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Influence of bluestain fungi on laboratory rearing of pinewood nematode infested beetles

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Summary – This study compared the number of *Bursaphelenchus xylophilus* carried by *Monochamus carolinensis* beetles which developed in fungus-inoculated and non-inoculated pine bolts. Beetles that emerged from pine bolts inoculated with the bluestain fungus *Ophiostoma minus* carried a greater mean number (8198) of *B. xylophilus* dauer juveniles than beetles that emerged from non-fungal bolts (5570). Nematode density was higher in the fungal bolts than in the control bolts but the difference was not significant. The proportion of emerged beetles that carried nematodes was not dependent on inoculation treatment. Fungal inoculation did not affect the number of days required for beetle development from oviposition to adult emergence. The method used for the laboratory culture of *B. xylophilus* infested beetles in this study allows for laboratory production of beetles suitable for use in studies on the interspecific association between *B. xylophilus* and its vectors in the genus *Monochamus*.

Résumé – ***Influence du champignon agent du bleuissement du bois de pin sur l'élevage au laboratoire d'insectes infestés par* Bursaphelenchus** – Au cours de cette étude sont comparés les nombres de *Bursaphelenchus xylophilus* transportés par *Monochamus carolinensis* lorsque l'insecte se développe dans des bûchettes de pin inoculées avec le champignon *Ophiostoma minus*, cause de la coloration du bois et dans des bûchettes non inoculées. Les insectes émergeant des bûchettes inoculées contiennent un plus grand nombre (8198) de « dauer » juvéniles de *B. xylophilus* que les insectes émergeant de bûchettes non inoculées (5570). La densité des nématodes est plus élevée dans les bûchettes infectées par le champignon que dans les témoins, mais la différence n'est pas significative. La proportion d'insectes émergeant transportant des nématodes ne dépend pas de l'inoculation du champignon. Cette inoculation n'affecte pas le nombre de jours requis pour le développement de l'insecte, de l'oviposition à l'émergence des stades adultes. La méthode décrite dans cette étude pour l'élevage au laboratoire d'insectes vecteurs de *B. xylophilus* permet d'obtenir des insectes utilisables en vue d'études d'association entre *B. xylophilus* et ses hôtes vecteurs du genre *Monochamus*.

Key-words : *Bursaphelenchus, Monochamus, Ophiostoma,* pinewood nematode, rearing.

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, is vectored by adult pine sawyers in the genus *Monochamus* (Linit, 1988). The nematode is introduced into pine trees when adult beetles in the genus *Monochamus* feed on branches of healthy trees (Luzzi *et al.*, 1984; Linit, 1990) or oviposit on stressed pine trees (Wingfield, 1983; Luzzi *et al.*, 1984; Edwards & Linit, 1992). The nematode population increases rapidly within susceptible trees feeding on epithelial cells of resin canals (Mamiya, 1984) and fungi introduced by secondary insects. *Monochamus* larvae hatch from eggs deposited in the phloem of dying or recently cut pine trees. Late instar larvae tunnel into the xylem and form a pupal cell near the wood surface (Anon., 1985 *b*). Fourth-stage dauer juveniles enter the respiratory system of teneral adult *Monochamus* spp. and are carried to new host trees or logs.

The number of dauer juveniles carried by an individual beetle varies within and between populations of beetles (Togashi, 1985; Linit, 1988). Mamiya (1984) reported that the number of dauer juveniles carried by a beetle is related to environmental conditions within the pupal chamber. The number of nematodes carried by a beetle may also be related to the presence of fungi in dying trees. Several genera of fungi have been identified as potential food sources for the pinewood nematode in dying pine trees. Kobayashi *et al.* (1974) found that *B. xylophilus* would multiply on four genera of fungi isolated from dying pine trees. Of these, *Ophiostoma* was the predominant genus found in pupal chambers and on adults of *Monochamus alternatus* Hope.

Studies of the interspecific relationship of the nematode and its vector require a continuous supply of nematode infested beetles. *Monochamus carolinensis* (Olivier), the principal vector of the nematode in the midwestern United States (Linit, 1988), can be reared in the laboratory using artificial diet (Pershing & Linit, 1989) or in pine bolts (Linit, 1985). The pine bolt method was modified to obtain nematode infested beetles for previous studies on the nematode-beetle relationship (Linit, 1990; Edwards & Linit, 1992). We report here on the influence of *Ophiostoma minus* (Hedgc.) Syd. & P. Syd.

inoculation of pine bolts on the process of rearing *B. xylophilus* infested beetles.

Materials and methods

Bolts used in this experiment were taken from a 20-year-old (approx.) stand of jack pine (*Pinus banksiana* Lamb.) at the Thomas A. Baskett Wildlife Research and Education Area, Boone County, Missouri, USA. Wood samples were taken from each tree to ensure that no *B. xylophilus* were present. Two bolts, 35-cm long by 12 to 15-cm diameter were cut from each of fifteen trees. A 5-cm section was removed from each bolt to determine moisture content, and the ends of each bolt were coated with paraffin to retard desiccation. Each section was weighed to the nearest 0.1 g, put in an oven (125 °C) to dry, then weighed daily until the reduction from the previous day's weight was less than 1 g. Initial moisture content was expressed as [(wet weight - dry weight)/ wet weight *100].

One bolt from each pair was randomly chosen for inoculation with the bluestain fungus, *O. minus*. A 5 mm diameter circular plug of agar with *O. minus* from a laboratory culture maintained on malt dextrose agar was placed in each of two, 1.25 diameter by 5-cm deep, holes : one hole on each end and opposite side of the bolt. The holes were plugged with styrofoam and sealed with petroleum jelly. The remaining bolt of each pair received no fungal inoculum although each bolt was naturally infested with *Ophiostoma* spp. and other fungi from the field. The purpose of inoculation was to enhance the colonization of *Ophiostoma* in the treatment bolts. No quantitative measure of fungal colonization was made.

One week after fungal inoculation, approximately 500 *B. xylophilus* (all life stages) suspended in 0.5 ml distilled water were inoculated into each of two similar holes drilled on opposite sides of each of the 30 bolts. These holes were placed opposite the fungal inoculation sites or at a similar location on the control bolts. Inoculation holes were plugged and sealed as described above. Nematodes used in this study were isolated from a Scots pine (*P. sylvestris* L.) at the Thomas A. Baskett Wildlife Research and Education Area and reared on *Botrytis cinerea* Pers. on potato dextrose agar using a technique adapted from Southey (1986). Nematodes were extracted from the agar using the modified Baermann technique (Southey, 1986).

After nematode inoculation, all bolts were placed in a screened cage with *M. carolinensis* adults. Individual bolts remained in the cage until at least 20 beetle oviposition sites were found or for a maximum of three days. Bolts were held at 27 °C and 75-80 % relative humidity during beetle development.

Beetles were collected upon emergence from the bolts and macerated. Nematodes were extracted from each beetle using a modified Baermann technique and counted. The number of days from the beginning of oviposition on a particular bolt to emergence of each beetle from the bolt was recorded. Beetle emergence from each bolt was monitored daily until a period of two weeks passed during which no beetles emerged. Five randomly located wood samples (1.25 by 5 cm) were then removed from the bolt with a brace and bit. The samples were weighed and nematodes extracted. The number of third-stage dispersal juveniles, fourth-stage dauer juveniles and other *B. xylophilus* life stages were determined for each sample under a stereo microscope. Dispersal and dauer juveniles were distinguished from other life stages by the dark body coloration due to the presence of lipids. Third-stage dispersal juveniles have a stylet and the abdominal tip is rounded. Dauer juveniles have no stylet and the abdominal tip is pointed.

After nematode extraction each wood sample was dried at 125 °C for approximately 48 hours and percentage moisture content of the wood was calculated as above. Nematode counts were expressed as the number of nematodes per gram of dry wood for statistical comparisons.

Chi-square analysis was used to determine if the proportion of beetles that emerged carrying nematodes was dependent on bolt inoculation treatment. Analysis of variance was used to determine if the number of nematodes carried per beetle differed between inoculation treatments. A similar analysis was conducted to determine if developmental time, the number of days between initiation of oviposition and emergence of the first adult beetle from each bolt, differed between inoculation treatments or among trees. Analysis of the mean developmental time of all beetles that emerged during the study was not conducted because of the arbitrary emergence cutoff (two weeks) criteria used for inclusion of beetles in the study. Correlation analysis was used to determine if there was a relationship between initial or ending moisture content of wood with the time necessary for beetle development, nematode population densities in wood at the end of beetle emergence, or the number of nematodes carried per beetle. Analysis of variance was used to determine if inoculation treatment affected within-wood population density of *B. xylophilus* (all life stages) and within-wood density of third-stage dispersal juveniles and fourth-stage dauer juveniles. A logarithmic transformation, $log\ (x + 1)$, was performed to insure normality of nematode density data. Statistical analyses were conducted using the Statistical Analysis System (Anon., 1985 *a*).

Results

A total of 213 beetles emerged from the 30 bolts used in the study : 99 beetles from fungus-inoculated bolts, 114 from control bolts (Table 1). The proportion of emerged beetles that carried nematodes was not dependent on inoculation treatment ($\chi^2 = 0.15$; $df = 1$;

Table 1. *Number of pinewood nematodes per beetle emerging from bolts inculated with* Bursaphelenchus xylophilus *and* Ophiostoma minus, *and from bolts inoculated with* B. xylophilus *only.*

Treatement	No of beetles		No of nematodes		
	Total	With nematodes	Mean (sd)	Minimum	Maximum
B. xylophilus + *O. minus*	99	86	8198 (9266)	0	37 750
B. xylophilus	114	101	5570 (7670)	0	48 100

P = 0.70). Beetles that developed within the fungus inoculated bolts carried a significantly *(F = 5.00; df = 1.14; P = 0.04)* greater mean number of nematodes than beetles that developed within control bolts (Table 1).

Nematode population density (all life stages) was higher in the fungal bolts than in the control bolts but the difference was not significant *(F = 0.14; df = 1.14; P = 0.71)* (Table 2). Similar trends were noted for third-stage dispersal juveniles and fourth-stage dauer juveniles combined *(F = 0.16; df = 1.14; P = 0.70)* and for dauer juveniles alone *(F = 0.13; df = 1.14; P = 0.73).*

Mean developmental time until first beetle emergence differed significantly among the fifteen trees *(F = 3.01; df = 14.14; P = 0.02)* but not between fungal inoculation treatments *(F = 1.22; df = 1.14; P = 0.29).* First beetle emergence from each fungus inoculated bolt (n = 15) occurred 50-73 days after egg deposition. First beetle emergence from the control bolts (n = 15) took 51-71 days.

Beetle developmental time was negatively correlated *(r = – 0.27; P < 0.01)* with initial moisture content of the wood and the number of nematodes carried per beetle was positively correlated with final moisture content of the wood *(r = 0.27; P < 0.01).* There was no relationship between final moisture content and within-wood density of *B. xylophilus* life stages : all life stages *(r = 0.11; P = 0.17),* third-stage dispersal juveniles and fourth-stage dauer juveniles combined *(r = 0.08; P = 0.31),* dauer juveniles only *(r = 0.02; P = 0.84).*

Conclusions

Dwinell (1986) reported that bluestain fungi enhanced the growth of *B. xylophilus* populations in pine wood chips : the population density of *B. xylophilus* in pine wood chips with bluestain was *ca* 1.3 times greater than in chips without the fungus. In the present study, total nematode population density and the population density of third-stage dispersal juveniles and fourth-stage dauer juveniles were 1.5 times greater in *O. minus* inoculated bolts, although the differences were not significant. Beetles that emerged from bolts inoculated with *O. minus* carried a significantly higher number of nematodes than beetles from non-inoculated bolts. The increase in the number of nematodes carried by beetles from fungus inoculated bolts compared to those from non-inoculated bolts (1.5 ×) was similar to the increase observed in the within-wood *B. xylophilus* densities.

Linit *et al.* (1983) reported the range in number of nematodes per *M. carolinensis* adult was 0-79 000 ($\bar{x}$ = 19 151) for field collected beetles. Similar values were reported by Malek and Appleby (1984) for field-collected *M. carolinensis* in Illinois. Wingfield and Blanchette (1983) reported mean nematode numbers of 4538-10 516 for *M. carolinensis* collected from field grown Austrian and red pines in Minnesota. Mean nematode numbers for beetles in the present study were within the range of means reported above, with the mean for beetles reared in fungus inoculated bolts closer to the midrange of means reported in other studies. The frequency of beetles that carried nematodes did not differ between treatments and was similar to those reported for *M. carolinensis* captured during emergence from

Table 2. *Number of pinewood nematodes, all life stages and dispersal stages, recovered from bolts inoculated with* Bursaphelenchus xylophilus *and* Ophiostoma minus, *and from bolts inoculated with* B. xylophilus *only.*

Nematode life stage(s)[a]	*Treatment*	*Mean*[b] *(sd)*	*Minimum*	*Maximum*
All life stages	*B. xylophilus* & *O. minus*	34.43 (70.83)	0	433
	B. xylophilus only	22.66 (35.09)	0	182
Dispersals and dauers	*B. xylophilus* & *O. minus*	30.96 (68.42)	0	433
	B. xylophilus only	19.57 (31.50)	0	182
Dauers only	*B. xylophilus* & *O. minus*	2.14 (11.17)	0	94
	B. xylophilus only	1.19 (4.75)	0	32

[a] Dispersals = third-stage dispersal juveniles, Dauers = fourth-stage dauer juveniles.
[b] Each mean is based on 75 wood samples. All means expressed as number of nematodes per gram of dry wood.

naturally infested trees (Linit *et al.*, 1983; Wingfield & Blanchette, 1983).

The method used for the laboratory culture of *B. xylophilus* infested beetles in this study allows for laboratory production of beetles suitable for use in studies on the interspecific association between *B. xylophilus* and its vectors in the genus *Monochamus*.

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