論文の内容の要旨

森林科学　　専攻

平成18年度博士課程　進学

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論文題名　Migration of *Bursaphelenchus xylophilus* and *B. mucronatus* in tissues of susceptible and resistant pine species

（感受性および抵抗性マツ組織内におけるマツノザイセンチュウとニセマツノザイセンチュウの移動）

The first country suffering from pine wilt disease (PWD) is Japan, and the disease has recently spread to some other countries including Korea and China. Although devastated areas by PWD are now gradually decreasing through continual applications of various control methods, a great amount of pine trees still are lost. For effective control of this disease, we need to know the mechanism of symptom development more precisely.

After invasion of a pathogenic pine wood nematode (PWN), *Bursaphelenchus xylophilus*, into a pine tree, PWNs are thought to migrate to every branch through pine tissues, proliferate, kill pine cells, and thereby lead the pine tree to death. Since PWN migration is a prerequisite to development of all symptoms, it is important to know how PWNs migrate within pine tissues. PWN migration has been investigated using the Bearmann funnel technique by which the number of PWNs in a branch segment could be counted, and migration patterns within pine trees after PWN inoculation have been outlined. Such kind of investigation, however, could not directly reveal migration routes of invading PWNs on the scale of each tissue because segments for the Bearmann funnel technique can be prepared only as assemblages of various tissues. Although some investigators infer migration routes on the scale of each tissue from the distribution of discolored and damaged regions on the assumption that pine tissue damage after PWN inoculation coincides with PWN presence, none of them provide direct evidence for PWN migration routes. Migration of individual PWNs in each tissue needs to be traced more precisely to clarify the causal relationship between PWN presence and pine symptom. Recently, a new staining technique in which fluorescein isothiocyanate-conjugated wheat germ agglutinin (F-WGA) intensely stains PWNs in

pine tissues without staining background has been developed and enabled us to know PWNs distribution within each pine tissue much easily.

In this thesis, I investigated time-course changes in distribution of pathogenic and nonpathogenic PWNs in PWD-susceptible and resistant pines after PWNs inoculation using the F-WGA staining technique and inferred PWN migration routes.

Distribution and migration routes of *Bursaphelenchus xylophilus* in *Pinus thunbergii*

Distribution of a pathogenic PWN (*B. xylophilus*) in the tissues of susceptible *Pinus thunbergii* seedlings was investigated. After PWNs were inoculated into current-year stems of pine seedlings, 1-cm blocks excised from the stems at about 5 cm below the inoculation site were thin sectioned and stained with F-WGA. Distribution of PWNs in each tissue of cross thin sections was observed under epifluorescence microscope. PWN distribution was confined only to cortical resin canals at 1 day after inoculation (dai), and then spread to xylem axial resin canals, pith and resin canals of short branches at 3 dai. PWNs were newly detected in cortical tissues and tracheids at 7 dai. Lots of PWN were additively distributed in cortical tissues and cambial region at and after 14 dai. A new finding that PWNs invade cortical resin canals of short branches at as early as 3 dai may suggest that the leaf wilting appearance specific to PWD results from damaging of the leaf bases by the PWNs.

To estimate vertical or horizontal migration speed of PWNs, PWNs were inoculated onto the cross or tangential cut surfaces of *P. thunbergii* stem segments and time-course changes in PWN distribution were chased using F-WGA staining technique. Maximal speed of PWN migration was estimated to be much faster through cortical and xylem axial resin canals (more than 6.7 and 2.2-2.3 mm per hour, respectively) than through cortical tissues both vertically and horizontally (1.0-1.2 and 0.2 mm per hour) at 6 hours after inoculation (hai).

To examine whether PWNs in resin canals could invade surrounding tissues, pulse-chase experiments were performed. Segments in which PWNs resided only in cortical resin canals were prepared by removing 2 cm portions including the inoculation site (top cross-cut surface) from 5 cm stem segments at 6 hai. Additional incubation of residual segments caused extended PWN distribution to xylem axial resin canals and then to other tissues. Segments in which PWNs resided only in xylem axial resin canals and pith were also prepared by removing 2 cm portion including the inoculation site at 12 hai from 5 cm stem segments girdled just before PWN inoculation at 1-2 cm from the top cross-cut surface. Additional incubation of those segments also caused extended PWN distribution to cortical resin canals and then to other tissues. These results indicate that PWNs have an ability to migrate from cortical resin canals and xylem axial resin canals to other tissues. The present pulse-chase experiments provided direct evidence for the routes of PWN migration in susceptible *P. thunbergii*: PWNs migrate quickly through

cortical and xylem resin canals from an infected site to remote site in a pine tree and thereafter PWNs within resin canals invade surrounding tissues.

Migration of *Bursaphelenchus xylophilus* in resistant pine species

It is known that some pine species and some families of susceptible pine species have resistance to PWD. Such host resistance may reflect the difference in PWN behavior in each pine tissue. Therefore, I tried to obtain information on resistance mechanisms against PWD from PWN migration patterns in each tissue of resistant pines.

PWNs (*B. xylophilus*) were inoculated onto top cross-cut surfaces of 20 cm stem cuttings of resistant pines, i.e. *P. strobus*, *P. rigida* and a *P. thunbergii* resistant family Namikata, as well as susceptible *P. thunbergii* as a control. Time-course changes in PWN distribution within these resistant pines were traced using the F-WGA staining technique up to 8 dai. PWNs were absent from entire tissues in a remote region (11 to 19 cm from the top surfaces) of *P. strobus* cuttings at 6 and 24 hai, and still absent from almost all tissues in the region except for cortical resin canals containing a few PWNs at 3 and 8 dai, when many PWNs already spread to the remote region in susceptible *P. thunbergii*. In *P. rigida*, PWNs were absent from both cortex and xylem in the remote region during entire experiment time. In a resistant family of *P. thunbergii*, PWNs were absent from entire tissues in the remote region at 6 hai, and thereafter remained absent form xylem with a few PWN in pith at 8 dai. These results indicate that PWN migration were completely inhibited in xylem and highly restrained in cortex of resistant pine species. Since the complete inhibition of PWN migration in xylem prevents PWNs widely dispersing in a pine tree, it may be at least one reason for resistance of resistant pines to PWD.

Migration in pine species and ability to kill pine cells of *Bursaphelenchus mucronatus*

A nonpathogenic PWN, *B. mucronatus*, has been isolated from pine trees. Differences in pathogenicity between *B. xylophilus* and *B. mucronatus* may reflect difference in behavior in pine tissues. Information on the differences would provide a hint on the mechanism of PWD symptom development.

B. mucronatus was inoculated onto top cross-cut surfaces of 20 cm stem cuttings of susceptible *P. thunbergii*, *P. strobus*, *P. rigida* and a *P. thunbergii* resistant family Namikata. Time-course changes in distribution of *B. mucronatus* in tissues of those pines were investigated using the F-WGA staining technique up to 8 dai. Distribution patterns of *B. mucronatus* in tissues of susceptible and resistant pines were essentially the same as those of *B. xylophilus*; *B. mucronatus* spread to the remote region in susceptible *P. thunbergii* at 8dai, whereas migration of *B. mucronatus* was inhibited in xylem of *P. strobus*, *P. rigida* and a *P. thunbergii* resistant family Namikata. This result indicates that nonpathogenicity of *B. mucronatus* may not be derived from weakened migration ability.

To estimate the ability of PWN to kill pine cells, I developed the following method. A pine cutting is inoculated with PWNs onto top cross-cut surface. Several days after inoculation, the cutting is divided into small segments (about 2.5 cm long) and tangentially cut with a razor blade to expose epithelial cells inside cortical resin canals longitudinally. Distribution of dead epithelial cells inside cortical resin canals is observed after staining with Evans' blue which stains dead cells but not living ones. While almost no dead cell in non-inoculated *P. thunbergii* cuttings throughout the experiment for 7 days, dead cells were distributed sparsely and in an isolated manner among epithelial cells in *B. xylophilus*-inoculated cuttings even at 1 dai. The sparse distribution of isolated dead cells may interestingly mean that a PWN attacks one epithelial cell at once and randomly. Although density of dead cells increased at 3 dai, the dead cells were still distributed in a sparse and isolated manner. At 7 dai, dead epithelial cells increased, and in some resin canals, all cells were dead. The pattern of cell death caused by *B. mucronatus* inoculation was essentially the same as that by *B. xylophilus* inoculation. The results suggest that *B. mucronatus* has the same ability to kill epithelial cells and probably other pine cells as *B. xylophilus*.

Discoloration of pine bark peelings caused by PWN inoculation on its cambium side was also compared between *B. xylophilus* and *B. mucronatus*. Discoloration rates of non-inoculated and *B. mucronatus*- and *B. xylophilus*-inoculated bark peelings were 13%, 27% and 100% at 7 dai, and 20%, 60% and 100% at 11 dai, respectively. This result also indicates that *B. mucronatus* has ability to discolor pine cells although it is weaker than that of *B. xylophilus*.

From the above results, I concluded that lack of pathogenicity in *B. mucronatus* is not derived from lack of migration ability or ability to kill pine cells, but deficiency of some other ability.

Conclusion

The present time-course chases of individual PWN distribution within each pine tissue provided new findings on PWN migration routes, PWD-resistance, and PWN-pathogenicity. Pulse-chase experiments first provided direct evidence for detailed migration routes of *B. xylophilus* in *P. thunbergii*; from cortical resin canals to xylem axial resin canals and vice versa, and thereafter from both cortical and xylem resin canals to surrounding tissues. Inoculation experiments of resistant pine species, *P. strobus*, *P. rigida* and a resistant family of *P. thunbergii*, showed that migration of *B. xylophilus* in these pine species was inhibited especially in xylem at the early days after inoculation. The inoculation experiment with nonpathogenic *B. mucronatus* showed that the PWN has ability to migrate in resin canals and spread to surrounding tissues. Moreover, I developed a new technique to estimate PWN attack to epithelial cells of cortical resin canals, and using it, found that *B. mucronatus* have ability to kill pine epithelial cells in the same manner as *B. xylophilus*.