

## Scanning Electron Microscopy of Pine Seedling Wood Tissue Sections Inoculated with the Pinewood Nematode *Bursaphelenchus xylophilus* Previously Prepared for Light Microscopy

YASUHARU MAMIYA

**Abstract:** Scanning electron microscopy (SEM) was applied to paraffin-embedded wood sections to study the histopathology of pine seedlings inoculated with the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. The sections, which had been previously prepared and observed by light microscopy (LM) on glass slides, were originally obtained from experiments in which pine seedlings had been inoculated with PWN. The cover glass was removed by soaking the glass slide in xylene for 3 to 5 days. The glass slides were cut into small pieces so that each piece contained one wood section. Each piece of the glass slide was attached with double adhesive tape to an aluminum stub. The specimens were sputter-coated with gold and examined with a scanning electron microscope (JEOL-JSM 5200). Compared to LM (as documented in previous reports) SEM provided greater depth of focus and resolution of the damaged wood tissues, nematodes and associated bacteria. SEM made it possible to observe the relationship between bacterial distribution and nematode distribution in wood tissues. SEM observations also suggested the possibility of documenting the death of ray cells and other parenchyma cells in relation to disease development. Finally, the current study of PWN in pine seedlings demonstrated that glass slides prepared for LM observations more than 25 years earlier could be successfully processed for examination by SEM.

**Key words:** bacterial mass, inoculation experiments, *Pinus* sp., scanning electron microscopy, technique.

Pine wilt disease caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Bührer, 1934) Nickle, 1970 is devastating pine forests not only in Japan but also in other countries, such as China, Korea and Portugal (Futai, 2008; Mota and Vieira, 2008; Shin, 2008; Zhao, 2008). To elucidate the behavior of PWN in wood tissues and pathological effects on pine trees, histological studies were previously conducted on nematode-infested trees (Mamiya, 1980, 1982, 1984, 1985; Myers, 1986; Fukuda et al, 1992; Ishida et al., 1993; Ichihara et al, 2000; Hara and Futai, 2001). In these studies, wood samples of diseased trees were embedded in paraffin and sectioned with a microtome. Serial-sectioned tissues affixed on glass slides and stained were examined by light microscopy (LM). The current study examined the possibility that additional information could be obtained from these sections by subsequent examination with scanning electron microscopy (SEM). A method for using SEM on paraffin-embedded plant tissues affixed on a cover glass was described by Gaudet and Kokko (1984). Kuroda and Mamiya (1986) applied this method to the histological observation on 2-month-old pine seedlings inoculated with PWN, which is useful for exposing the internal structures of tissues. It is simple and combines the advantages of great depth of focus, three-dimensional views, and high magnification. The prior use of LM facilitated the selection of material for observation by SEM.

In the present study, a modification of Gaudet and Kokko's method was used to study the histological

aspects of pine seedlings infested with PWN. SEM observations were made on deparaffinized sectioned tissues affixed to glass slides. The slides used in this study had been prepared and examined by LM more than 25 years ago for investigations into the histology of PWN-inoculated pine seedlings (Mamiya, 1980, 1982, 1984, 1985).

### MATERIALS AND METHODS

**Specimens prepared for LM:** The slides used in this study were previously used for LM observations of 5-month-old, 3-year-old, and 4-year-old Japanese red pine (*Pinus densiflora* Siebold & Zucc.) and Japanese black pine (*P. thunbergii* Parl.) seedlings inoculated with PWN (Mamiya, 1980, 1982, 1984, 1985). The nematode inoculum was obtained from *Botrytis cinerea* (De Bary) Whetzel cultures. The 5-month-old seedlings had been inoculated by pipetting a drop of water suspension of PWN onto a piece of filter paper inserted into the seedling stem. The 3-year-old and 4-year-old seedlings had been inoculated by injecting a water suspension of PWN into a vinyl tube firmly attached to the cut end of a 2-year-old branch. Wood samples were collected from seedlings at regular time intervals after inoculation, fixed in formalin-acetic acid-alcohol (FAA) or formalin-propionic acid-alcohol (FPA), and embedded in paraffin or celoidine. Serial sections, 12 µm thick, were cut on a microtome affixed to 76 x 26 mm glass slides with albumen-glycerin adhesive and double-stained with safranin and fast green. Sections were sealed with Canada balsam under a 50 x 24 mm cover glass. Serial wood sections of uninoculated control seedlings were made by the same method as inoculated seedlings. All slides used in this study had been kept at room temperature and humidity after the LM observations were made.

Received for publication April 24, 2012.

Formerly Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687 and Tamagawa University, Machida, Tokyo 194-8610, Japan.

The author thanks Dr. Kyoko Watanabe, Tamagawa University, for her valuable advice and for providing technical guidance regarding SEM and Dr. B. A. Jaffee for reviewing the manuscript before submission.

E-mail: CBL01545@nifty.com

This paper was edited by Nancy Kokalis-Burelle.

*SEM observations:* Slides made for LM observations were immersed in xylene for 3 to 5 days to dissolve the Canada balsam and remove the cover glasses. After the cover glasses were removed, the glass slides were rinsed in absolute ethanol and air-dried. A glass cutter was then used to cut the slide into small pieces so that there was one wood section per piece. The pieces of glass slides with a wood section were attached with double-adhesive tapes to aluminum stubs and sputter-coated with gold and examined with a scanning electron microscope (JEOL-JSM 5200).

RESULTS

*SEM observations on nematode distribution in wood tissues:* Just after the seedlings were inoculated, most PWNs were located at the inoculation site (Fig. 1A). The nematodes then spread quickly in wood tissues through the cortex, ray and resin canals. Nematode movement was evident through the axial resin canal to the ray, from the ray to the axial resin canal, and from the ray to the tracheid via the window-like pits of the ray (Fig.1B). Nematode distribution was associated with

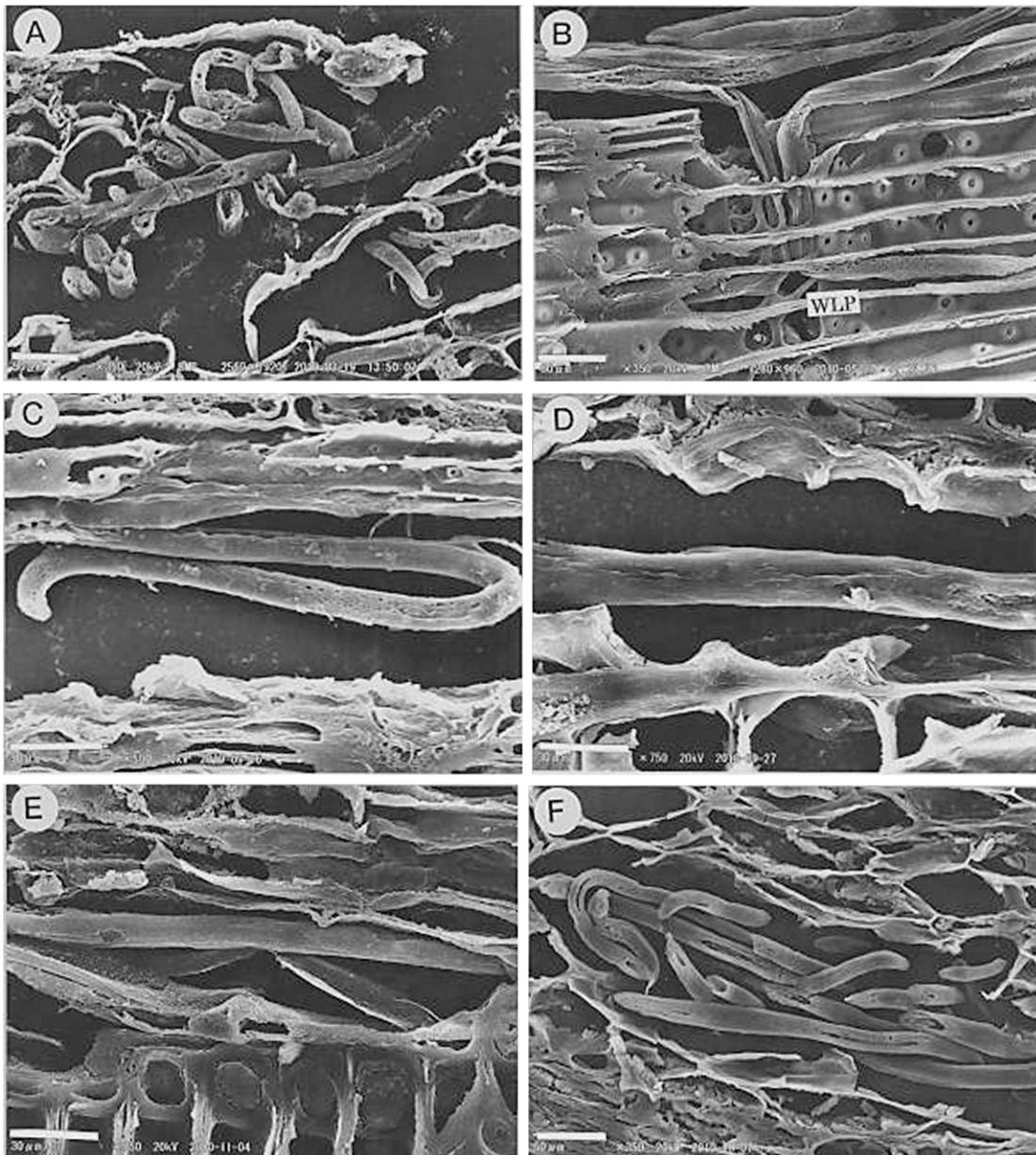


FIG. 1. Scanning electron micrographs of pine (*Pinus densiflora* Siebold & Zucc.) wood tissues showing the radial or tangential face infested with the pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhner, 1934) Nickle, 1970. A: PWN in the destroyed cortex at the inoculation site. (5-month-old seedling, 24 h after inoculation) B: PWN in the axial resin canal (ARC) and the ray (R). Nematodes were moving to tracheids via the window-like pits (WLP) (4-year-old seedling, 6 days after inoculation) C: PWN in the axial resin canal (3-year-old seedling, 3 days after inoculation) D: PWN destroying cortical cells (5-month-old seedling, 12 days after inoculation) E: PWN destroying ray cells (4-year-old seedling, 22 days after inoculation) F: PWN in the destroyed cortex (5-month-old seedling, 12 days after inoculation). Scale: A, B, C, F = 50  $\mu$ m; D, E = 30  $\mu$ m.

the destruction of parenchyma cells (Fig. 1C, D, E). With the passage of time after inoculation, nematodes distributed throughout the wood tissues was evident in the cortex, phloem, cambium, and rays; destruction of the parenchyma cells of the radial and axial resin canals of the xylem was also evident (Fig. 1F, Fig. 2A, B).

*Distribution of bacteria in wood tissues:* Bacterial masses were observed in cavities formed as a consequence of tissue destruction, in the cortex, rays and resin canals (Fig. 2C, D). Bacterial cells were commonly observed on

the body surface of nematodes shortly after inoculation (Fig. 2E). Bacterial masses were evident in destroyed tissues of 5-month-old seedlings 6 days after inoculation and then became more common as the disease developed. Although bacterial masses were less common in 3- or 4-year-old seedlings than in 5-month-old seedlings, bacterial cells were widely observed in destroyed tissues and on the surface of nematodes in the destroyed tissues of the 3- or 4-year-old seedlings. The distribution of bacteria was not observed in the wood tissues of uninoculated seedlings.

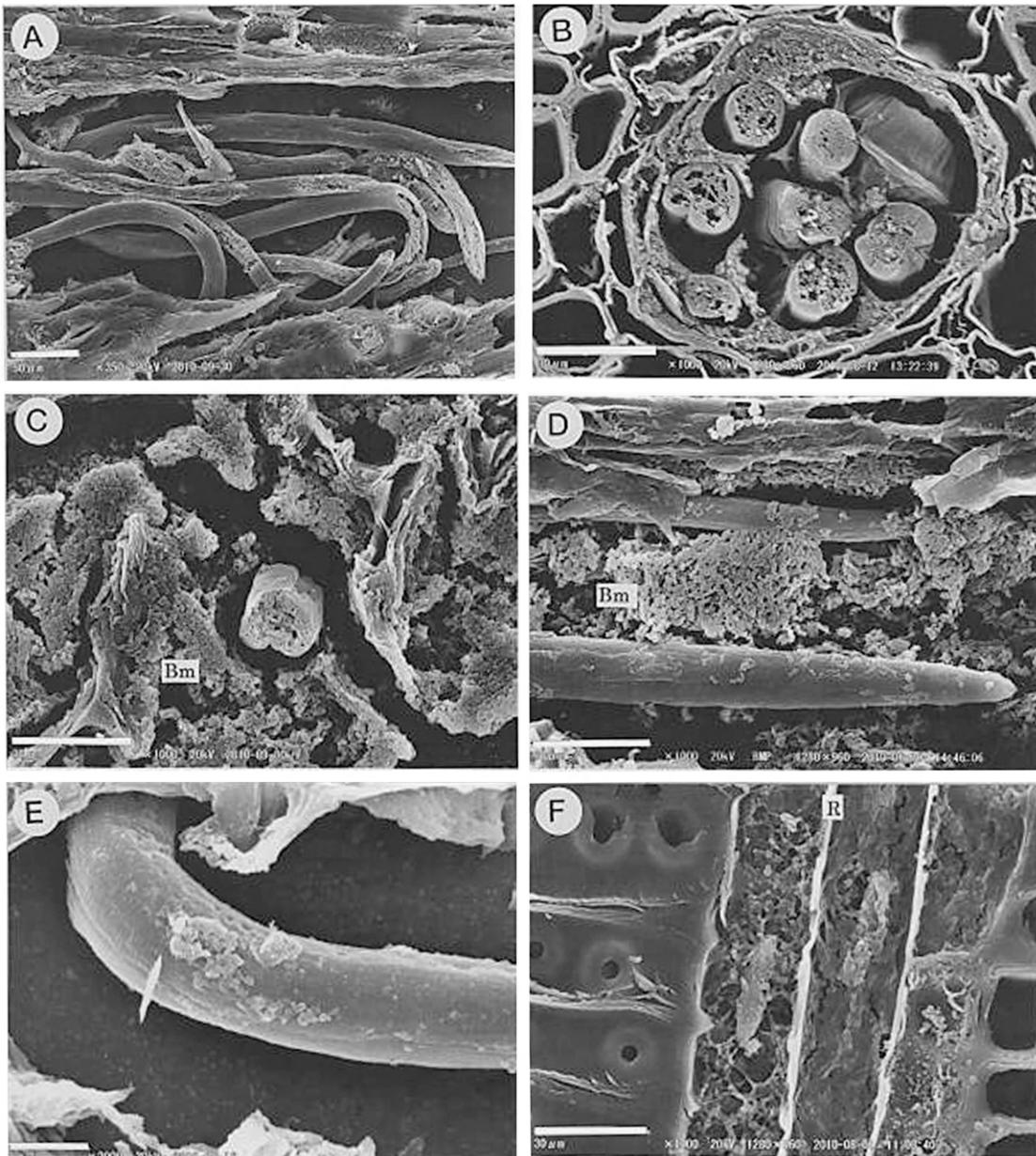


FIG. 2. Scanning electron micrographs of the pine (*Pinus densiflora* Siebold & Zucc.) wood tissues showing the cross or radial face infested with PWN, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970. A: PWN in the axial resin canal (5-month-old seedling, 12 days after inoculation). B: PWN in the axial resin canal (3-year-old seedling, 19 days after inoculation). C: PWN and bacterial mass (Bm) in the destroyed cortex. (5-month-old seedling, 18 days after inoculation). D: PWN and bacterial mass (Bm) in the axial resin canal. (5-month-old seedling, 12 days after inoculation). E: PWN in the axial resin canal and bacterial cells attached to PWN. (5-month-old seedling, 3 days after inoculation). F: Denatured ray cells. (4-year-old seedling, 9 days after inoculation). Scale: A = 30  $\mu$ m; B, C, D, F = 30  $\mu$ m; E = 10  $\mu$ m.

*Deterioration of ray cells:* SEM revealed that the contents of the ray cells were denatured in wood tissues of both 5-month-old seedlings and 3- or 4-year-old seedlings as the result of the nematode inoculation (Fig. 2F). SEM observations confirmed that the contents of ray cells were destroyed early in disease development (as soon as 3 days after nematode inoculation) and that the deterioration spread widely in wood tissues over time.

#### DISCUSSION

This study confirmed that plant tissue sections originally affixed to glass slides for LM observation could also be examined by SEM, as first demonstrated by Gaudet and Kokko (1984). The preparation of material for LM made it possible to use LM observation to select material to be examined at higher resolution and with great depth of field with SEM.

A comparison of the results of LM observations of PWN in inoculated seedlings (Mamiya, 2008) with the SEM observations in the current study indicates that SEM reveals greater detail than LM. SEM observations on wood sections collected from pine trees infested with PWN have also been made using the standard technique for SEM, i.e., without prior preparation for LM (Yik and Birchfield, 1981; Kusunoki, 1987). As mentioned by Gaudet and Kokko (1984), SEM of deparaffinized sections offers several advantages over standard techniques used for SEM. In particular, LM is useful for selecting the specimens to be examined with SEM.

LM observations demonstrated that bacterial masses occupied cavities formed by PWN in wood tissues, including the cortex, cambium, and ray and resin canals (Mamiya, 1980). The presence and distribution of these bacterial masses were confirmed by the SEM observations in the current study. In addition, SEM made it possible to observe bacteria as separated colonies rather than as masses in the damaged wood tissues. SEM confirmed that bacteria are widely distributed in PWN infested pine seedlings. Researchers have hypothesized that bacteria might be the causal agents of pine wilt disease (Kawazu et al., 1998; Han, et al., 2003; Zhao et al., 2003), and several species of bacteria isolated from dead pine trees were considered as potential causal agents of pine wilt disease (Kawazu et al., 1998; Han, et al., 2003; Zhao et al., 2003). Kusunoki (1987) observed bacteria in the wood of pine seedlings inoculated with PWN using SEM. Detailed information, however, has been lacking concerning the distribution of bacteria in wood tissues in relation to symptomatic progression of PWN infested diseased trees. The SEM observations in the current study demonstrated common distribution of bacteria in wood tissues and revealed that bacteria propagated and distributed in association with nematode movement in wood and destruction of wood tissues.

The source of the bacteria in sections examined here is unknown. In the experiments that produced the sections examined in this study, nematode inoculum was prepared from the monoxenic culture of PWN and *B. cinerea* on barley (*Hordeum vulgare* L.) grains. However, nematode sterility was not maintained in the preparation of the water suspensions used as inoculum. Further studies are necessary to determine the significance of the bacteria observed in wood of pine trees infested with PWN.

Denatured parenchyma cells are considered to be a typical histopathological symptom of pine wilt disease (Mamiya, 1982; Fukuda et al., 1992; Hara et al., 2006). Although SEM observations in the current study revealed degeneration of cell contents of the ray, the current study could not provide the detailed information concerning the process of degradation of cell contents. The combination of LM observation and SEM observation as applied in this study will be useful for the further investigations that follow the development of denatured parenchyma cells in relation to disease development.

#### LITERATURE CITED

- Fukuda, K., Hogetu, T., and Suzuki, K. 1992. Cavitation and cytological changes xylem of pine seedlings inoculated with virulent and avirulent isolates of *Bursaphelenchus xylophilus* and *B. mucronatus*. *Journal of the Japanese Forestry Society* 74:289–299.
- Futai, K. 2008. Pine wilt in Japan: From first incidence to the present. Pp. 5–12 in B. G. Zhao, K. Futai, J. R. Sutherland and Y. Takeuchi, eds. *Pine wilt disease*: Springer.
- Gaudet, D. A., and Kokko, E. G. 1984. Application of scanning electron microscopy to paraffin-embedded plant tissues to study invasive process of plant-pathogenic fungi. *Phytopathology* 74:1078–1080.
- Han, Z. M., Hong, Y. D., and Zhao, B. G. 2003. A study on pathogenicity of bacteria carried by pine wood nematode. *Journal of Phytopathology* 151:683–689.
- Hara, N., and Futai, K. 2001. Histological changes in xylem parenchyma cells and the effects of tracheids of Japanese black pine seedlings inoculated with pine wood nematode, *Bursaphelenchus xylophilus*. *Journal of the Japanese Forestry Society* 83:285–289.
- Hara, N., Takeuchi, Y., and Futai, K. 2006. Cytological changes in ray parenchyma cells of seedlings of three pine species infected with the pine wilt disease. *Japanese Journal of Nematology* 36:23–32.
- Ichihara, Y., Fukuda, K., and Suzuki, K. 2000. Early symptom development and histological changes associated with the migration of *Bursaphelenchus xylophilus* in seedling tissues of *Pinus thunbergii*. *Plant Disease* 84:675–680.
- Ishida, K., Hogetu, T., Fukuda, K., and Suzuki, K. 1993. Cortical response in Japanese black pine to attack by the pine wood nematode. *Canadian Journal of Botany* 71:1399–1405.
- Kawazu, K., Yamashita, H., Kobayashi, A., and Kanzaki, H. 1998. Isolation of pine-wilting bacteria accompanying pine wood nematode, *Bursaphelenchus xylophilus*, and their toxic metabolites. *The Scientific Reports of the Faculty of Agriculture, Okayama University* 87:1–7.
- Kuroda, K., and Mamiya, Y. 1986. Behavior of the pine wood nematode in pine seedlings growing under aseptic conditions. *Transactions of 97<sup>th</sup> Meeting of the Japanese Forestry Society*: 471–472.

- Kusunoki, M. 1987. Symptom development of pine wilt disease - histopathological observations with electron microscopes. *Annals of the Phytopathological Society of Japan* 53:622-629.
- Mamiya, Y. 1980. Inoculation of the first year pine (*Pinus densiflora*) seedlings with *Bursaphelenchus lignicolus* and the histopathology of diseased seedlings. *Journal of the Japanese Forestry Society* 62: 176-183.
- Mamiya, Y. 1982. Pine wilt and the pine wood nematode: histopathological aspects of disease development. Pp. 153-160 in H. M. Heybroek, B. R. Stephan and K. von Weissenberg, eds. *Resistance to disease and pests in forest trees*: Center for Agricultural Publishing and Documentation, Wageningen, The Netherlands.
- Mamiya, Y. 1984. Behavior of the pine wood nematode, *Bursaphelenchus xylophilus*, associated with the disease development of pine wilt. Pp. 14-25 in V. H. Dropkin, eds. *Proceedings of the United States-Japan Seminar*: East-West Center, Honolulu, Hawaii.
- Mamiya, Y. 1985. Initial pathological changes and disease development in pine trees induced by the pine wood nematode, *Bursaphelenchus xylophilus*. *Annals of the Phytopathological Society of Japan* 51: 546-555.
- Mamiya, Y. 2008. Histopathological observations of *Bursaphelenchus xylophilus* in symptomatic tissues of pinewood. Pp. 321-334 in M. M. Mota and P. Vieira, eds. *Pine wilt disease: a worldwide threat to forest ecosystems*: Springer.
- Shin, S. C. 2008. Pine wilt disease in Korea. Pp. 26-32 in B. G. Zhao, K. Futai, J. R. Sutherland and Y. Takeuchi, eds. *Pine wilt disease*: Springer.
- Yik, C. P., and Birchfield, W. 1981. Observations on the morphology of the pine wood nematode, *Bursaphelenchus xylophilus*. *Journal of Nematology* 13:376-384.
- Zhao, B. G. 2008. Pine wilt disease in China. Pp. 18-25 in B. G. Zhao, K. Futai, J. R. Sutherland and Y. Takeuchi, eds. *Pine wilt disease*: Springer.
- Zhao, B. G., Wang, H. L., Han, S. F., and Han, Z. M. 2003. Distribution and pathogenicity of bacteria species carried by *Bursaphelenchus xylophilus* in China. *Nematology* 6:899-906.