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Influence of a Foliar Endophyte and Budburst Phenology on Survival of Wild and Laboratory-Reared Eastern Spruce Budworm, *Choristoneura fumiferana* on White Spruce (*Picea glauca*)

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Abstract: A manipulative field study was carried out to determine whether the foliar endophyte fungus, *Phialocephala scopiformis* DAOM 229536, decreased the performance of eastern spruce budworm, *Choristoneura fumiferana* larvae developing on white spruce trees. Overwintered second-instar budworm larvae from a laboratory colony or from a wild population were placed on endophyte positive or negative trees one or two weeks before budburst. The presence of the endophyte in the needles reduced the survival of *C. fumiferana* from both a wild population and a laboratory colony. Survival for budworm juveniles up to pupation and to adult emergence was 13% and 17% lower, respectively, on endophyte positive trees. The endophyte did not influence the size or sex of survivors and budworm survival was not influenced by any two- or three-way interactions. Budworm survival was higher for wild than for laboratory-reared budworm and for budworm placed on trees a week before budburst. This may be the first field study to demonstrate the efficacy of an endophytic fungus against wild individuals of a major forest insect pest. The efficacy of the endophyte at low larval densities suggests that it could be a useful tactic to limit spruce budworm population growth in the context of an early intervention strategy.

Keywords: Pinaceae; endophytic fungi; plant tolerance; *Phialocephala scopiformis*; *Picea glauca*; spruce budworm; phenology; insect susceptibility

1. Introduction

Mutualistic interactions between fungi living within leaf tissues (endophytes) and their host plants are common [1]. Plant tissues provide endophytes with nutrients [2] and some endophytes provide plants with protection from herbivores and fungal diseases [1,3]. Although most previous work on endophyte–plant interactions has been carried out in grasses and other agricultural crops [1,4], endophytic fungi are common in foliage of many conifers and may play similar roles in these large, long-lived plants [5,6].

Previous studies carried out with potted seedlings under laboratory [7–9] and field conditions [10] demonstrated that the native rugulosin-producing endophyte, *Phialocephala scopiformis* DAOM

229536 Kowalski & Kehr (Helotiales:Ascomycota) reduced the growth of eastern spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera:Tortricidae). Under nursery conditions, most of the effect was attributed to the presence of the anti-insect toxin rugulosin [10]. Building on those studies, we recently demonstrated a similar effect on budworm developing on white spruce trees that had been inoculated with the endophyte more than 10 years earlier [11]. The reduction in budworm survival was highest for larvae developing in the mid and upper crown of trees, the most important crown region for photosynthesis and tree growth. These results suggest that inoculation of white spruce trees with *P. scopiformis* could reduce tree susceptibility to spruce budworm during outbreaks.

In our previous study [11], laboratory-reared second-instar budworm were placed on trees in the field on a single date. Consequently, we do not know if the endophyte is as effective on wild as lab-reared budworm or during years when spring synchrony between larval emergence and budburst varies. Manipulative field studies carried out with lab-reared budworm reported that budworm survival is highest when second-instar budworm larvae emerge one to three weeks before budburst [12].

Here, we report results from a manipulated field study carried out to investigate the independent and interacting effects of the endophyte, *P. scopiformis*, larval source (wild or laboratory-reared), and budworm spring emergence–host plant budburst synchrony on the performance of spruce budworm. As in our previous study [11], the present study was carried out with relatively low budworm densities, and subsequently low levels of defoliation. The objective was to determine whether the endophyte would reduce budworm survival before a large outbreak occurred.

2. Materials and Methods

2.1. Study Site, Tree Selection, and Experimental Design

Field experiments were carried out near Havelock, New Brunswick in two adjacent "test plots" (45°587" N, 65°26" W) of approximately 10-year-old white spruce, *Picea glauca* (Moench) Voss, trees planted by JDI Limited from seedling stock in 2003. Test plots are described in our previous study [11]. Briefly, both untreated control and endophyte positive trees were interplanted at 2 m × 2 m spacing in each of two adjacent 0.12 ha plots. Study trees were grown in 2000 and 2001 at Sussex Tree Nursery and endophyte-inoculated trees were wound inoculated as described by Miller et al. [8] with cultures of *P. scopiformis*. Trees were tested for the presence of the endophyte prior to planting in the field in 2003 with a polyclonal antibody for mycelium, and by measuring the insect toxin rugulosin by HPLC [13].

In mid-April 2012, we selected 14 pairs of trees. Each tree pair consisted of one endophyte-inoculated and one control tree; trees within a pair were located <8 m from each other. Trees with noticeable browsing, defoliation, mechanical damage, or deformation due to spruce gall midge (*Mayetiola piceae* (Felt)) or spruce bud midge (*Rhabdophaga swainei* (Felt)) (Diptera:Cecidomyiidae) were excluded from the study. Presence of the endophyte in endophyte-inoculated trees and absence of the endophyte in uninoculated control trees was verified using the polyclonal antibody test [13]. We placed 15 wild larvae on one branch in the mid-crown on 21 April 2012 and another 15 larvae on an adjacent mid-crown branch on 28 April 2012, approximately 9 and 2 days before budburst started on the most phenologically advanced trees, and enclosed them within a sleeve cage. Fifteen laboratory-reared larvae were placed on an adjacent branch on each of the two dates, for a total of 4 sleeve cages per tree (i.e., 2 sources of larvae × 2 dates). Two of the 112 cages (i.e., 4 cages per tree × 28 trees) were damaged by winds and were not included in analyses. As the majority of buds burst 3–5 days after the first buds burst, most larvae in the phenology treatments were placed on trees approximately one or two weeks before budburst.

2.2. Insect Sources

The study was carried out with wild larvae collected in eastern Quebec and with laboratory-reared larvae. Disease-free second-instar budworm larvae were obtained from the rearing facility of the

Canadian Forest Service in Sault Ste. Marie, Ontario [14] and stored at 4 °C for 1–2 weeks before placement in the field.

To obtain overwintered wild second-instar larvae for use in experiments in spring 2012, we used pole pruners to collect branches from highly defoliated natural spruce/fir stands close to Baie Comeau, Quebec, in late July and early August 2011. Egg-bearing shoots were cut from branches and transported in coolers to the University of New Brunswick (UNB). Egg-bearing shoots were placed in metal trays and reared at 22 ± 1 °C and 65% ± 5% RH under a 14 h light: 10 h dark photoperiod. A piece of Parafilm™ with a smaller piece of cheesecloth attached to it, had been placed on the bottom of each tray and another larger piece was used to seal the top of each container. A black piece of cardboard, with an approximately 12 cm × 6 cm rectangular hole in the center, was placed over each tray. Following egg hatch, first instar budworm larvae spun hibernacula on the cheesecloth. The pieces of cheesecloth were removed two weeks later and placed in sleeve cages. We fixed the sleeve cages to the lower bole of spruce trees in the UNB woodlot in Fredericton where they overwintered. The cages and enclosed larvae were collected when needed for experiments.

2.3. Insect Rearing Procedures

The protocols were similar to those described in Quiring et al. [11]. Briefly, in spring 2012, pieces of cheesecloth on which the wild and lab-reared second instars had previously spun hibernacula were placed at 20 ± 1 °C, 75% RH under a 14 L/10 D photoperiod until the first larva emerged. Cheesecloth pieces with 15 hibernacula each were cut under a binocular microscope and transported to the field in a cooler. These were attached to each experimental branch with a pin. The branches were enclosed in a sleeve cage which then was attached to the branch. The cheesecloth pieces were removed from the cages two weeks later and the number of dead, second-instar larvae that had not left the cheesecloth recorded. Those remaining were not included in the survival calculations. We reattached the sleeve cages and monitored them weekly until the first pupa was observed. Juveniles were removed once most larvae had pupated, placed in aerated containers on moistened vermiculite, and reared under natural light in the laboratory at 20 ± 1 °C, 65% ± 5% RH. The few remaining larvae were provided foliage from the same branch on which they developed and pupated within several days of collection. All emerged adults were killed by freezing and sexed. One forewing of each female was measured under a binocular microscope with a micrometer. Female forewing length is positively correlated with fecundity [15]. At the end of summer, defoliation on current-year branches was visually estimated, as in [16].

2.4. Statistical Analysis

The independent and interacting effects of the endophyte, phenology, and larval source on larval survival (i.e., second instar to pupation), total survival (i.e., second instar to adult emergence), and adult sex ratio was evaluated using generalized linear mixed effects models with logit link functions and binomial probability distributions. Tree was included as a random factor. All generalized linear mixed effect models were carried out using the *glmer* function from the lme4 package (version 1.1.12) [17] of R (version 3.3.2) [18]. For these and subsequent analyses described below, we inspected residual plots of all models and found no obvious trends or heteroscedasticity. We used the *dispersion glmer* procedure from the blmeco package (version 2.1) [19] of R to verify that statistical models were not overdispersed.

We used likelihood ratio (LR) tests, obtained through the *anova* function in R, to evaluate the contribution of fixed effects. First, we evaluated the contribution of the interaction between endophyte and either larval source or phenology. When an interaction was not significant, the significance of main effects was determined by comparing models with one of the fixed effects to models with both fixed effects.

The effects of endophyte, phenology or larval source on the wing length of female survivors were examined using linear mixed effects models, with tree included as a random factor, using the *lmer* function in the lme4 package [17] of R. We subjected defoliation estimates, which were

non-count proportion data, to logit transformation before analysis; we used the "empirical logit", log[(y + ε)/(1 − y + ε)], where ε is the smallest non-zero proportion observed because our data included values of 0 and 1 [20]. LR tests, described above, were used to test the significance of fixed factors. As expected, defoliation was very low (18.2 ± 1.2%, $N = 110$) and neither the independent nor interacting effect of endophyte was significant ($p \geq 0.3760$).

3. Results

Survival of second-instar larvae until pupation or adult emergence (i.e., larval and total survival, respectively) was significantly influenced by the main effects of endophyte, budburst phenology and insect source but not by any two- or three-way interactions (Table 1). Total survival of larvae developing on endophyte-inoculated trees was lower than that of larvae on control trees (Figure 1b). A similar trend is evident for larval survival (Figure 1a) but the effect of endophyte was marginally insignificant (Table 1). Larval and total survival was reduced by approximately 12% and 17% when developing on endophyte-inoculated compared to endophyte-free trees (Figure 1).

Table 1. Summary of generalized linear mixed models evaluating the influence of a native endophytic fungus, larval source, and phenology on larval (i.e., second instar to pupa) and total (i.e., second instar to adult emergence) survival, adult sex ratio and female wing lengths of eastern spruce budworm reared on 14 white spruce trees with and 14 trees without the endophyte in 2012. Second-instar larvae from a laboratory colony or field population (insect source) were placed in the mid-crown of study trees approximately one or two weeks before budburst (phenology). Tree was included as a random variable in the mixed effects models (either GLMM with logit link or LMM).

Response Variable	Source of Variation	df	X^2	p
Larval Survival	Endophyte	1	3.3672	0.0665
	Insect source	1	21.3920	**<0.0001**
	Phenology	1	9.6566	**0.0019**
	Endophyte:Insect source	1	0.5317	0.4659
	Endophyte:Phenology	1	1.3282	0.2491
	Insect source:Phenology	1	0.0090	0.9244
	3-way interaction	1	1.0769	0.2994
Total Survival	Endophyte	1	9.0715	**0.0026**
	Insect source	1	25.1450	**<0.0001**
	Phenology	1	9.2577	**0.0023**
	Endophyte:Insect source	1	0.0045	0.9468
	Endophyte:Phenology	1	2.4841	0.1150
	Insect source:Phenology	1	0.6497	0.4202
	3-way interaction	1	3.3404	0.0676
Sex Ratio	Endophyte	1	0.0367	0.8480
	Insect source	1	0.3793	0.5380
	Phenology	1	1.104	0.2942
	Endophyte: Insect source	1	0.1281	0.7204
	Endophyte:Phenology	1	0.4997	0.4797
	Insect source:Phenology	1	0.9684	0.3251
	3-way interaction	1	2.8481	0.0915
Female Wing Length	Endophyte	1	0.7665	0.3813
	Insect source	1	7.9002	**0.0049**
	Phenology	1	0.0073	0.9321
	Endophyte:Insect source	1	2.6551	0.1032
	Endophyte:Phenology	1	1.0289	0.3104
	Insect source:Phenology	1	1.1638	0.2807
	3-way interaction	1	0.0809	0.7761

Note: Significant p values are presented in bold type.

Both larval and total survival of wild budworm was significantly higher than that of lab-reared budworm (Figure 2, Table 1). Larval and total survival were approximately 26% and 33% higher for wild than lab-reared budworm (Figure 2). Larval and total survival of budworm placed on trees approximately a week before budburst was approximately 15% and 16.5% higher, respectively, than that for larvae placed on trees two weeks before budburst (Figure 3, Table 1).

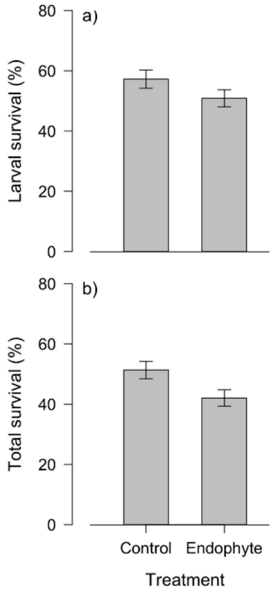

Figure 1. Mean (± SE) survival of second-instar eastern spruce budworm (**a**) to pupation (i.e., larval survival) and (**b**) to adult emergence (total survival) on white spruce trees with (Endophyte) or without (Control) a native endophytic fungus. n = 14 control and 14 endophyte trees.

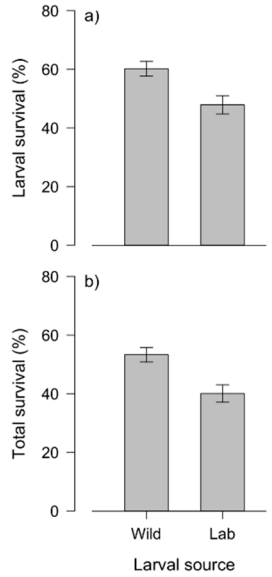

Figure 2. Influence of larval source on mean (± SE) survival of second-instar eastern spruce budworm (**a**) to pupation (i.e., larval survival) and (**b**) to adult emergence (total survival) on white spruce trees. Second instars were obtained from a wild population (Wild) or from a laboratory colony (Lab). n = 14 control and 14 endophyte trees.

The sex ratio of emerged adults was not influenced by the main or interacting effects of endophyte, phenology or insect source (Table 1). Similarly, the wing lengths of emerged females was not influenced by the main or interacting effects of endophyte and phenology. However, the wing lengths of

wild budworm females were slightly but significantly longer than those from the lab-reared colony (1.24 ± 0.01 versus 1.21 ± 0.01 cm, Table 1).

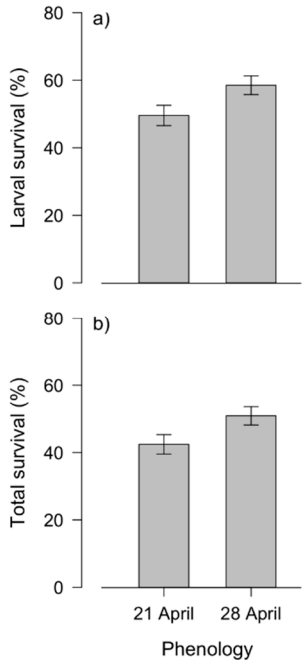

Figure 3. Influence of spring phenological synchrony between the date of emergence of eastern spruce budworm and of budburst of white spruce trees on mean (± SE) survival (**a**) to pupation (i.e., larval survival) and (**b**) to adult emergence (total survival) on white spruce trees. Second-instar larvae were placed on 28 trees approximately one (28 April 2012) or two (21 April 2012) weeks before budburst.

4. Discussion

Inoculation of study trees with a native endophytic fungus >10 years prior to the current study increased tree defense against a major forest pest. Most importantly, the endophyte was as effective against larger, wild budworm as it was against budworm from a laboratory colony. Reductions of approximately 17% in total survival of wild and lab-reared budworm, under two different phenological conditions, was similar to that reported in a study carried out with lab-reared budworm in the same study plots the two previous years [11]. The majority of budworm mortality attributable to the endophyte occurred during larval development and the presence of the endophyte did not influence adult size or sex ratio.

The present data indicate that budworm survival was not influenced by interactions between the endophyte and budburst phenology or insect source. In the earlier study, in which budworm were placed on the tree at one time point, interactions between the endophyte and crown level or insect density influenced budworm survival. The lack of an interaction in the present study is presumably not due to a lack of sufficient variation in these two variables because both insect source and budburst phenology independently influenced budworm survival.

The endophyte was as efficient in reducing the survival of wild larvae as it was in reducing the survival of laboratory-reared larvae, as indicated by the lack of an interaction between insect source and endophyte. This suggests that the endophyte may be effective against a range of budworm phenotypes. Wild and lab-reared budworm in the current study originated from different budworm populations and had experienced different environmental conditions prior to the field study.

Higher survival for larvae that were placed on study trees approximately one week before budburst than for those placed two weeks before budburst is probably due to either reduced success choosing and mining old needles or reduced nutritional quality of old needles until budburst. Second-instar budworm mine into old foliage in spring, where they feed and obtain some nutritive benefit [21],

and remain there until budburst, when they move to feed on the bursting buds [12]. The study plots received approximately 2 cm of rain on 21 April 2012, the first date that budworm were placed on study trees, and 2 cm the next day, but did not receive any precipitation on 28 April 2012, the second date when budworm were placed on study trees [22]. Second-instar budworm are very small, and driving rain against the sleeve cages may have dislodged some from the branch surface or water entering the cages may have drowned others.

Following a manipulated field study carried out with laboratory-reared budworm on white spruce, Lawrence et al. [12] reported that budworm survival was highest when second instars were placed on buds 1–3 weeks before budburst, and that the survival of individuals was slightly higher when placed on trees two rather than one week before budburst. Thus, we speculate that the lower survival of larvae placed on trees two, as opposed to one, week before budburst was primarily due to reduced needle colonization success, due to inclement weather.

5. Conclusions

The present study extends previous field experiments carried out with lab-reared larvae and demonstrates that a native endophytic fungus reduces the survival of wild individuals of the major pest of coniferous trees in eastern North America. Interestingly, although budworm survival was influenced by spring larval emergence/host tree budburst synchrony and whether juveniles were wild or from a laboratory colony, the endophyte reduced budworm survival regardless of spring emergence/budburst synchrony and regardless of whether individuals were wild or laboratory-reared. Importantly, the endophyte was effective at relatively low larval densities and, thus, could offer a complementary tactic for hindering spruce budworm population growth in the context of an Early Intervention Strategy [23].

Author Contributions: D.Q., S.E., L.F. and J.D.M. contributed to the general study conceptualization; L.F., J.D.M., S.E. and D.Q. supervised and conducted the laboratory and/or field observations and measurements; D.Q. and S.E. carried out the statistical analyses and made figures; and D.Q. was responsible for funding acquisition and project administration. All authors interpreted the data and contributed substantially to manuscript preparation, development and revision.

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