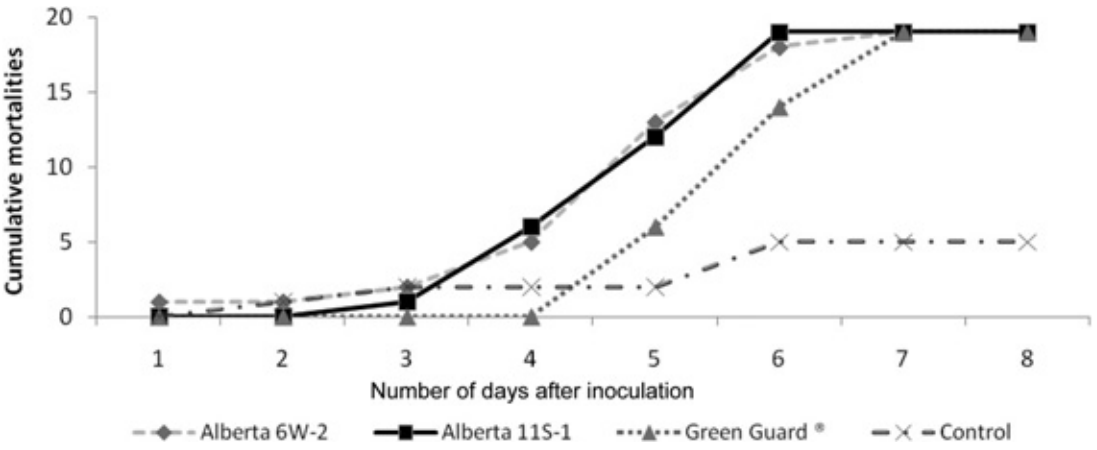


Table 1. Adjusted (Abbott 1925) cumulative percent mortalities (total for two bioassays) of *Melanoplus bivittatus* exposed to three isolates of *Metarhizium anisopliae*.

	Number of days after inoculation			
Treatment	5	6	7	8
Alberta 6W-2	60.9	87.5	93.8	93.8
Alberta 11S-1	55.1	93.8	93.8	93.8
Green Guard	23.6	66.7	100	100

Table 2. Adjusted (Abbott 1925) cumulative percent mortalities (total for two bioassays) of *Tenebrio molitor* larvae exposed to three isolates of *Metarhizium anisopliae*.

	Number of days after inoculation			
Treatment	5	6	7	8
Alberta 6W-2	−5.6	10.0	11.1	16.7
Alberta 11S-1	10.6	10.6	11.1	16.7
Green Guard	−5.6	−0.6	5.6	5.6

glass vials. Treatment insects were each challenged with one dose of 10^5 *M. anisopliae* conidia from one of the fungal isolates suspended in 2 μL of sunflower oil on a lettuce wafer. Control insects were each confined with a lettuce wafer and 2 μL of sunflower oil only. All insects were incubated at 24 °C with a 16L:8D photoperiod. On day 1, inoculum consumption, activity level, and molting activity of all insects were assessed prior to transferring them to larger plastic containers containing wheat bran as feed. On all subsequent days, activity level, molting activity, and feeding activity of each insect were evaluated. Insect cadavers were removed daily, placed in petri dishes on filter paper moistened with 350 μL of sterile water, and then monitored for 14 days for fungal growth and sporulation. Each petri dish was sealed with laboratory film and placed in a dark environment.

Table 3. Adjusted (Abbott 1925) cumulative percent mortalities (total for two bioassays) of *Tenebrio molitor* adults exposed to three isolates of *Metarhizium anisopliae*.

	Number of days after inoculation			
Treatment	5	6	7	8
Alberta 6W-2	−0.6	−5.6	14.3	33.3
Alberta 11S-1	20.6	25.4	35.7	40.5
Green Guard	10.0	19.8	7.1	9.5

Results and discussion

The two new Canadian isolates and Green Guard caused greater than 90% mortality of *M. bivittatus* nymphs by day 7 (Table 1) when adjusted against control mortality. Survival

Fig. 2. Total cumulative mortalities, from two bioassays ($n = 20$), of *Tenebrio molitor* larvae and adults challenged with one dose of 10^5 conidia from each of three isolates of *Metarhizium anisopliae*.

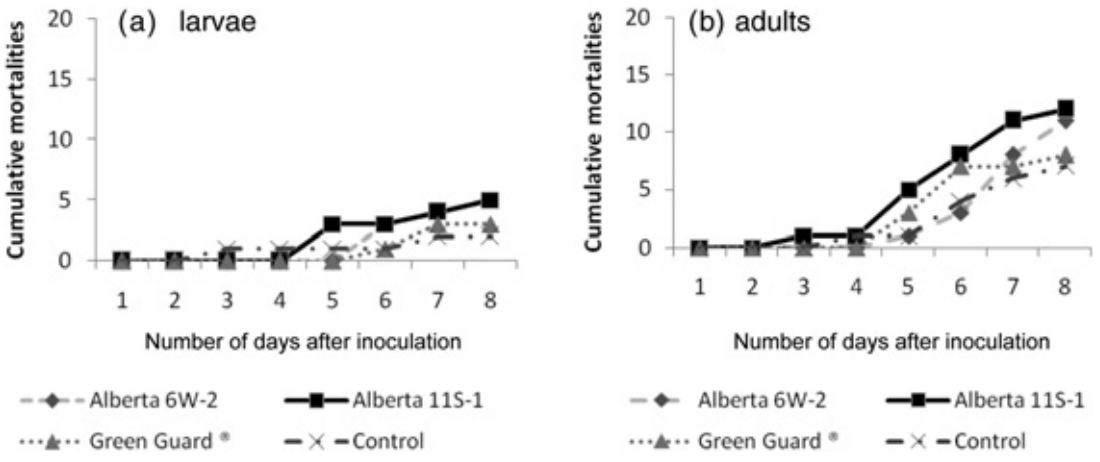
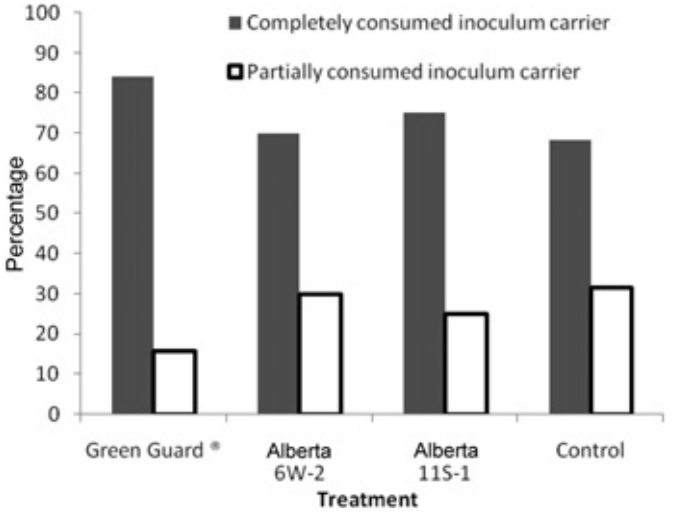


Fig. 3. Percentages of *Melanoplus bivittatus* nymphs that completely or partially consumed inoculum carrier, averaged for two bioassays with three isolates of *Metarhizium anisopliae*.



rates (proportion remaining alive) of grasshoppers treated with the three isolates were not statistically different (based on either ANOVA or Cox regression). Analysis of the number of mortalities by date showed no significant differences among isolates (Fig. 1). ANOVA of the number of mortalities on day 5 indicated a significant treatment effect ($P < 0.01$) and a significant difference between the control group and the treatment groups but no significant differences among the three isolates (Tukey's HSD, $\alpha = 0.05$) or between the replicate runs of the experiment.

The Canadian isolates and Green Guard were less pathogenic to *T. molitor*. The average adjusted mortality of *T. molitor* larvae (average of two bioassays) peaked at about 17% for Alberta 11S-1 and Alberta 6W-2 and 5.5% for Green Guard (Table 2); the average adjusted mortality of the adults (average of

Fig. 4. Percentages of *Tenebrio molitor* larvae that completely consumed, partially consumed, or did not consume inoculum carrier, averaged for two bioassays with three isolates of *Metarhizium anisopliae*.

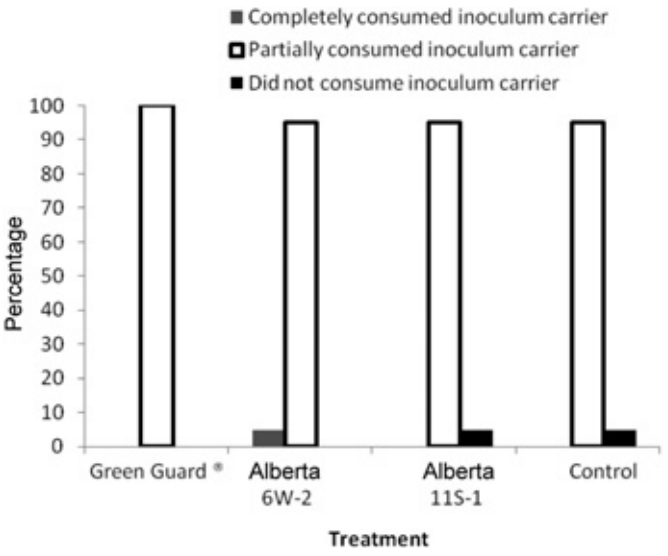
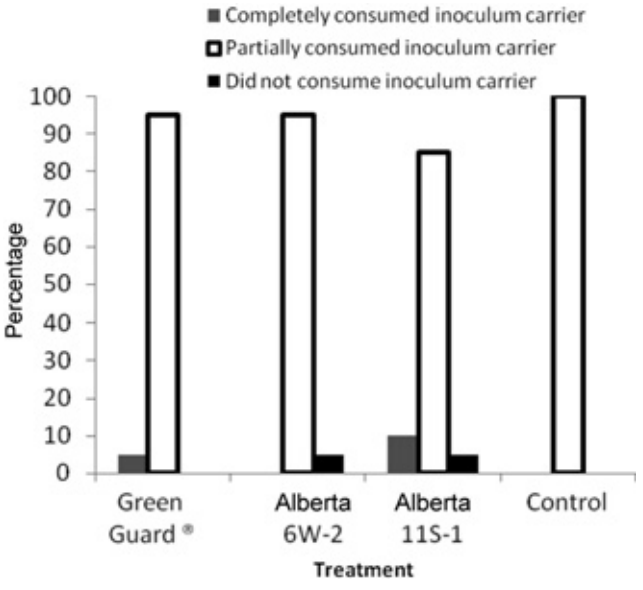


Fig. 5. Percentages of *Tenebrio molitor* adults that completely consumed, partially consumed, or did not consume inoculum carrier, averaged for two bioassays with three isolates of *Metarhizium anisopliae*.



both bioassays) peaked at approximately 40% for Alberta 11S-1, 33% for Alberta 6W-2, and 20% for Green Guard (Table 3).

All grasshoppers and 93% of *T. molitor* larvae and adults consumed, at least partially, the inoculum (Figs. 3, 4 & 5). Subsequent activity levels of individually confined grasshoppers and adult *T. molitor*, including feeding rates and movement response to the stimulus, did not vary significantly among the treated groups. The pupation rate for *T. molitor* larvae appeared to have been

somewhat reduced by the pathogen treatments, but a larger experiment is required to confirm, and precisely estimate the degree of, this effect. Overall, by day 4 after treatment, 20% of *T. molitor* controls (no pathogen) had pupated, compared with 10% of the treated groups (Fig. 2).

These experiments show that the two recently discovered, previously untested isolates (Alberta 11S-1 and Alberta 6W-2) of *M. anisopliae* var. *anisopliae* from Canadian soil appear to be capable of causing a high degree of mortality in pest grasshoppers within 7 days of treatment, and could serve as candidate control agents. *Tenebrio molitor* is a cosmopolitan insect species that is a post-harvest pest in some situations because it may attack stored grain. We consider it a model for nontarget darkling (tenebrionid) beetles whose typical habitats in Canadian grasslands include areas likely to be sprayed with *M. anisopliae* during grasshopper outbreaks. As such, we consider the lower impact of these newly discovered isolates on *T. molitor*, relative to the target pest, *M. bivittatus*, to be an advantage because it may indicate a degree of specificity that would support the environmental sustainability of these new isolates as grasshopper control agents.

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