Effect of Clonal Variation Among Hybrid Poplars on Susceptibility of Gypsy Moth (Lepidoptera: Lymantriidae) to *Bacillus thuringiensis* subsp. *kurstaki*

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ABSTRACT Trees in the genus *Populus* can provide substantial commercial and ecological benefits, including sustainable alternatives to traditional forestry. Realization of this potential requires intensive management, but damage by defoliating insects can severely limit productivity in such systems. Two approaches to limiting these losses include cultivation of poplar varieties with inherent resistance to pests and application of microbial pesticides. Little is known about the interaction between host resistance and the ability of poplars to support the efficacy of biocontrol agents. The research described here was conducted to survey the effect of hybrid poplar clones on gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), a pest on these trees. We assessed the effect of various poplar clones on larval performance and susceptibility to *Bacillus thuringiensis* subsp. *kurstaki*. Larvae were reared from hatching on the foliage of 25 hybrid poplar clones and we monitored larval survival, development time, and weight at fourth instar. Eight of these clones showed high resistance against gypsy moth. The remaining clones showed high variation in their effect on larval performance. We evaluated the susceptibility of third-instar larvae to *B. thuringiensis* subsp. *kurstaki* when reared on the 17 remaining clones. There was a significant effect of poplar clone on time to death after ingestion of *B. thuringiensis* subsp. *kurstaki*. The susceptibility of gypsy moth larvae to *B. thuringiensis* on various clones was not correlated with the effects of these clones on larval performance in the absence of *B. thuringiensis*, suggesting this interaction is more complex than merely reflecting higher mortality to previously stressed larvae.

KEY WORDS *Populus*, *Lymantria dispar*, short rotation trees, biomass production, microbial pesticides

Trees of the genus *Populus* are important components of many natural ecosystems worldwide, including some of the most widely distributed species (Mitton and Grant 1996). They are also cultivated extensively, owing to their rapid growth, abundant genetic and adaptive diversity, and amenability to vegetative propagation and transgenic transformation (McCown et al. 1991, Johnson 2000). These attributes have contributed to their increasing ecological and economic value as a renewable source for pulpwood, lumber, and biofuels (Ball et al. 2005). They are also of high environmental importance as tools for promoting carbon sequestration and phytoremediation (Newman et al. 1997, Abrahamson et al. 1998, Schoene and Netto 2005). The value of *Populus* species is enhanced by the ease with which various species can be hybridized, which has permitted the selection of trees with improved growth and adaptability (Stettler et al. 1996). This feature also has facilitated the selection of trees with increased resistance to pathogens and insect herbivores, a necessary requirement for intensive management (Bisoffi and Gullberg 1996, Netzer et al. 2002, Coyle et al. 2005).

Poplars are most severely affected by two major insect groups, Coleoptera and Lepidoptera (Harrell et al. 1981, Mattson et al. 2001). Poplar resistance to insect herbivores is due largely to production of phenolic compounds, including phenolic glycosides, flavonoids, and tannins (Hemming and Lindroth 1995, Hwang and Lindroth 1997). Phenolic compounds are known to deter feeding and reduce growth of generalist herbivores feeding on *Populus* (Lindroth and Peterson 1988; Appel 1993; Robison and Raffa 1994; Havill and Raffa 1999, 2000; Hemming and Lindroth 2000). However, some specialist herbivores, such as the cottonwood leaf beetle, *Chrysomela scripta* F. (Coleoptera: Chrysomelidae), are attracted to and use

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phenolics as feeding stimulants and sequester them for defense against predators (Bingaman and Hart 1992, 1993; Coyle et al. 2001; Donaldson and Lindroth 2004; Kendrick and Raffa 2006). Thus, it is unlikely that any one poplar clone could confer resistance to both *C. scripta* and lepidopteran pests, which comprise the two most damaging defoliator groups in managed settings (Ramachandran 1994, Raffa 2004). Resistance to Coleoptera and Lepidoptera is complemented by the application of pesticides, including the microbial *Bacillus thuringiensis* Berliner, with subspecies *tenebrio* and *kurstaki* (Btk) used against beetles and moths, respectively.

The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), is an invasive polyphagous folivore, and despite extensive management, it remains an important pest of native and cultivated tree species (Barbosa 1978, Lechowicz and Maufette 1986, Mattson et al. 2001). However, as gypsy moth continues its westward expansion in North America it has moved into major regions of poplar production. Given the high susceptibility of *Populus* trees to this pest, gypsy moth is becoming an increasingly important factor in the production of both hybrid poplar and aspen (*Populus tremuloides* Michaux) (Montgomery and Wallner 1989, Mattson et al. 2001).

The bacterial insecticide *B. thuringiensis* is commonly used to control gypsy moth, including in managed settings. Gypsy moth susceptibility to *B. thuringiensis* varies with host diet and past studies have shown that larvae may be more susceptible to *B. thuringiensis* subsp. *kurstaki* when feeding on some species such as aspen than on others such as willow (*Salix fragilis* L.) (Appel and Schultz 1994, Farrar et al. 1996, Broderick et al. 2003). Additional studies have shown that some phenolic compounds can increase susceptibility of gypsy moth to *B. thuringiensis* subsp. *kurstaki* (Lindroth and Weisbrod 1991, Hwang et al. 1995, Lindroth and Hwang 1996, Kleiner et al. 2003). *B. thuringiensis* affects susceptible larvae through a multistep process, which ultimately leads to epithelial cell lysis. One consequence of this process is that soon after ingestion larvae cease feeding due to paralysis of the midgut (Heimpel and Angus 1959). Some phenolic compounds also are reported to cause lysis of larval midgut epithelial cells (Lindroth and Peterson 1988). Because phytochemistry is known to vary widely among *Populus* spp., it seems likely that gypsy moth feeding on different hybrids will exhibit various degrees of both innate resistance and susceptibility to the addition of *B. thuringiensis.*

The objectives of this study were to 1) identify fitness effects of hybrid poplar clones on gypsy moth larvae and 2) determine whether hybrid poplar clones differentially affect susceptibility of gypsy moth to *B. thuringiensis* subsp. *kurstaki.*

Materials and Methods

Insect Rearing. Egg masses were obtained from the USDA–APHIS laboratory at the OTIS Air National Guard Base, Cape Cod, MA. Eggs were sterilized in 40–50 ml of a solution of 10.2 ml of Tween 80 (polyoxyethylene sorbitan monooleate) and 19.9 ml of bleach per liter of distilled water for 5 min, rinsed three times with distilled water, and dried under a vacuum hood for 30 min.

Neonate larvae were reared on artificial diet for 24 h after emergence before being separated into groups of 60 and reared on one of 25 different clones (most of which are still in the experimental phase of development) of hybrid poplar until 16 individuals reached second instar. All larval cohorts were reared in 17-cm plastic petri dishes and maintained at 25°C under a photoperiod of 16:8 (L:D) h with relative humidity at ≈60%. One or two leaves were placed in each dish, with the petioles inserted into water-picks filled with distilled water to prevent desiccation. Each dish was lined with filter paper and moistened with distilled water before larval transfer. Larvae were transferred to clean petri dishes with new foliage every 48 h. Mortality was monitored daily throughout rearing.

Foliage Collection. Leaves were selected randomly from hybrid poplar clones grown at the University of Wisconsin-Madison, Arlington Research Station (Arlington, WI). Leaves were removed from trees by cutting at the petiole using a sharp razor blade, and cut ends were immediately inserted into a water-pick filled with distilled water to prevent desiccation. They were stored in unsealed plastic zip-lock bags at 4°C until use. All assays used leaves of similar phenological age.

Effect of Poplar Clonal Variety on Larval Fitness. Initial assays to assess larval success on the 25 poplar clones were performed in June 2004. Seventeen poplar clones were selected from a pool of 25 to be tested as a diet for gypsy moth fitness. These clones were selected for further testing because enough larvae ($n > 20$ per cohort) developed on them for use in assays. Larvae were reared as described above, with 16 larvae per rearing group. Two groups of larvae also were reared on artificial diet for comparison. Each larva was fed a 4-mm leaf disc daily until it molted to third instar. Third instars were then fed 8-mm leaf discs. Larvae feeding on artificial diet were provided an amount of diet equivalent to the average mass of the leaf disks (0.018 g), which was increased to 0.036 g for third-instar larvae to ensure sufficient nutrition for normal development. Individual larvae were provided food as required until their removal from the assay due to death or upon molting to fourth instar. Mortality and date of molting to fourth instar were recorded for each larva. Wet weight of each larva was recorded upon molting to fourth instar by using a Mettler AE100 microbalance (sensitivity 100 μg).

Effect of Poplar Clonal Variety on Larval Susceptibility to *B. thuringiensis* subsp. *kurstaki*. Assays to assess larval susceptibility to *B. thuringiensis* were performed in June 2004, with a cohort of larvae reared along with the above-mentioned assay, and repeated with a second cohort in July 2004. Larvae were reared as described above. Upon molting to third instar, larvae were transferred to assay trays. Individual larvae were placed in cells (4 by 2.5 by 1.5 cm) of assay trays

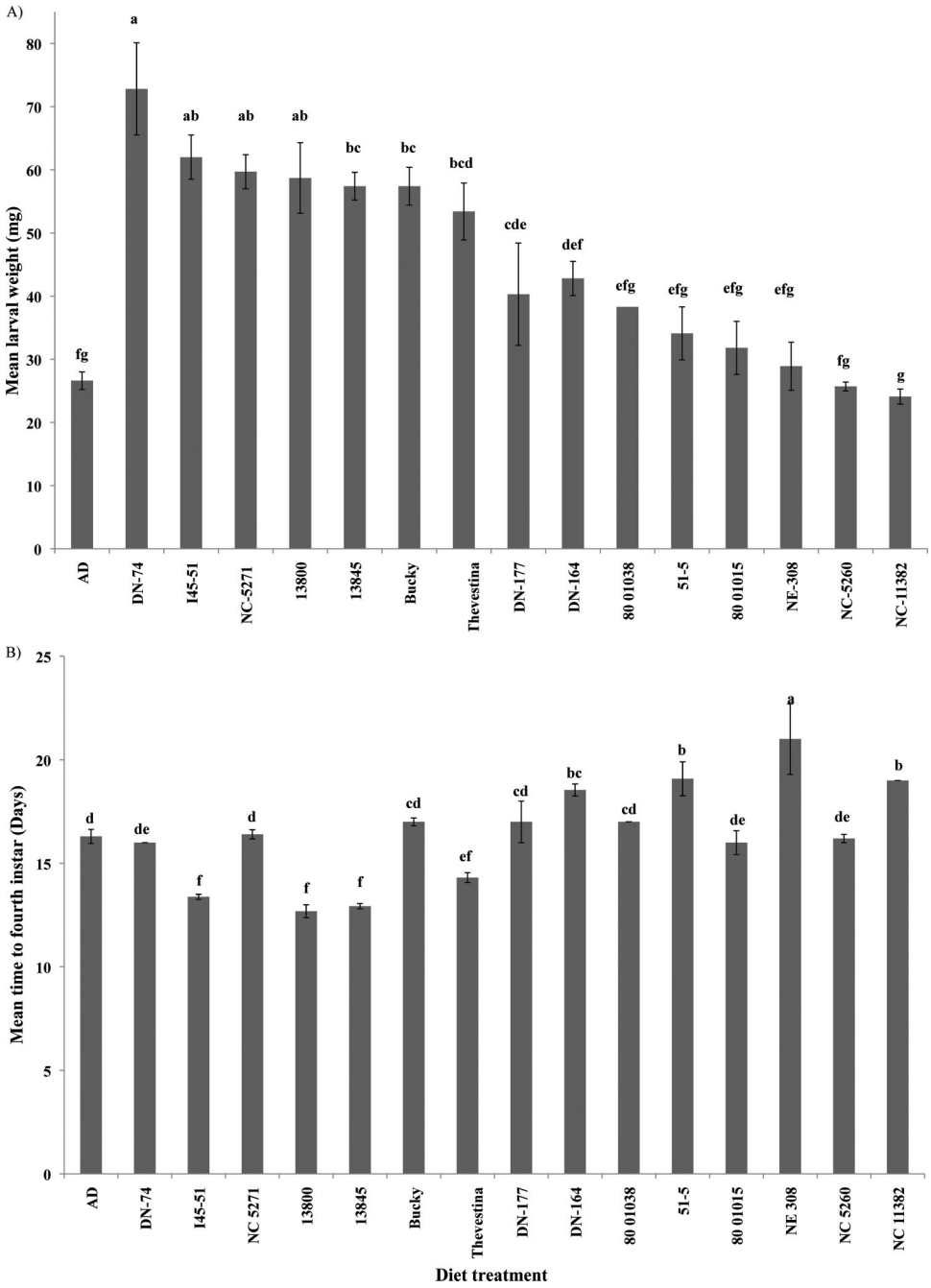

Fig. 1. Effect of hybrid poplar clone on mean weight of gypsy moth larvae and length of time to fourth instar. Each bar represents mean ± SEM of larvae reared on diet from hatching; data were analyzed by analysis of variance (ANOVA) and means were separated for significance according to Fisher protected LSD at $P < 0.05$. Clones NE-264 and DN-154 were excluded from performance analyses as larvae died before molting to fourth instar. (A) Mean weight at fourth instar ($F = 18.35$, df = 15, $P < 0.0001$). (B) Mean time (days) to fourth instar ($F = 26.55$, df = 15, $P < 0.0001$).

and starved for 24 h, at which time each larva was provided either an untreated leaf disk (4 mm in diameter, with an approximate mass of 0.018 g) or with a leaf disk to which 1 μl of a sporulated culture of *B. thuringiensis* subsp. *kurstaki* strain HD-1 ($\approx 2.5 \times 10^4$ colony-forming units/ml) had been applied. Treatments were administered for two consecutive days, after which larvae were provided untreated leaf disks for 3 d. Mortality was recorded daily over the 5-d period. For comparison, the larvae reared on artificial

Table 1. Larval success of gypsy moth reared on artificial diet and various hybrid poplar clones[a]

Diet treatment	Parentage	% larvae molt to L3
Artificial diet 1		96
Artificial diet 2		98
Clone DN-74	*P. deltoides* × *P. nigra*	98%
Clone I45-51	*P. deltoides* × *P. nigra*	89
Clone NC-5271	*P. nigra* var. *charkowiensis* × *P. nigra* var. *caudina*	100
Clone 13800		90
Clone 13845		77
Clone Bucky	Unknown	60
Clone Thevestina	*P. nigra*	96
Clone DN-177	*P. deltoides* × *P. nigra*	98
Clone DN-164	*P. deltoides* × *P. nigra*	75
Clone 80 01038	*P. deltoides*	84
Clone 51-5	*P. deltoides*	50
Clone 80 01015	*P. deltoides*	93
Clone NE-308	*P. nigra* var. *charkowiensis* × *P. nigra* var. *incrassata*	42
Clone NC-11382	*P. nigra* × *P. berolinensis*	67
Clone NC-5260	*P. tristis* × *P. balsamifera* 'Tristis #1'	47
Clone NE-264	*P. deltoides* × *P. nigra*	89
Clone DN-154	*P. deltoides* × *P. nigra*	44
Clone 91.05-02	*P. deltoides*	0
Clone 91.08-09	*P. deltoides*	7
Clone 7300501	*P. deltoides*	5
Clone 80 00601	*P. deltoides* × *P. deltoides*	0
Clone D124	*P. deltoides*	0
Clone D121	*P. deltoides*	4
Clone NC 13460	(*P. trichocarpa* × *P. deltoides*) × *P. deltoides*	0
Clone NC 13550		0

[a] Larvae were reared from hatching on test diet ($n = 60$ each). The percentage of larvae molting to third instar by day 14 is shown.

diet were provided an artificial diet disk of approximately the same mass as the leaf disks with and without *B. thuringiensis* subsp. *kurstaki*.

Statistical Analysis. Mean larval mortality, mean larval weight, and SE were determined using PROC MEANS (SAS Institute 2006). Means were separated using Fisher protected least significant difference (LSD) at $P = 0.05$. The weight-by-time interaction (larval development) was analyzed using the SAS general linear modeling (GLM) procedure (SAS Institute 2006). Correlations between larval weight and mortality attributable to *B. thuringiensis* subsp. *kurstaki* were analyzed by regressing mean mortality or mortality ratio on larval weight for each diet treatment. The effect of poplar clone on time to death of *B. thuringiensis* treated larvae was analyzed using PROC PROBIT (SAS Institute 2006). Significant differences in LT_{50} and LT_{95} values between treatments were determined based on probit values with nonoverlapping 95% fiducial limits (FL).

Results

Effect of Hybrid Poplar Clone on Larval Growth and Development. Hybrid poplar clones substantially affected larval growth ($F = 18.35$, df = 15, $P < 0.0001$; Fig. 1), rate of development ($F = 26.55$, df = 15, $P < 0.0001$; Fig. 1), and overall survival (Table 1). In the initial screen, eight of the 25 poplar clones showed very strong resistance against gypsy moth, with >90% of larvae feeding on them dying before third instar, and larvae reared on two others (NE-264 and DN-154) died before molting to fourth instar (Table 1). Mean

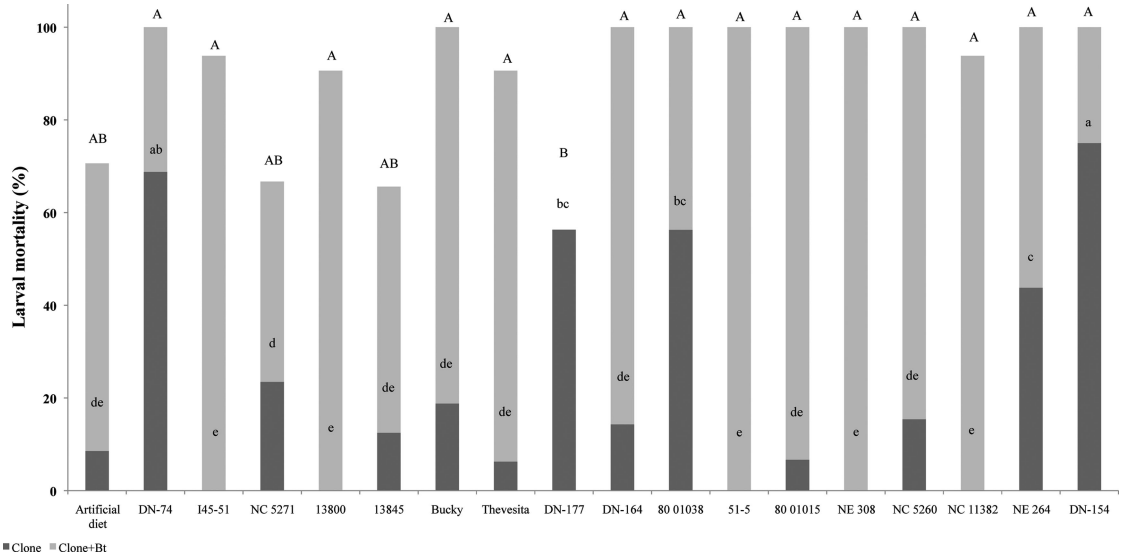

Fig. 2. Contribution of hybrid poplar clone to mortality of third-instar gypsy moth, alone and in combination with *B. thuringiensis* subsp. *kurstaki*. Larvae were reared and assayed on the same poplar clone. Larvae reared on artificial diet served as a control. Mortality values of larvae assayed on poplar clone alone or in combination with *B. thuringiensis* subsp. *kurstaki* were analyzed separately by ANOVA. Means were separated for significance according to Fisher protected LSD at $P < 0.05$ [Clone (lowercase) $F = 32.10$, df = 17, $P = 0.0002$; Clone + *B. thuringiensis* subsp. *kurstaki* (Bt) (uppercase) $F = 1.65$, df = 17, $P = 0.1293$)].

Table 2. Effect of poplar clone on mean mortality and time to death of third-instar gypsy moth larvae[a]

Clone	Bt (IU/larva)	LT_{50} (95% FL) (d)	LT_{95} (95% FL) (d)	Larval mortality, mean % (SE)	Mortality attributable to Btk	
					Difference in mean (%) mortality	Mortality ratio [with Btk/without Btk][b]
Artificial diet	0	261 (31–>100)	35306 (>100)	9 (3)		
Artificial diet	2	3 (3–3)	10 (8–12)	71 (8)	62	8
DN-74	0	4 (3–5)	8 (6–16)	69		
DN-74	2	<1[c]	<1[c]	100 (0)	32	1
I45-51	0	nc[d]	nc	0		
I45-51	2	1 (1–2)	4 (3–8)	94	94	94
NC 5271	0	7 (6–14)	13 (8–71)	24 (21)		
NC 5271	2	2 (2–3)	22 (12–80)	67 (33)	43	3
13800	0	nc	nc	0		
13800	2	2 (1–2)	5 (4–7)	91 (9)	91	91
13845	0	12 (nc)	40 (nc)	13		
13845	2	1 (0–2)	139 (19–>100)	66 (28)	53	5
Bucky	0	5 (nc)	6 (nc)	19		
Bucky	2	<1[c]	<1[c]	100 (0)	81	5
Thevestia	0	nc	nc	6		
Thevestia	2	1 (1–2)	6 (4–10)	91 (9)	84	14
DN-177	0	4 (4–5)	8 (6–21)	56		
DN-177	2	5 (4–9)	41 (16–>100)	50 (31)	−6	1
DN-164	0	7 (nc)	13 (nc)	14		
DN-164	2	<1[c]	<1[c]	100 (0)	86	7
80 01038	0	4 (3–6)	17 (9–182)	56		
80 01038	2	<1[c]	<1[c]	100 (0)	44	2
51-5	0	nc	nc	0		
51-5	2	1 (0–1)	3 (2–5)	100 (0)	100	100
80 01015	0	nc	nc	7		
80 01015	2	1 (1–2)	2 (2–3)	100 (0)	93	15
NE 308	0	nc	nc	0		
NE 308	2	1 (nc)	1 (nc)	100 (0)	100	100
NC 5260	0	nc	nc	15		
NC 5260	2	1 (1–2)	3 (3–4)	100 (0)	85	6
NC 11382	0	nc	nc	0		
NC 11382	2	1 (1–2)	6 (5–13)	94 (6)	94	94
NE 264	0	8 (4–>100)	126 (19–>100)	44		
NE 264	2	1 (0–1)	2 (2–4)	100 (0)	56	2
DN-154	0	3 (3–5)	14 (8–75)	75		
DN-154	2	<1[c]	<1[c]	100 (0)	25	1

[a] Larval mortality rates were analyzed by PROC PROBIT. Estimates of the time (day) at which 50 and 95% of larvae died for each treatment are listed. A cut-off of >100 was assigned to upper FL estimates (note these are computational outputs, not actual estimates of larval life span).
[b] Where control mortality = 0%, it was adjusted to 1%.
[c] 100% mortality by day 1.
[d] Not calculated; value could not be computed statistically.

weight of larvae feeding on the remaining 15 clones varied strongly with genotype, ranging from 24.1 to 72.8 mg at fourth instar (Fig. 1A). Approximately half of these clones produced larvae with average weights that were significantly higher than those reared on artificial diet. In no case in which larvae survived to fourth instar were the weights of larvae fed any of the clones significantly lower than artificial diet. The average number of days for larvae to reach the fourth instar also varied significantly among poplar clones (Fig. 1B). Overall, larvae molted to fourth instar between 12 and 21 d after hatching, with larvae reared on artificial diet averaging 16 d. The weight × time interaction was highly significant ($F = 5.97$, df = 1, $P = 0.0265$).

Mortality of larvae feeding on clones DN-74, DN-177, 80 01038, NE264, and DN154 was significantly higher than larvae reared on artificial diet or the remaining clones (Fig. 2). Larvae reared on four of these (DN-74, DN-177, 80 01038, and DN-154), as well as NC5271 died more quickly than larvae reared on artificial diet or on most of the other poplar clones (Table 2).

Effect of Hybrid Poplar Clone on Larval Susceptibility to *B. thuringiensis* subsp. *kurstaki*. There was a significant effect of poplar clone on the rate of larval mortality after ingestion of *B. thuringiensis* subsp. *kurstaki* ($\chi^2 = 74.17$, df = 17, $P < 0.0001$; Table 2). Larvae fed *B. thuringiensis* subsp. *kurstaki* died faster when they were reared on all but one of the poplar clones (DN177) than when reared on artificial diet. When fed six of the 17 poplar clones (DN-74, Bucky, DN-164, 80 01038, NE308, and DN-154), all larvae died within 1 d of treatment, whereas larvae fed artificial diet died within 3–5 d (Table 2). There was no correlation between weight of larvae at fourth instar in the absence of *B. thuringiensis* subsp. *kurstaki* among control larvae and mortality attributable to *B. thuringiensis* subsp. *kurstaki* among treated larvae (Table 2), compared as either the difference in mean mortality ($F = 0.03$, df = 1, $P = 0.8679$) or mortality ratio ($F = 0.05$, df = 1, $P = 0.8333$). The clones also differed

significantly in their effect on total mortality of gypsy moth larvae when fed alone or in conjunction with *B. thuringiensis* subsp. *kurstaki* (Fig. 2).

Discussion

This study indicates that hybrid poplar clones differ in their inherent susceptibility to gypsy moth and in the extent to which they augment the toxicity of *B. thuringiensis* subsp. *kurstaki.* The mechanistic basis for the interaction between *B. thuringiensis* subsp. *kurstaki* and tree defense is unknown. However, there was no relationship between growth rate of larvae feeding on a given clone alone and larval susceptibility to *B. thuringiensis* subsp. *kurstaki* when fed that clone, indicating that the enhanced susceptibility to *B. thuringiensis* subsp. *kurstaki* was not due simply to larvae being sickly from feeding on certain clones.

We speculate that variations in tree chemistry are probably responsible for the differences we observed between clones. Phenolic compounds are known to affect larval susceptibility to *B. thuringiensis* and vary significantly among poplar clones. In particular, higher concentrations of phenolic glycosides are correlated with both reduced larval performance and increased susceptibility to *B. thuringiensis*, whereas the levels of tannins are not correlated with significant effects (Kleiner et al. 1998, Hemming and Lindroth 2000, Hale et al. 2005, Milanovic et al. 2006, Barbehenn et al. 2009). However, poplar clones also affect levels of midgut enzymes, such as esterase and glutathione transferase, as well as glucose and cholesterol in the midgut of gypsy moth larvae (Hemming and Lindroth 2000, Barbehenn et al. 2009, Daryaei et al. 2009), which also could influence their susceptibility to *B. thuringiensis.* Future work should analyze the levels of total phenolics, phenolic glycosides, and tannins of the clones used in this study to determine whether there is a correlation between tree chemistry and larval susceptibility.

These results raise the possibility that some clones might require substantially less *B. thuringiensis* subsp. *kurstaki* for protection than others. If so, this could improve the economics of hybrid poplar production and delay biotype evolution (Tabashnik 2008). Future work is needed to determine the minimum required application rate. It would likewise be useful to conduct similar studies with coleopteran pests, especially cottonwood leaf beetle, given their importance in poplar management. The eight clones that exhibited a high level of innate resistance against gypsy moth should likewise be prioritized for evaluation of resistance to other pests and general growth characteristics. These evaluations could benefit from knowledge of the phytochemistry of these clones, which is currently lacking.

In addition to studying these interactions in intensely managed systems, it would be useful to quantify interactions between native *Populus* species and *B. thuringiensis* subsp. *kurstaki.* Variation in susceptibility of larvae to *B. thuringiensis* subsp. *kurstaki* could partially explain why the rate of spread of gypsy moth varies among regions despite intense and coordinated national programs (Johnson 2000). Such variation in plant genotype has been shown to affect interactions with biocontrol agents in other systems and is proposed as a mechanism to improve insect and pathogen management (Price et al. 1980, Bottrell et al. 1998, Smith et al. 1999, Cortesero et al. 2000). Identification of genes that modify the effect of biocontrol agents could provide a useful and manipulable tool to enhance plant protection. In this manner, this study suggests that exploitation of host genotype to support biocontrol agents may be a fruitful and largely unexplored avenue for protecting intensively managed short rotation trees from insects and pathogens.

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