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Effect of *Bursaphelenchus xylophilus* on the Assimilation and Translocation of ^{14}C in *Pinus sylvestris*

H. Melakeberhan and J. M. Webster[1]

Abstract: The effect of wound, wound + water, wound + *Bursaphelenchus xylophilus* culture filtrate, or wound + lethal *B. xylophilus* doses on the assimilation and translocation of ^{14}C by 8-month-old *Pinus sylvestris* seedlings was tested. In two separate experiments, pine seedlings were exposed to 28.35 μCi of $^{14}CO_2$ for 20 minutes below or above (to the pine shoot leader) the point of nematode inoculation. After 2 and 4 hours of dark adaptation, 80% ethanol soluble ^{14}C tissue extracts were determined by liquid scintillation counting. Nematode infection significantly ($P = 0.05$) decreased ^{14}C assimilation. Treatments translocated less than 6% of the total amount of the fixed ^{14}C and translocation generally decreased with increasing size of nematode inoculum. However, nematode-infected pines translocated a greater proportion of the amount of ^{14}C fixed per gram of exposed plant tissue than did the control pines. The lower levels of photoassimilate entering the plant system probably resulted in a reduced metabolic capacity in *B. xylophilus*-infected pine seedlings. The effect on photosynthesis could be one of the key factors leading to death of pines through starvation, and it is possible that it was preceded by an effect on related physiological processes such as water uptake.

Key words: Bursaphelenchus xylophilus, $^{14}CO_2$ photosynthesis, pinewood nematode, *Pinus sylvestris*, translocation.

Chlorosis and wilting are two major symptoms of pine wilt disease caused by *Bursaphelenchus xylophilus* (Steiner & Buhrer) before death of the trees. In addition to the presence of the nematode, certain environmental stress conditions must prevail for symptoms to occur or for the trees to die. The stress conditions that have been suggested and (or) demonstrated include high temperature (23), low relative humidity (25), lack of water (26), and (or) nutrients, and infection by other pathogens (4). Under such conditions *B. xylophilus* may function as a primary pathogen. Reports on the timing of the physiological response and mortality of *B. xylophilus*-infected pines vary from that of observations at the cellular level at 3 days postinfection (17) to decreased transpiration rate and pine wilt at 20–30 days (7,13,18) to pine mortality at several months (5,15) postinfection.

The formation of girdling cankers (1) and the physical blockage of the vascular tissue (7,13), either by the presence of the nematodes or the resin production in the tissues (26), are commonly held hypotheses on the mechanisms of nematode-induced mortality in pine trees. A partial or full blockage of the vascular tissue probably would decrease the upward movement of water and minerals which, in turn, would affect photosynthesis, and could result in nonsynchronous shoot and root development (10). *Bursaphelenchus xylophilus* is mainly a shoot pathogen that may affect the translocation of photosynthate beyond the infected tissues, leading to death of trees through lack of energy for growth and health. However, how and when the nematode affects photosynthesis, and whether it is an effect on the upward or downward translocation of substances that influences the disease syndrome is not known. The objectives of this study were to determine the effect of *B. xylophilus* on 1) the photosynthesis of *Pinus sylvestris* L. and 2) the translocation of photosynthate below and above the point of nematode inoculation.

Materials and Methods

Plant growth and nematode inoculations: Growth conditions for pines and culturing and inoculation techniques for *B. xylophilus* (a British Columbia isolate) were as described previously (20) except that the Scots pines (*Pinus sylvestris* L.) were 8-month-old seedlings and nematodes were inoculated

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[1] Department of Biological Sciences, Simon Fraser University, Burnaby, Vancouver, British Columbia, Canada V5A 1S6.

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through a 1.5-cm vertical slit cut in the bark with a razor blade at 12–14.5 cm from the base of the stem.

Experiments: Two experiments, each with four replicates, were done using pine seedlings 22.7 ± 1.4 cm tall. In the first experiment, the $^{14}CO_2$ fixation and downward translocation in each of 28 seedlings was measured at 64–88 ($\bar{x} = 76 \pm 12$) hours after treatment as follows: 1) wound alone, 2) wound + water, 3) wound + nematode culture filtrate, 4) wound + 10,000, 5) wound + 20,000, 6) wound + 40,000 *B. xylophilus* per plant, and 7) untreated checks. The nematode culture filtrate was a nematode-free, filtered aqueous suspension washed from the lids of agar plates on which *B. xylophilus* had been cultured. Nematode inocula selected to represent a range of lethal doses (20) were applied 12–13.5 cm from the base of the stem. The upper 8.5 cm of the leader of each seedling starting ca. 1 cm above the point of nematode inoculation was exposed to $^{14}CO_2$.

In the second experiment, one nematode level, 20,000 *B. xylophilus* per plant, and the same control treatments as in experiment 1 were compared for fixation of $^{14}CO_2$ and the translocation of photosynthate above and below the point of $^{14}CO_2$ exposure and nematode inoculation at 116–136 ($\bar{x} = 126 \pm 10$) hours after inoculation. The nematode inoculation point was at 13–14.5 cm from the base of the stem; the $^{14}CO_2$ was introduced into 5.7 ± 0.8 cm of the stem of each of 20 seedlings starting ca. 1 cm below the point of nematode inoculation.

$^{14}CO_2$ labelling and dark adaptation: The required amount of $^{14}CO_2$ was produced by mixing aqueous ^{14}C sodium bicarbonate (56.6 mCi/mmole specific activity) with 2.5 ml 1 N H_2SO_4 in a closed photosynthesis measuring system (11,28). In order to determine the exact amount of $^{14}CO_2$ being released within 20 minutes (an arbitrarily determined time of exposure to $^{14}CO_2$) and to ensure that $^{14}CO_2$ was not a limiting factor, 18.9 μCi of $^{14}CO_2$ was released into the photosynthesis measuring system. After 15 minutes of stabilization, the $^{14}CO_2$ was circulated for 20 minutes through a flask containing 250 ml 20-methoxyethylamine (Carbo-Sorb, Canberra Packard, Canada), a high capacity $^{14}CO_2$ absorber. The procedure was repeated three times. An average of 6.125 μCi (32.41% of the amount introduced into the system) was recovered from the absorber in that time. To account for what may have been lost in the system, the amount of $^{14}CO_2$ was increased to 28.35 μCi for the rest of the experiments.

Before being placed in the $^{14}CO_2$ feeding system, all plants were removed from the growth chamber, watered to saturation, and kept for 2 hours on a laboratory bench under fluorescent light (300 μE m^{-2} s^{-1}). The $^{14}CO_2$ was mixed in the system for 15 minutes, and the portion of each plant to be exposed to $^{14}CO_2$ was sealed in a plexiglass chamber under a sodium-iodide lamp (550 μE m^{-2} s^{-1}) and exposed to $^{14}CO_2$ for 20 minutes.

To account for the predetermined location of $^{14}CO_2$ exposure and nematode inoculation and for the time required for the ^{14}C translocation beyond the point of inoculation, dark adaptation periods of 2–8 hours were tested. Six pines of the same age and similar size as the experimental pines were exposed to $^{14}CO_2$ above the point of nematode inoculation (as in experiment 1) and six were exposed to $^{14}CO_2$ below the point of inoculation (as in experiment 2), and the two groups were dark adapted for 4 and 8 hours and 2 and 4 hours, respectively. A measurable amount of ^{14}C activity was present at the earliest sampling times in the unexposed plant parts. Hence, the plants were dark adapted for 4 hours (experiment 1) and 2 hours (experiment 2) after exposure to $^{14}CO_2$ and before extraction of ^{14}C.

Quenching coefficients and extraction of ^{14}C labelled assimilate: Quenching coefficients and specific activity (amount of $^{14}CO_2$ per unit plant weight) and optimum scintillation sample volume were simultaneously determined from uninfected pines (9) by adding 20 μCi $^{14}CO_2$ to shoot and root extracts of the same age pines as the experimental pines after boiling in 80% ethanol

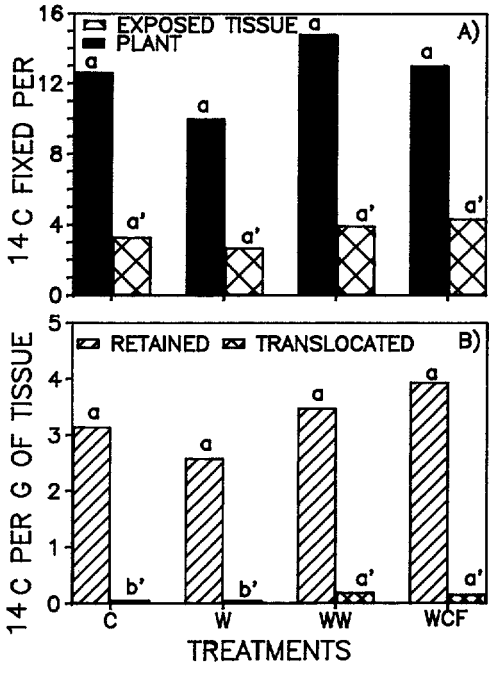

FIG. 1. Comparison of the influence of different types of control treatments of untreated check (C), wound (W), wound + water (WW), and wound + *Bursaphelenchus xylophilus* culture filtrate (WCF) on the amount of ^{14}C (μCi) assimilated by 8-month-old *Pinus sylvestris*. A. Per plant and per gram of exposed tissue. B. Retained at the exposed site or translocated to below the point of treatment. Comparisons apply to bars of the same shade. Bars with the same letter are not significantly different ($P = 0.05$) from each other.

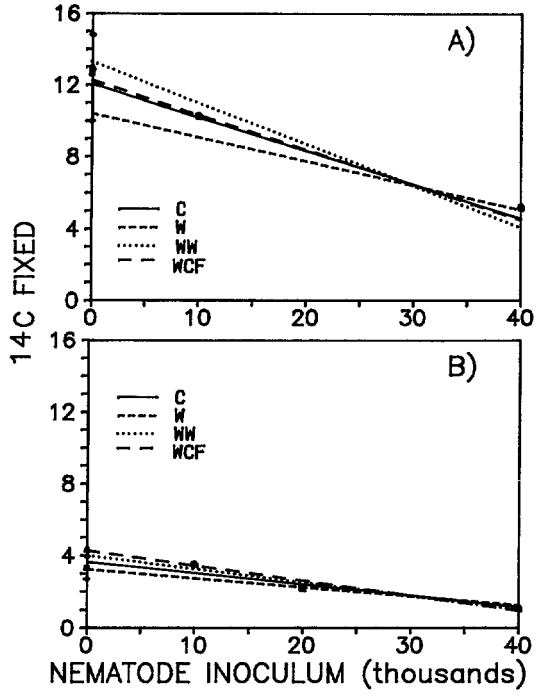

FIG. 2. Comparison of untreated control (C), wound (W), wound + water (WW), or wound + nematode culture filtrate (WCF), with increasing *Bursaphelenchus xylophilus* inoculum in the amount of ^{14}C (μCi) assimilated by 8-month-old *Pinus sylvestris*. A. Per plant. C = 12.03 − 0.0002X, r^2 = 0.22; W = 10.45 − 0.00013X, r^2 = 0.17; WW = 13.335 − 0.00023X, r^2 = 0.36*; WCF = 12.25 − 0.0002X, r^2 = 0.29*. B. Per gram of exposed tissue. C = 3.62 − 0.00006X, r^2 = 0.33**; W = 3.254 − 0.00005X, r^2 = 0.29*; WW = 4.003 − 0.00007X, r^2 = 0.51**; WCF = 4.25 − 0.00008X, r^2 = 0.55**.

(27) for 8 minutes. Based on a series of extractions that received 20 μCi of $^{14}CO_2$, an 800-μl scintillation sample volume was used. Each sample containing ethanol soluble ^{14}C tissue extracts was analyzed for 15 minutes using a liquid scintillation counter (9).

After dark adaptation, roots were gently washed free of soil and each plant was cut and separated into $^{14}CO_2$-exposed (source) shoot, unexposed shoot, and root samples. Each sample was further cut into ca. 1-cm-long pieces and separately boiled as described in the previous section. The aqueous suspensions were saved and the plant tissues were discarded. Total specific activity of ^{14}C per sample was converted into μCi for data analyses (9).

Data analyses: Data from experiment 2 and from the control treatments of experiment 1 were analyzed by ANOVA and the means were compared by Duncan's multiple-range test. Each of the control treatments of experiment 1 was compared with increasing nematode treatment by regression.

RESULTS

Experiment 1: There were no visible disease symptoms on any treated pine seedling over the duration of the experiment. The control treatments did not differ in the total ^{14}C fixed per plant or per gram of $^{14}CO_2$-exposed tissue (Fig. 1A) retained in the exposed tissue or translocated to below the point of nematode inoculation (Fig. 1B). However, all control treatments fixed more ($P = 0.05$) $^{14}CO_2$ per plant (Fig. 2A) and per gram of exposed tissue (Fig. 2B)

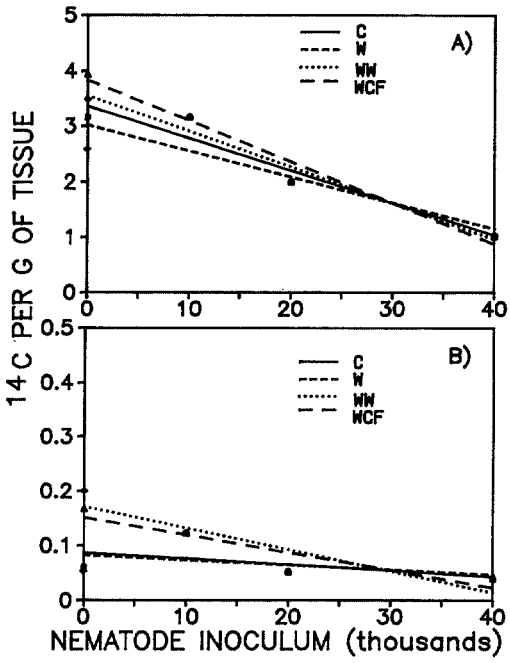

FIG. 3. Comparison of untreated control (C), wound (W), wound + water (WW), or wound + nematode culture filtrate (WCF) with increasing *Bursaphelenchus xylophilus* inoculum in the translocation of ^{14}C (μCi) by 8-month-old *Pinus sylvestris*. A. Retained at the exposed site. C = 3.534 − 0.000058X, r^2 = 0.33*; W = 3.013 − 0.000047X, r^2 = 0.28*; WW = 3.553 − 0.000065X, r^2 = 0.44**; WCF = 3.828 − 0.000074X, r^2 = 0.51**. B. Translocated to below the point of inoculation. C = 0.088 − 0.000001X, r^2 = 0.09; W = 0.0835 − 0.0000009X, r^2 = 0.08; WW = 0.1716 − 0.0000039X, r^2 = 0.55**; WCF = 0.1515 − 0.000003X, r^2 = 0.47**.

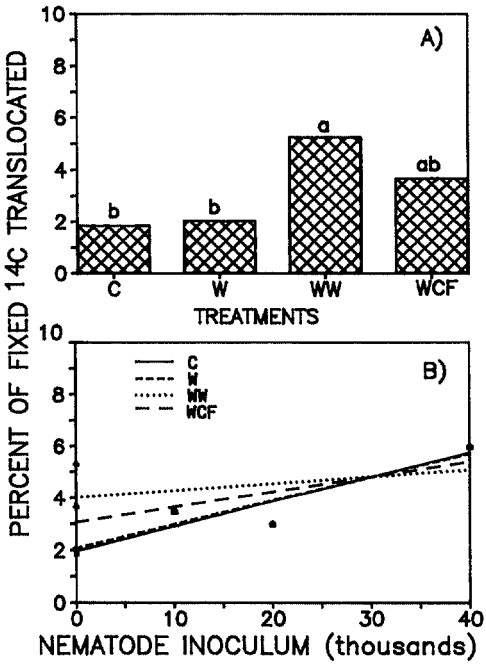

FIG. 4. The percentage of fixed ^{14}C translocated in 8-month-old *Pinus sylvestris*. A. In untreated control (C), wound (W), wound + water (WW), and wound + nematode culture filtrate (WCF). B. Comparisons between C, W, WW, or WCF with increasing *Bursaphelenchus xylophilus* inoculum. C = 1.919 + 0.0000953X, r^2 = 0.40**; W = 2.027 + 0.0000917X, r^2 = 0.38*; WW = 3.967 + 0.0000271X, r^2 = 0.03; WCF = 3.016 + 0.0000588X, r^2 = 0.20.

than did nematode-infected pine seedlings. The amount of ^{14}C retained in the $^{14}CO_2$-exposed tissues decreased (P = 0.05) with increasing nematode inoculum (Fig. 3A). Although the amount of ^{14}C translocated through the site of nematode inoculation decreased with increasing inoculum levels, only the water and culture filtrate treatments were different (P = 0.01) from nematode treatments (Fig. 3B). The maximum ^{14}C translocated below the point of nematode inoculation was less than 6% of the total ^{14}C fixed. Relative to the amount of ^{14}C fixed per gram of exposed tissue, however, the water (Fig. 4A) and nematode treatments (Fig. 4B) translocated significantly (P = 0.05) more than the check and wound treatments.

Experiment 2: The nematode-infected pines began to show signs of needle wilting and discoloration around the inoculation sites at the time of sampling. For the same age plants, the amount of ^{14}C fixed in this experiment (Fig. 5A) was about one-third that of the first experiment (Fig. 2). On a per gram of exposed tissue basis, the check and filtrate treatments, and on a per plant basis, all the controls except the water treatment fixed more (P = 0.05) $^{14}CO_2$ than nematode treatment (Fig. 5A). Although nematode treatment resulted in ca. 30–90% decrease in translocated photosynthates, treatments did not differ in the amount of ^{14}C translocated to above or below the point of $^{14}CO_2$ exposure (Fig. 5B). The amount of ^{14}C retained at the exposed site was 71–93% less in nematode-infected pines, and was significantly lower (P = 0.05) than for the wound and culture filtrate treatments

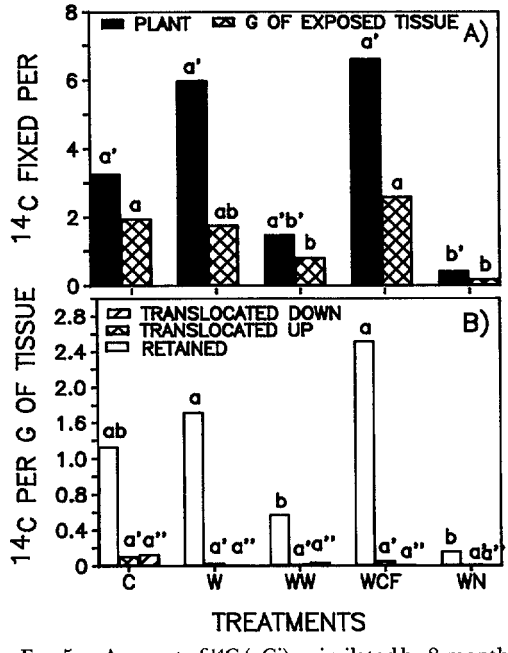

FIG. 5. Amount of ^{14}C (μCi) assimilated by 8-month-old *Pinus sylvestris*. A. Per plant and per gram of exposed tissue. B. Retained at the exposed site or translocated to above and below the point of untreated check (C), wound (W), wound + water (WW), wound + *Bursaphelenchus xylophilus* culture filtrate (WCF) treatment, and wound + *Bursaphelenchus xylophilus* (WN). Comparisons apply to same shape bars only. Bars with the same letter are not significantly different ($P = 0.05$) from each other.

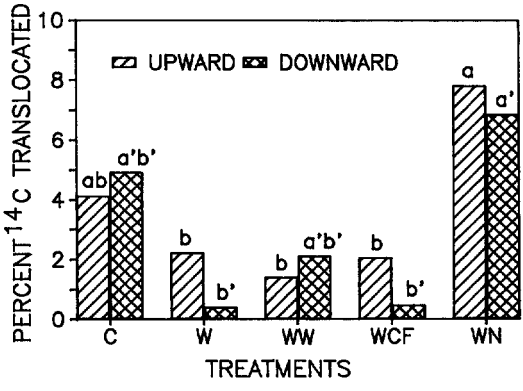

FIG. 6. Comparison of untreated check (C), wound (W), wound + water (WW), wound + *Bursaphelenchus xylophilus* culture filtrate (WCF) treatment, and wound + *Bursaphelenchus xylophilus* (WN) treatment on the percent translocation of total ^{14}C fixed per gram of tissue above and below the point of inoculation by 8-month-old *Pinus sylvestris*. Comparisons apply to same shape bars only. Bars with the same letter are not significantly different ($P = 0.05$) from each other.

(Fig. 5B). Relative to the amount of ^{14}C fixed per gram of exposed tissue, nematode-infected pines translocated upward a larger ($P = 0.05$) percentage than did all the controls except the check and translocated downward more than the wound and culture filtrate treatments (Fig. 6).

DISCUSSION

Bolla et al. (2) speculated that an effect of *B. xylophilus* on photosynthesis of pines might lead to starvation of the pine and might be responsible for the pine wilt disease itself. The data resulting from this study show the effect of *B. xylophilus* on $^{14}CO_2$ fixation and the translocation of photosynthate in pine seedlings. The amount of ^{14}C fixed by all treatments in the first experiment was higher than in the second experiment. In addition, the pine tissue that was exposed to $^{14}CO_2$ was more actively growing in the first than in the second experiment. Hence, the difference between the two sets of experiments in the amount of ^{14}C fixed may reflect the difference in type and activity of tissue exposed to the $^{14}CO_2$ (14). In both experiments, however, the amount of ^{14}C fixed declined with nematode infection, more so in the second experiment which lasted longer. Therefore, *B. xylophilus* appears to decrease photosynthesis, and this might be a factor limiting growth of the host and one of the main causes of death of pine trees. The timing and sequence of events leading to this decreased photosynthesis and translocation process seem complex. Although the exact mechanisms of how *B. xylophilus* affects photosynthesis are not known, the decrease in ^{14}C fixation when $^{14}CO_2$ was introduced above the point of nematode inoculation and when there were no visible disease symptoms suggests a possible effect on water uptake (7,25). The decrease in photosynthesis in experiment 2 indicates an effect on water uptake as well as nematode-induced host physiological changes (16,21) and (or) toxicity, possibly originating from the nematode (22,24) or the result of cavitation (12,13), which led to visible disease expression.

The amount of ^{14}C retained at, or translocated from, the source had a similar trend to the amount of ^{14}C fixed. In experiment 1, a high retention of ^{14}C at the source probably indicated a demand for photosynthate by actively growing tissues (28) whereas in experiment 2 it may have reflected a slow rate of translocation, possibly because of the physiological age of the $^{14}CO_2$ exposed tissue (11). In comparing the treatments, however, the decreased ^{14}C translocation with increasing duration of nematode infection emphasizes the importance of the early effects of nematodes on the physiological processes and of our understanding of the sequence of events before total host-plant destruction occurs.

Whether it is nematode-produced toxins (3,24), cavitation (12,13) leading to blockage of nematode-reduced water uptake (25,26), the massive number of nematodes, or the reduced photosynthesis that results in the death of a pine is not clear. All these factors could play significant roles in the death process and, although not independent of each other, they can be divided into primary and secondary factors. For example, when *B. xylophilus* causes vascular destruction, water uptake and translocation are the primary factors directly affected which, in turn, secondarily affect photosynthesis. The present study further indicates an effect on photosynthesis that is likely to affect the energy demand and partitioning process as well as host physiological efficiency (19). For example, in response to *B. xylophilus* infection the host synthesizes monoterpenes, such as β-myrcene, which increase the transmigration of the nematode from the sawyer into the pine (8) and its multiplication in the pine (6) and which are toxic to the plant (24). The synthesis of the monoterpenes and other carbohydrates required for defence mechanisms, together with a decrease in photosynthesis, would place high stress on the energy reserve of the host and weaken it (2). Moreover, while the total ^{14}C translocated from the source generally decreased with increasing infection, the nematode-infected pines translocated proportionally larger percentages than the controls. This suggests that the host may be mobilizing its resources for either defense mechanisms (2) or to compensate for nematode-induced damages.

A decreased photosynthetic capacity of the host limits its normal performance. In conclusion, decreased photosynthetic capacity and the diminished translocation of photosynthate products in these young pine seedlings probably point to starvation as one of the primary factors causing pine death, and that the effect on photosynthesis may be preceded by an effect on physiological processes such as water uptake.

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