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Phylloplane Sterilization With Bleach Does Not Reduce Btk Toxicity for *Papilio Glaucus* Larvae (Lepidoptera: Papilionidae)

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PHYLLOPLANE STERILIZATION WITH BLEACH DOES NOT REDUCE BTK TOXICITY FOR *PAPILIO GLAUCUS* LARVAE (LEPIDOPTERA: PAPILIONIDAE)

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ABSTRACT

Neonate tiger swallowtail larvae (*Papilio glaucus*) were used to bioassay the effects of Btk (*Bacillus thuringiensis* var. *kurstaki*) at 4 doses (0.268, 0.034, 0.008, and 0.004 BIU per cm^2 leaf surface) with an untreated control. Larvae, obtained from females captured in Geogria and North Carolina, were fed leaves of either tulip tree (*Liriodendron tulipfera*) or black cherry (*Prunus serotina*) in experiments that either included a pre-treatment dip and rinse in 5% chlorox bleach or not, before the application of Btk (dipping leaves in serially diluted solutions of Foray 48B).

The results show no difference between North Carolina and Georgia *P. glaucus* larval dosage sensitivities, but do illustrate a clear dosage effect for all 4 treatments (cherry with and without bleach pre-treatment; tulip tree with and without bleach pre-treatment). The larvae on the bleached leaves do not do better (for cherry or tulip tree host plant) as would be expected if microbial symbionts on the phylloplane synergize the Btk toxicity. These results show that Btk (at doses several thousand-fold less than aerial sprays across forests for gypsy moth control) will kill *P. glaucus*, with or without microbial synergism on leaf phylloplanes.

The microbial mediation of plant-herbivore interactions has recently been recognized as a major and pervasive influence on community dynamics (Martin 1979, Jones 1984, Barbosa et al. 1991). The ecological and evolutionary effects of microorganisms on modifying plant resource suitability for insect herbivores are both direct and indirect (Jones 1991, Martin et al. 1993, Bauer 1995). These may involve plant pathogens, nitrogen-fixers, endophytes, ectophytes and phylloplane microflora (Andrews and Hirano 1991, Benedict et al. 1991). The interaction of *Bacillus thuringiensis* var. *kurstaki* (Btk) with leaf chemistry can be enhanced or inhibited depending on pH, nutrient levels, condensed tannins and phenolic glycosides (Reichelderfer 1991, Meade and Hare 1993, 1994; Moldenke et al. 1994, Hwang et al. 1995, Farrar et al. 1996). For example, simple phenolics can increase Bt toxicity to Lepidoptera (Brewer and Anderson 1990, Sivamani et al. 1992) whereas tannins can reduce the toxicity to Lepidoptera (Krischik et al. 1988).

Extensive areas of North America have recently received sprays of Bt which has a major impact on pests such as gypsy moth, but also non-target species. It is surprising that the ecology of Btk on leaf surfaces is basically

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unknown (Smith and Couche 1991, Lambert and Perferoen 1992) and essentially no information is presented in a treatise on the microbial ecology of plant phylloplanes (Andrews and Hirano 1991).

Earlier research with several species of "non-target" Lepidoptera has shown that Btk sprays remain toxic for 30–40 days post-spray under field conditions (Johnson et al. 1995). It has also been noted that naturally occurring *B. thuringiensis* populations on the leaf surface of temperate deciduous trees could serve as an insect feeding deterrent as well as a toxin (Smith and Couche 1991).

We also have noted differential disease in our laboratory mass-rearing operations with these Lepidoptera. In order to evaluate the role of leaf surface microbes which could directly cause insect disease, or synergize Btk toxicity, we conducted a series of experiments in which leaves to be fed to larvae in controlled environmental studies were sterilized by immersion in chlorine bleach solutions.

METHODS

A 10 minute chlorine bleach (5% concentration) dipping and 10 minute leaf rinsing with subsequent application of *Bacillus thuringiensis* var. *kurstaki* (Btk) in serial dilutions was used to examine the potential synergistic interactions of the microbial community for *Papilio* larvae. The original full strength formulation (Foray 48B) has 1.27×10^{-2} BIU/ml, which gives billion international unit equivalents per cm^2 leaf surface of 0.268, 0.034, 0.008, 0.004, for doses #1–#4 (Tables 1 and 2).

The tulip tree, *Liriodendron tulipifera* and black cherry, *Prunus serotina* leaves were collected from 2–4 trees near the MSU campus in Okemos/East Lansing. These experiments were conducted in a controlled environment chamber maintained at 20°C (with a 16:8 photo:scotophase). Larvae were maintained in groups of 3–5 per petri dish with 42 and 43 per treatment dose on cherry and tulip tree, respectively. A total of 660 larvae from Georgia from 46 different females and 200 larvae from 12 different North Carolina females were used in these experiments with different concentration of Btk on the leaves.

Source of Larvae. Larvae were obtained from eggs laid by field captured female *P. glaucus* using an oviposition arena (Scriber 1993) from Clark County, Georgia and Raleigh, North Carolina. They were, by location, separately mixed and randomly selected from both the Georgia and North Carolina populations to be distributed across the various treatments: sterilized and unsterilized leaf surfaces of black cherry and tulip tree leaves with differing Btk dosages.

Dosage Studies (*Bacillus thuringiensis*). We used a commercial formulation of Btk which is used across all of Michigan's gypsy moth suppression program, Foray 48B (Novo Nordisk, Danbury, CT). A serial dilution sequence of the original stock was made and leaves of black cherry and tulip tree were dipped in these solutions and allowed to dry in the lab before placement into the feeding dishes for bioassay. The laboratory doses were at solution concentrations of 0.002, 0.00025, 0.00006, 0.00003 ml Foray per mL of solution, and a control. These correspond to a leaf surface (cm^2) concentration of 0.268, 0.034, 0.008, and 0.004 BIU's (doses of 1–4, respectively). We mixed 4 ml in 1996 ml of distilled water, concentration of 0.002 ml of Foray per 1 ml solution. The recommended application rate for GM in the field for Foray 48B is at least several orders of magnitude greater (Reardon et al 1994). Half of the leaves for each tree species were immersed in 5% chlorine

Table 1. 6-day survival of neonate *P. glaucus* larvae fed black cherry (*Prunus serotina*) leaves with different doses* of Btk. Leaves were either treated with bleach (top) or not (bottom).

		Initial	% Survival (leaves bleached)				
Replicate	Source	(n)	Dose 1	Dose 2	Dose 3	Dose 4	Control
1	GA	(5)	0	0	0	60	20
2	GA	(5)	0	0	40	20	60
3	GA	(5)	0	0	60	20	40
4	GA	(5)	0	0	60	40	20
5	GA	(5)	0	0	20	0	60
6	GA	(5)	0	0	0	0	80
7	GA	(3)	0	0	33	33	0
	(GA mean)		(0.0)	(0.0)	(30.3)	(24.7)	(40.0)
1	NC	(4)	0	0	25	25	25
2	NC	(5)	0	0	20	50	80
	(NC mean)		(0.0)	(0.0)	(22.5)	(37.5)	(52.5)
			Survival (leaves normal)				
1	GA	(5)	0	0	20	40	60
2	GA	(5)	0	0	40	60	20
3	GA	(5)	0	0	20	0	40
4	GA	(5)	0	0	20	40	40
5	GA	(5)	0	0	0	60	60
6	GA	(5)	0	0	20	60	60
7	GA	(3)	0	0	0	33	33
	(GA mean)		(0.0)	(0.0)	(17.1)	(41.7)	(44.7)
1	NC	(4)	0	0	50	50	75
2	NC	(5)	0	0	40	20	100
	(NC mean)		(0.0)	(0.0)	(45.0)	(35.0)	(87.5)

*Doses of Foray 48B presented as BIU (billion international units; see Reardon et al. 1994) per cm^2 of leaf surface (dose 1 = .268, dose 2 = .034, dose 3 = 0.008, dose 4 = .004 IU/cm^2, control = 0). No significant differences (t-test p = 0.05) between NC and GA

bleach solution for 10 minutes and throughly rinsed under running water for 10 minutes and allowed to dry before receiving their Btk dip treatment. The other half of the leaves were not dipped in chorine bleach nor rinsed before dipping in Bt. These leaves simulated the normal field condition for August of 1995.

RESULTS

A dose response to Btk dipping was observed in all treatments for *P. glaucus* fed black cherry and tulip tree both with and without phylloplane sterilization by chlorine bleach. Studies in the last 20 years show 20–30% mortality of neonates reared on field collected cherry leaves to be normal. Six days after the larvae were allocated to their treatments, there were no survivors on the two highest Btk doses for black cherry in either treatment (chlorine bleach pretreatment, no chlorine bleach; Table 1, Figure 1, 2). While no survivors were observed on the tulip tree treatments for the highest Btk dose, there were some survivors in the next highest dose. Twenty percent

Table 2. 6-day survival of neonate *P. glaucus* larvae fed tulip tree (*Lirodendron tulipfera*) leaves with different doses of Btk. Leaves were either treated with bleach (top) or not (bottom).

Replicate	Source	Initial (n)	% Survival (leaves cloroxed) Dose 1	Dose 2	Dose 3	Dose 4	Control
1	GA	(5)	0	0	60	20	0
2	GA	(5)	0	60	40	40	60
3	GA	(5)	0	0	0	40	80
4	GA	(5)	0	40	0	40	0
5	GA	(5)	0	40	20	20	60
6	GA	(5)	0	0	40	40	60
7	GA	(3)	0	0	33	33	33
	(GA mean)		(0.0)	(20.4)	(27.6)	(33.3)	(33.3)
1	NC	(5)	0	0	0	60	60
2	NC	(5)	0	0	60	20	40
	(NC mean)		(0.0)	(0.0)	(30.0)	(40.0)	(50.5)
					Survival (leaves normal)		
1	GA	(5)	0	0	60	60	60
2	GA	(5)	0	0	100	80	60
3	GA	(5)	0	80	100	80	100
4	GA	(5)	0	20	0	60	60
5	GA	(5)	0	80	0	40	40
6	GA	(5)	0	0	20	40	40
7	GA	(3)	0	0	0	67	67
	(GA mean)		(0.0)	(25.7)	(40.0)	(61)	(61.0)
1	NC	(4)	0	0	40	40	80
2	NC	(5)	0	0	40	20	80
	(NC mean)		(0.0)	(0.0)	(40.0)	(30.0)	(80.0)

*Doses of Foray 48B presented as BIU (billion international units; see Reardon et al. 1994) per cm^2 of leaf surface (dose 1 = .268, dose 2 = .034, dose 3 = 0.008, dose 4 = .004 IU/cm^2, control = 0). No significant differences at any dose between GA and NC larvae.

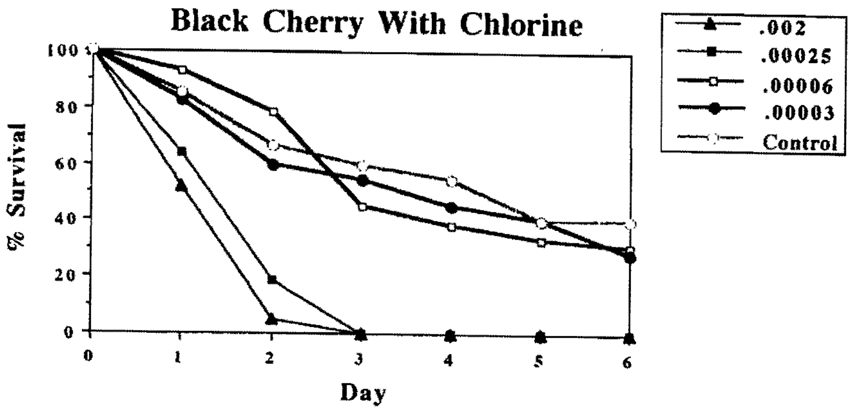

Figure 1. Differential 6-day larval survival on black cherry leaves with phylloplane sterilization (chlorine bleach dip) and subsequent Btk applications at different doses.

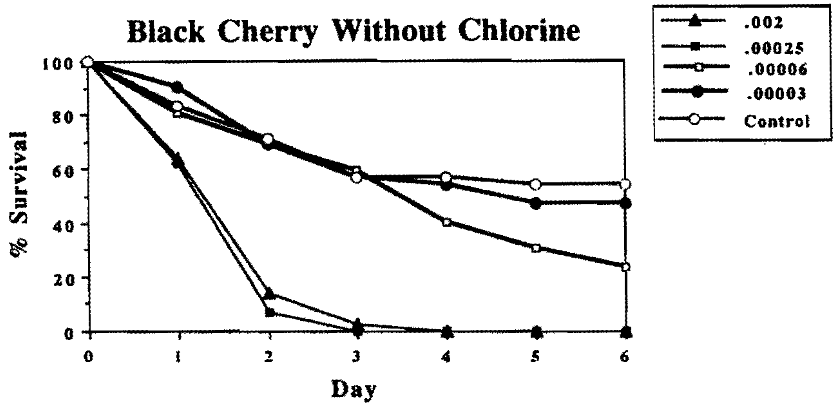

Figure 2. Differential 6-day larval survival of *P. glaucus* on black cherry leaves without phylloplane sterlization (Btk doses as in Fig. 1).

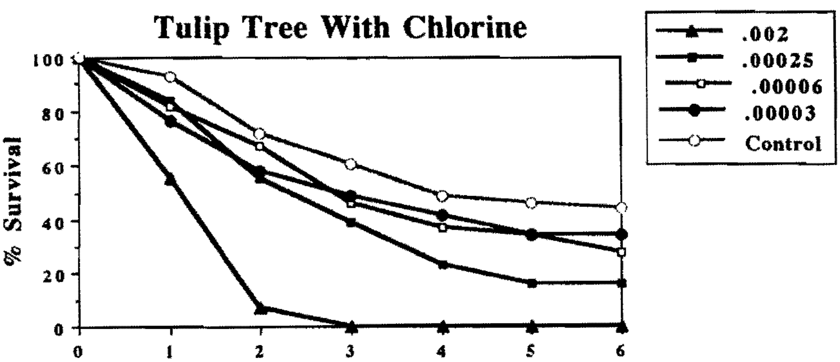

Figure 3. Differential 6-day larval survival of *P. glaucus* on tulip tree leaves with phylloplane sterilization (chlorine bleach dip) and subsequent Btk applications at different doses.

of the larvae survived the chlorine bleach treatment and twenty-five percent without chlorine bleach pretreatment in dose 2 on tulip trees (Table 2, Figure 3, 4). Progressively higher survival was observed for both host plants in all 4 treatments with lowest 3 doses. Of the survivors, the larvae on the lowest dose and controls were larger than the higher doses, implying that a chronic suppression of larval growth could be mediated by the Btk. This could be due to suppressed consumption rates, reduced conversion efficiencies, or both.

In the control (no Btk) treatments for both tulip tree and black cherry, we observed significantly smaller 7-day larvae sizes (Fig. 5) on the chlorine bleach dipped leaves compared to the control (non-dipped leaves). This sug-

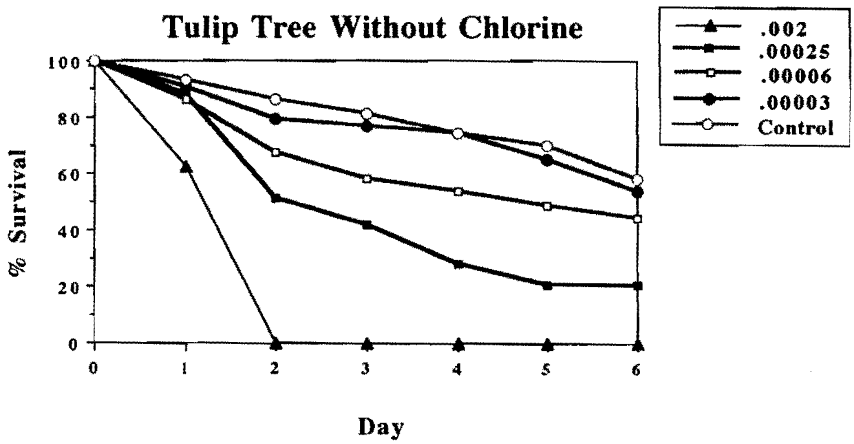

Figure 4. Differential 6-day larval survival of *P. glaucus* on tulip tree leaves without chlorine bleach treatment. (Btk doses as in Fig. 3.

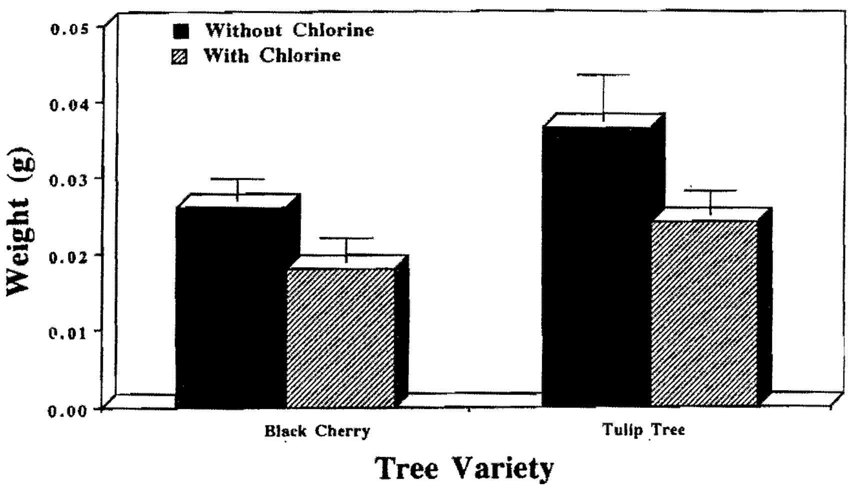

Figure 5. Larval live weights at 7-days fed black cherry and tulip tree leaves with and without phylloplane sterilization. Significant differences exist between the chlorine treated and untreated for both host plants (t-test, p=0.01).

gests that the chlorine bleach treatment (phylloplane sterilization process) itself had some deleterious effect on *P. glaucus* larvae, independent of the interaction with Btk. Variable results were observed for the next two lowest Btk doses with regard to chlorine-treatment and none.

DISCUSSION

The main focus of this research was to compare larval survival and growth under different Btk dosages on black cherry and tulip tree leaves with and without phylloplane sterilization. Dose dependent responses were observed over all of the 4 treatments. Even at very low doses used in our studies, the *Papilio* were found to be be very sensitive. The non-target implications of this Btk dose-toxicity (Scriber and Haas 1996) and the long term 30-day persistence of Btk under field conditions (Johnson et al 1995) are very significant in gypsy moth management programs (Reardon et al 1994). Our study shows that in the susceptible forests of North Carolina and Georgia (Liebhold et al. 1997), *Papilio* are of proven vulnerability to Btk sprays at several thousand-fold lower concentrations than aerial application.

Removing the microbial community from the leaf surface did not decrease the toxicity of Btk to *Papilio* in our studies, as would have been predicted if microbes synergize the toxicity of Btk (Dubois and Dean 1995). In fact, the bleaching procedure used to remove the microbes appears to actually increase mortality and reduced growth rates compared to untreated leaves. These findings could result from the leaf surface being altered upon application of the chlorine bleach. We speculate that: 1) Removal of microbial flora by chlorine bleach could have lowered the nutritional value of the diet, 2) Sub-detectable tissue damage on the leaf surface when dipping leaves in chlorine bleach (or rinsing) could have caused induction of secondary chemicals leading to an increase in mortality rates in larvae treated with chlorine bleach, 3) The wax layer could have been partially removed by chlorine bleach, perhaps allowing the leaves to desiccate at a higher rate, and somehow causing larvae to lower their leaf intake, or 4) Chlorine bleach residues themselves may have persisted on the leaf even after 10 minutes of rinse. The causal mechanism of chlorine bleach-treated leaves on larval performance remains unresolved, however it is clear from our study that very low doses of Btk will kill *Papilio glaucus* larvae, whether or not there are leaf microbes persent in the phylloplane.

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