マツノザイセンチュウによるセルラーゼの分泌とその運動軌跡における検出

誌名	日本林學會誌 = Journal of the Japanese Forestry Society
ISSN	0021485X
著者	山本, 直樹 小谷, 圭司 佐々木, 恵彦 西山, 嘉彦
巻/号	68巻6号
掲載ページ	p. 237-240
発行年月	1986年6月

日林誌 68(6)'86 237～240

論 文

Cellulase Exudation by the Pine Wood Nematode ——Detection of Activity in Its Crawling Track

Naoki YAMAMOTO,* Keiji ODANI,*
Satohiko SASAKI,* and Yoshihiko NISHIYAMA**

YAMAMOTO, Naoki, ODANI, Keiji, SASAKI, Satohiko, and NISHIYAMA, Yoshihiko: **Cellulase exudation by the pine wood nematode—Detection of activity in its crawling track J. Jpn. For. Soc. 68: 237～240, 1986** Cellulase activity was detected in the supernatant of suspension of pine wood nematodes kept at 0°C for 12 hours, and the amount of the enzyme in the supernatant increased gradually in the following 36 hours. The isozyme pattern of the cellulase observed was similar to that detected in the homogenates of the nematodes. Nematodes placed on a polyacrylamide gel plate containing carboxymethyl cellulose left their crawling track on the gel plate by exuding cellulase outside. Feeding of the supernatant to pine seedlings induced partial necrosis of the needles. These results suggest that the pine wood nematodes exude cellulase, and induce the early symptom of the pine wilt disease.

山本直樹・小谷圭司・佐々木恵彦・西山嘉彦: **マツノザイセンチュウによるセルラーゼの分泌とその運動軌跡における検出** 日林誌 **68: 237～240, 1986** セルラーゼ活性をマツノザイセンチュウの 0℃, 12 時間けん濁液上清に認めた。その活性は，けん濁開始後 36 時間の間，徐々に増加した。セルラーゼのアイソザイムパターンは，線虫のホモジネートからえられたものと同じであった。CMC をふくむポリアクリルアミドゲルのプレート上をはわせた線虫の運動軌跡は，セルラーゼ活性により検出できた。けん濁液上清をマツ苗木に投与すると，針葉の部分的枯死をひきおこした。マツの材線虫はセルラーゼを外部に分泌してマツの材線虫病の初期症状をひきおこすことを示唆している。

I. Introduction

In localized regions around resin canals of stems infected by the pine wood nematodes, abnormal leakage of the oleoresin and its diffusion into adjacent tracheids were observed. The lesions widened as the disease developed, and finally complete blockage of the sap flow was observed (SASAKI *et al.*, 1984). This oleoresin leakage was caused by the destruction of parenchymatous tissues in the resin canals. We hypothesized that cell walls and membrane system of these parenchymatous cells were disturbed by certain agents, and tested various surfactants or cellulases for such destructive activity. Among them, a commercial cellulase, Cellulase Onozuka R-10, and Macerozyme (products of Kinki Yakult), caused symptoms very similar to those of the pine wilt disease, that is, oleoresin leakage to tracheal elements, blocking of water conductance in xylem, and rapid necrosis of the needles (SASAKI *et al.*, 1984). Therefore, cellulase activity was examined along with the nematode homogenates, and great activity of the enzyme was found in the homogenates by the method of polyacrylamide gel electrophoresis using sodium carboxymethyl cellulose as a substrate (ODANI *et al.*, 1985).

To detect enzyme activity, homogenates can be used as starting material for an enzyme analysis, but as pointed out by DEUBERT and ROHDE (1971), the analytical results obtained from homogenates sometimes provide a distorted picture of the real pathological features. To obtain evidence of the role of nematode cellulase in developing the early symptoms of the pine wilt disease, it is necessary to show that the pine wood nematodes release cellulase into the host cells. Thus experiments were conducted, and it was proved that the nematode, *Bursaphelenchus xylophilus* exuded cellulase into the supernatant of their distilled water suspension. They also left their crawling tracks in dissolvings

* For. and Forest Prod. Res. Inst., Ibaraki 305 林業試験場
** Kyushu Br., For. and Forest Prod. Res. Inst., Kumamoto 860 林業試験場九州支場

carboxymethyl cellulose mixed in a polyacrylamide gel plate on which they crawled.

II. Materials and Methods

Pine wood nematodes (*Bursaphelenchus xylophilus*, Isolate No. S-10) supplied by the Laboratory of Nematology of the Forestry and Forest Products Research Institute were subcultured on *Botrytis cinerea* grown on barley medium. To collect living nematodes only, the nematode-medium mixture was left on a sheet of filter paper (Toyo No. 2) submerged in water (NAKAGAWA, 1983). Only those passed through the filter paper were collected, and then suspended in a large volume of water. The suspension was left at 0°C until the nematodes sank completely, then they were collected by decantation. The nematodes were washed repeatedly, and then suspended in distilled water so as each milliliter of water contained about 10^6 nematodes. The nematode suspension was kept at 0°C in the dark, and at a 12-hour interval, supernatant of the suspension was collected, and cellulase activity in the supernatant was determined as described previously (ODANI *et al.*, 1985). One milliliter of 1% sodium carboxymethyl cellulose (CMC) in toluene-saturated 0.1 M, pH 6.0 acetate buffer was incubated with 200 μl of the supernatant at 37°C for one hour. The cellulolytic activity in the reaction mixture was assayed by determining the amount of reducing sugars liberated from CMC by the method of SOMOGYI-NELSON and expressed in equivalent μg of glucose. The polyacrylamide gel electrophoresis of cellulase, developed by GOREN and HUBERMAN (1976), was conducted as previously described (ODANI *et al.*, 1985). After the electrophoretic separation of the cellulase in the gel containing 0.2% CMC, the gel was incubated overnight at 37°C to digest CMC. Then the gel was stained with 60% H_2SO_4 and 2% KI+0.2% I_2 solution. The isozymes of cellulase were detected as transparent bands. Furthermore, to obtain direct evidence of cellulase exudation from the living nematodes, tracks of the nematodes movements were traced as follows (Fig. 1). Seven percent of acrylamide monomer mixed with 0.2% CMC was polymerized and solidified between a pair of the glass slides, and the solidified gel was washed intensively in distilled water to remove unpolymerized monomers. On this gel plate, a drop of the suspension containing 50,000 well-washed nematodes was placed. The glass slide with the nematodes was kept in a moist chamber to let the nematodes crawl freely for five hours. After the migration was marked, CMC degradation along the nematode crawling trace was detected by 60% H_2SO_4 and 2% KI+0.2% I_2 solution as in the case of the cellulase electrophoresis.

To compare the needle necrosis inducing activity of the supernatant of the nematode suspension with the commercial cellulase Onozuka R-10 or the nematodes, three-year-old seedlings of *Pinus densiflora* were divided into four groups, each consisting of six to eight seedlings. Their leader shoots were cut off just three centimeters above their base and fixed with a small piece of rubber tube. The nematodes tested were *B. xylophilus*, Isolate No. S-10, supplied by Dr. KIYOHARA, Laboratory of Forest Pathology, Kyushu Branch, Forestry and Forest Products Research Institute. About 9.8×10^6 nematodes were suspended in ten milliliters of distilled water and left under 0°C condition for 24 hours. The supernatant was filtered through a sterilizing filter (pore size : 0.22 μm), and 0.5 ml of the filtrate were fed to each pine seedling in one group through the rubber tubes. Ten thousands of the nematodes suspended in 0.5 ml of distilled water, 0.5 ml of 1% solution of cellulase Onozuka R-10, and 0.5 ml of distilled water as a control also were fed in the same way. After three weeks the developing necrotic symptoms of the needles were checked in the four groups.

III. Results and Discussion

Cellulase activity in the control supernatant of the distilled water suspension of the pine wood nematodes was determined. The suspension was kept at 0°C in the dark for 24 hours, and with various volumes of the supernatant of the suspension the cellulolytic activities in them were determined (Table 1). The enzyme activity was dose-dependent. The time course of the cellulase exudation by the nematodes into the supernatant of their freshly prepared suspension is shown in Table 2.

Cellulolytic isozymes also were separated by polyacrylamide gel electrophoresis with the same samples. The results are shown in Figure 2. Gradual increases in each cellulase isozyme similar to those detected in the homogenates of *B. xylophilus* were observed. Both results showed that the pine wood nematodes exude cellulase even at 0°C.

As pointed out by DEUBERT and ROHDE (1971), maintenance of the sterility of the nematodes and medium throughout the incubation is imperative for the study of the substances discharged by the nematodes into the medium, because microorganisms may grow in the medium and false results may be given. But complete sterilization of both the surface and inside of the nematodes is very difficult because they often carry some microorganisms internally (KOYAMA, 1975; KUSUNOKI, 1985). Addition of

Table 1. Cellulase activity in the supernatant of the suspension of the nematodes, *Bursaphelenchus xylophilus*

Amount of supernatant of the nematodes suspension (μl)	Amount of reducing sugar liberated from CMC (glucose equivalent μg)
0	8
50	38
100	75
200	150
400	280

Table 2. Time course of the cellulase exudation by *Bursaphelenchus xylophilus* to the supernatant of their freshly prepared suspension at 0°C

Time (hr)	Amount of reducing sugar liberated from CMC (glucose equivalent μg)
0	8
12	140
24	175
36	189
48	400

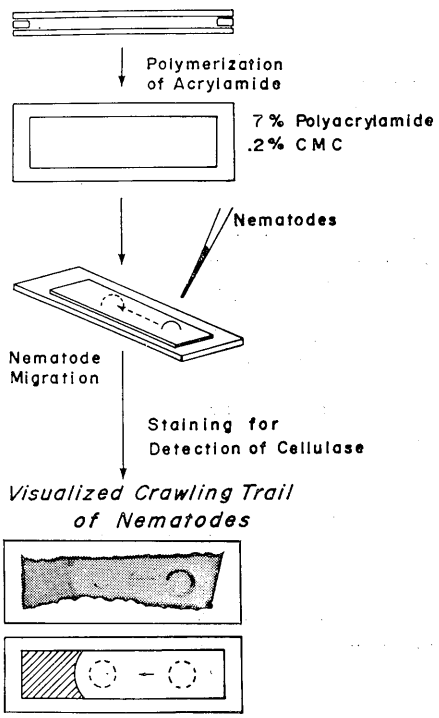

Fig. 1. Visualization of the crawling tracks of the pine wood nematodes marked by the exuded cellulase in a polyacrylamide gel plate containing 0.2% of carboxymethyl cellulose

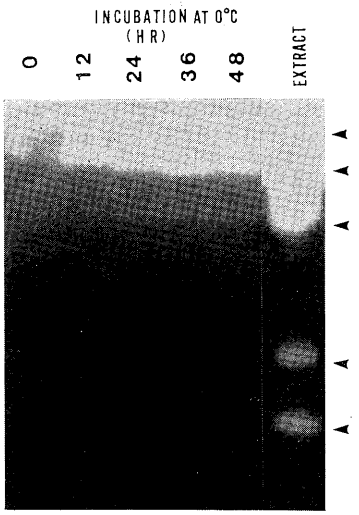

Fig. 2. Time course of the cellulase exudation by the pine wood nematodes analyzed by polyacrylamide gel electrophoresis

Ca. 10^6 nematodes were suspended in 1 ml of distilled water, 40 μl of the supernatant were sampled at a 12-hour-interval, and the gel electrophoresis for cellulase was carried out. The isozyme pattern of the cellulase of the nematode extract was shown as a reference.

antibiotics to the medium, however, may disturb the normal metabolism of the nematodes. For this reason, incubation was conducted at 0°C to suppress population increase of microorganisms without the addition of antibiotics.

The isozyme patterns obtained during 48 hours incubation of the nematode suspension at 0°C were very similar to the pattern of *B. xylophilus* cellulase prepared from the nematode homogenate (Fig. 2), suggesting no contamination of the cellulase was discharged by the microorganisms.

The detection of cellulase in the supernatant of the nematode suspension could be interpreted as the result of the death and lysis of the nematodes during incubation. Autolysis of the nematode after their death during incubation may result in discharging of their various hydrolytic enzymes into the supernatant. But this interpretation is not probable under 0°C condition where such lytic reactions by hydrolytic enzymes hardly take place. To obtain further evidence living nematodes exude cellulase, the crawling track of the nematodes was assayed for cellulolytic activity. A mass of the nematodes placed on a plate of polyacrylamide gel containing 0.2% CMC migrated about 30 mm in five hours. The gel was stained with H_2SO_4+KI+

Table 3. Effect of the supernatant of the nematode suspension, cellulase Onozuka R-10, and the nematodes on the induction of necrotic symptoms in the needles of *Pinus densiflora* seedlings

	Complete necrosis	Partial necrosis	No symptoms
Supernatant of the nematodes	0	3	5
1% cellulase	6	0	2
The nematodes	4	0	3
Distilled water control	0	0	6

The number of the seedlings for each symptom level is shown. The nematodes tested were *B. xylophilus* Isolate No. S-10. 9.8×10^6 nematodes were suspended in 10 ml of distilled water, and 0.5 ml of the supernatant of this suspension being filtered and fed to each pine seedling.

I_2 immediately after the nematodes were washed off from the gel plate. The gel developed the distinct mark of the nematode trace (Fig. 1). From this result, it is quite clear that the nematodes exude cellulase and leave cellulase behind in the host cells as they migrate.

In various kinds of plant parasitic nematodes, many hydrolyases have been reported in both nematode secretions and bodies (GIEBEL, 1982). Among them, cellulases were found with nematode species in more than ten genera, including the genus *Bursaphelenchus* (TRACEY, 1958; KRUSBERG, 1960; MORGAN and MCALLAN, 1962; DROPKIN, 1963; DROPKIN *et al.*, 1962; MYERS, 1965; MUSE and WILLIAMS, 1969; ODANI *et al.*, 1985), but these cellulases were found by the analysis of the homogenates and extracts of the nematodes. For discussion of roles of cellulase in the pathogenesis of the nematodes, the exudation of cellulase by the nematodes should be demonstrated. Only BIRD (1966) tried to demonstrate exudation of cellulase from sterilized *Meloidogyne* larvae, but failed to detect cellulase in the sterile larval exudates. Our evidences showed that the pine wood nematode exude cellulase outside and leaves cellulase in its migrating track.

The necrotic symptom-inducing activity of the supernatant of the nematode suspension, the authentic cellulase, and nematodes are shown in Table 3. No complete necrosis of the needles was observed in the seedlings treated with the supernatant, but sporadic distribution of the necrotic needles was observed.

Our present results strongly support our previous hypothesis that the nematode-originated cellulase may be one of the strong candidate of the pathogenic substances responsible for the development of the pine wilt disease.

Literature cited

BIRD, A. F.: Some observation on exudates from *Meloidogyne* larvae. Nematologica **12**: 471～482, 1966

DEUERT, K. H. and ROHDE, R. A.: Nematode enzymes. *In* Plant parasitic nematodes, Vol. II (ZUCKERMAN, B. M. and ROHDE, R. A., eds.). 73～90, 1971

DROPKIN, V. H.: Cellulase in phytoparasitic nematodes. Nematologica **9**: 444～454, 1963

———, MARCH, P. B., and SALDIND, D. H.: Cell-wall degrading enzymes in some plant parasitic, myceliophagus and free-living nematodes. Phytopathology **52**: 1218, 1962

GIEEEL, J.: Mechanism of resistance to plant nematodes. Ann. Rev. Phytopathol. **20**: 257～279, 1982

GOREN, R. and HUBERMAN, M.: A simple and sensitive staining method for the detection of cellulase isozymes in polyacrylamide gels. Anal. Biochem. **75**, 1～8, 1976

KOYAMA, R.: Chlamidia-like microorganism in *Bursaphelenchus lignicolus* MAMIYA et KIYOHARA, a causal agent of pine wilting disease. J. Jpn. For. Soc. **57**: 61～63, 1975

KRUSEERG, L. R.: Hydrolytic and respiratory enzymes of species of *Ditylenchus* and *Pratylenchus*. Phytopathology **50**: 9～22, 1960

KUSUNOKI, M.: Bacteria- and chlamidia-like bodies observed in *Bursaphelenchus xylophylus*. Ann. Phytopathol. Soc. Jpn. (in press)

MORGAN, G. T. and MCALLAN, J. W.: Hydrolytic enzymes in plant-parasitic nematodes. Nematologica **8**: 209～215, 1962

MUSE, B, D. and WILLIAMS, A. S.: A comparison of pectolytic and cellulolytic enzymes in two populations of *Ditylenchus dispaci*. J. Nematol. **1**: 19, 1969

MYERS, R. F.: Amylase, cellulase, invertase and pectinase in several free-living mycophagus, and plant parasitic nematodes. Nematologica **11**: 441～448, 1965

NAKAGAWA, S.: A method for separation of living and dead pine-wood nematodes. Trans. 94th Annu. Meet. Jpn. For. Soc.: 465～466, 1983**

ODANI, K., SASAKI, S., NISHIYAMA, Y., and YAMAMOTO, N.: Early symptom development of the pine wilt disease by hydrolytic enzymes produced by the pine wood nematodes—Cellulase as a possible candidate of the pathogen. J. Jpn. For. Soc. **67**: 366～372, 1985

SASAKI, S., ODANI, K., NISHIYAMA, Y., and HAYASHI, Y.: Development and recovery of pine wilt disease studied by tracing ascending sap flow marked with water soluble stains. J. Jpn. For. Soc. **66**: 141～148, 1984

TRACEY, M. V.: Cellulase and chitinase in plant nematodes. Nematologica **3**: 179～183, 1958

* In Japanese with English summary

** Only in Japanese

(Received May 23, 1985)