NOTE

Surface Disinfection Technique for *Plectris aliena* Grubs (Coleoptera: Scarabaeidae) Using Sodium Hypochlorite[1]

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J. Agric. Urban Entomol. 29: 10–15 (2013)

KEY WORDS white grubs, external disinfection, surface disinfection, *Plectris aliena*

Plectris aliena Chapin (Coleoptera: Scarabaeidae) has recently become established in North Carolina as a serious pest of sweetpotato, *Ipomoea batatas* (L.) Lam. (Convolvulaceae). Attempts to develop a laboratory colony of this insect for research studies were hampered by mortality of field-collected grubs brought into the laboratory. Approximately 20% of several hundred third-instar *P. aliena* grubs collected from fields in Columbus County, North Carolina, in February and March, 2009 and 2010, subsequently died in the laboratory due to pathogen infections. The exoskeleton of many of the dead grubs exhibited signs of patent infection, leading to the production of green fungal spores (asexual conidia). Samples of infected grubs were sent to the USDA-ARS, Biological Integrated Pest Management Research Unit, Robert W. Holley Center for Agriculture and Health, Ithaca, New York, where a positive identification of the fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae), was confirmed. Apparent bacterial infections were also observed in *P. aliena*, but these pathogen(s) were not identified.

Healthy, pathogen-free insects are required for laboratory research and rearing. Field-collected insects can carry a variety of microbial agents, including fungal spores on their body surfaces. Collected insects also can contact pathogens in the collection soil and become infected in the laboratory. Stressful conditions, such as handling during collection, storage, and transport, could cause grubs to be more susceptible to infection. In the present study, *P. aliena* grubs brought into the laboratory had been exposed to entomopathogens and harbored infectious conidia or carried a latent infection (Lomer et al. 2001). Because of the high rate of pathogen infection in *P. aliena* in the laboratory, a disinfection technique is needed for grubs collected from the field that are to be used to establish colonies or for research purposes.

[1]Accepted for publication 28 March 2013.
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Generic guidelines for surface disinfection of insects are provided by Lacey & Brooks (1997), but no specific disinfection methods are published for immature Scarabaeidae. Disinfection techniques using EtOH (ethanol), NaOCl (sodium hypochlorite), H_2O_2 (hydrogen peroxide), and CH_3CO_3H (peracetic acid) were evaluated for adult lesser mealworm beetles, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), in studies examining environmental transfer of bacteria by the beetles (Crippen & Sheffield 2006). Lesser mealworms were successfully disinfected with a combination of EtOH, H_2O_2, and CH_3CO_3H; however, NaOCl disinfection resulted in insect death. Leppla et al. (1974) and Connell (1981) successfully disinfected eggs of cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), using NaOCl, and the eggs of the shore fly, *Scatella picea* (Walker) (Diptera: Ephydridae), using benzalkonium chloride. Vail et al. (1968) surface disinfected the eggs of *T. ni* using a solution of formalin and NaOCl to prevent infection with a nuclear polyhedrosis virus. No disease developed on larvae reared from eggs washed with both agents, while 98% of the control group became infected with the virus disease. Sodium hypochlorite prevented the viability and infectivity of bacterial and fungal infections on *T. ni* larvae (Ignoffo & Dutky 1963).

The objective of this study was to develop a nonlethal surface disinfection technique for overwintering third instars of the white grub *P. aliena.*

Materials and Methods

Insect collection. Third-instar *P. aliena* grubs from the overwintering generation were collected on 31 March 2010 and 9 April 2011 (n = 95 grubs) from agricultural fields in Columbus County, North Carolina that had been planted to sweetpotato the previous year. A tractor-drawn, one-row disk plow was used to expose grubs by creating a furrow in the soil 25–31 cm deep. Grubs were collected by hand and placed in 19-liter buckets (10 grubs maximum per bucket) with field soil (7% soil moisture, sandy loam) and a layer of sod from the field. Buckets were transported to the Vegetable Entomology Laboratory, North Carolina State University, Raleigh, NC, where larvae remained in the buckets on the laboratory bench (23°C) for 12 (2010) and 29 (2011) days prior to experimentation.

Disinfection protocols. Grubs were removed from the field-collection buckets and placed individually into 59-ml plastic cups with non-sterilized, field-collected soil. Only grubs that had no visible signs of infection and could move normally (i.e., begin to immediately burrow when placed on the soil surface) were used in the experiments. Disinfection protocols were conducted in a Class II biological safety cabinet that was sterilized with UV light prior to use; all equipment was also exposed to UV light prior to use. A 6% solution of NaOCl (The Clorox Company, Oakland, CA) was diluted to working concentrations of 1% and 2% with sterile H_2O. Immediately before all treatments, grubs were individually submersed in 50 ml of 95% EtOH in 10-cm diameter Petri dishes for ten seconds to break surface tension and allow the disinfection agent improved access to the insect's exoskeleton, which has numerous setae and invaginations that can harbor microorganisms (Crippen & Sheffield 2006).

Treatment solutions were prepared in 10-cm Petri dishes with 50 ml of solution in each dish. The four experimental treatments were: (1) 1% NaOCl for 2 min (n = 5 grubs), (2) 1% NaOCl for 3 min (n = 21 grubs), (3) 2% NaOCl for

2 min (n = 21 grubs), and (4) 2% NaOCl for 3 min (n = 5 grubs). Concentrations of NaOCl and submersion times were selected based on the results of unpublished preliminary studies with *P. aliena*. Grubs were completely submersed in each treatment concentration. After each treatment, grubs were rinsed by completely submersing them for five seconds in each of three separate 10-cm Petri dishes with 50 ml of sterile H_2O. Three control groups were established as follows: (1) 95% EtOH with three autoclaved water rinses (to control for effects of an EtOH rinse alone on surface disinfection; n = 14 grubs), (2) three autoclaved water rinses (to control for effects of water alone on surface disinfection; n = 12 grubs), and (3) no immersion or rinse (to control for effects of rinses on grub behavior; n = 17 grubs).

Separate Petri dishes were used for each grub in each replication of every treatment. After treatment, grubs (n = 95) were placed individually into a sealed 59-ml plastic cup (Dart Container Corporation, Mason, MI) with 40 g of autoclaved soil collected from the same agricultural fields where the grubs were found. Cups with soil and grubs were placed one layer deep on 30.5 × 40.6 cm cafeteria trays on a laboratory bench (24°C) and covered with an inverted tray.

Microbial isolation. The sterile H_2O from the last (third) rinse Petri dish was streaked onto Trypticase Soy Broth (TSB) media (Difco 1984) in 10-cm Petri dishes to determine presence of microbes after surface sterilization treatments.

To confirm microbial presence on non-disinfected larvae, 14 field-collected grubs taken directly from unsterilized field soil were placed separately on TSB plates and allowed to move about freely for approximately two minutes and then removed. All inoculated plates were covered and sealed with Parafilm® (Pechiney Plastic Packaging Company, Chicago, IL), transferred to an incubator, maintained in darkness at 30°C for 2–3 days, and then examined for the presence of microbial colonies. Plates were rated (+) if microbial colonies were present or (−) if microbial colonies were absent.

Effect of disinfection on behavior. Grub burrowing behavior was used to estimate the effect of surface disinfection treatments on insect behavior. Methods used to evaluate grub burrowing behavior in this study were similar to those described by George et al. (2007) and Vernon et al. (2008). Three days after disinfection, the burrowing behavior of each grub was recorded for all treatments by removing the lids from the plastic cups, emptying the soil and grub, putting the same soil back in the cup, and replacing the grub on the top of the soil. Grubs that were alive and had completely buried themselves under the surface of the soil, or were actively burrowing at the time of observation, and had whole-body movement (head, mouthparts, legs, abdomen, etc.) without stimulus, were considered to have "normal" behavior. Live grubs that remained on the soil surface and either made writhing motions of body parts without moving and/or burrowing in the soil, or showed no movement of body parts, were considered moribund.

Statistical analysis. Each grub was considered a replicate (n = 85 grubs for all statistical analyses), and years were combined for analyses. Effects of surface disinfection on behavior were analyzed using SAS software procedure GLIMMIX (SAS Institute 2010). A logistic regression of "active burrowing" (Y = 1) or not "actively burrowing" (Y = 0) as the dependent variable and treatments as independent factors was fitted. Behavioral data were subjected to analysis of variance (ANOVA) to determine whether there were significant differences between treatment means (SAS Institute 2010). Only grubs from treatments

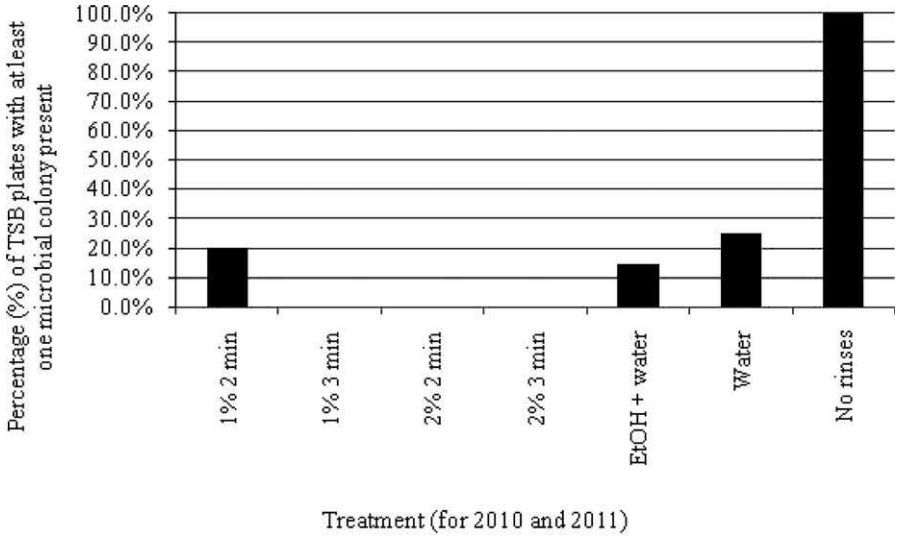

Fig. 1. Percentage of microbial plates with at least one microbial colony present for all treatments in 2010 and 2011. The treatment, "No rinses" includes grubs that were not rinsed but eventually placed in sterile soil (n = 17) to observe effects on behavior, and the control cohort (n = 14) that confirmed the microbial presence on non-disinfected, field-collected larvae.

where no microbial growth was observed on TSB plates and control group grubs were included in the analysis of the behavioral assay.

Results

The formation of colony forming units (CFU) on growth medium is the standard indicator of microbial growth. The only microbial CFU's observed on the TSB plates from surface disinfection treatments were from treatment 1 (1% NaOCl concentration and 2-min grub immersion time). Three microbial colonies were present in the streak marks of the rinse water. No microbial colonies were recovered from the other NaOCL treatments. All of the control group plates had microbial colonies present, as did all of the TSB plates on which non-disinfected, field-collected grubs were placed (Fig. 1).

Treatment 1 was omitted from the behavioral analysis because disinfection was not completely effective at that concentration and immersion time. Treatment 4 was omitted from the behavioral analysis because it was unnecessary as the lower concentration and shorter immersion times used in treatments 2 and 3 were effective. Grub burrowing behavior did not differ between treatments 2 and 3 and the controls (Table 1; df = 1; $P = 0.76$ for "Nonzero Correlation").

Discussion

Sodium hypochlorite has been used successfully as a surface disinfection to prevent microbial contamination on insects in laboratory bioassays (Vail et al.

Table 1. Effects of NaOCl concentration and controls on *Plectris aliena* white grub burrowing behavior in 2010 and 2011.

Treatment[a]	Immersion time (min)	No. of grubs	% Buried
1% NaOCl concentration	3	21	61.9 ns[b]
2% NaOCl concentration	2	21	57.1
EtOH + water (control)	-	14	64.3
Water (control)	-	12	33.3
No rinses (control)	-	17	64.7

[a]Only NaOCl concentrations and subsequent immersion times that were used for disinfection recommendations were chosen for burrowing behavior analyses.
[b]Nonsignificant F value in ANOVA, indicating no significant differences between treatments.

1968, Mangum et al. 1969, Lacey & Brooks 1997) and rearing facilities (Vanderzant & Davich 1951), and it is relatively inexpensive and easily accessible. In our tests, a disinfection solution with a 1% NaOCl concentration for 2 min was ineffective at removing all microbes from *P. aliena* grubs. Our results suggest that a 2- or 3-min immersion in a solution of 2% NaOCl, or a 3-min immersion in a solution of 1% NaOCl, following immersion in 95% EtOH for 10 sec, provide acceptable surface disinfection and do not negatively affect grub behavior. Because there was no microbial growth from surface washes or differences in grub behavior between immersion treatments of 2 or 3 min in 2% NaOCl, an immersion time of 2 min is recommended to minimize any potential disinfection treatment effects on the insect. Grubs exhibited normal burrowing behavior after surface disinfection treatments. This technique is efficient and provides an easy, low-cost method of surface disinfection for white grubs. The results observed in this study with *P. aliena* should have application for other white grub species used in laboratory settings when externally disinfected individuals are needed for experimental use. An effective surface disinfection technique will facilitate future studies that require field-collected grubs to examine the efficacy of entomopathogenic biological control agents (Lacey & Brooks 1987).

Acknowledgments

We would like to acknowledge the following people for their help with this project: Richard Humber, USDA-ARS Biological Integrated Pest Management Research Unit, Robert W. Holley Center for Agriculture and Health, Ithaca, New York for identification of *M. anisopliae*; Alana Jacobson, Steve Denning, and Rocio Davila for laboratory technical assistance; and Dr. Consuelo Arellano for statistical consulting. We would also like to acknowledge the North Carolina Agricultural Foundation for providing funding.

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