博士論文（要約）

Physiological and Molecular Biological Analyses of Induction Mechanism of Dispersal Stage in Pine Wood Nematode

（マツノザイセンチュウ分散型誘導メカニズムの生理学的・分子生物学的解析）

田中 克

Contents

Summary.

As spreading the pine wilt disease in the East Asian and the West European countries, the need to reveal the pathogenetic and epidemic mechanisms of the disease has been increased. *Bursaphelenchus xylophilus*, the pathogen of the pine wilt disease, has two different stages in the life cycle; one is the propagative stage, and the other is the dispersal stage. In the propagative stage, nematodes multiply inside the host pine tree; and as tree dies, nematodes change their stage to dispersal one. In the dispersal stage, nematodes tolerate under unfavorable condition inside the dead tree from the autumn to the next spring, and get in a *Monochamus* beetle to be transported to a healthy pine tree. This stage shift corresponds to the disease shift from the pathogenetic phase (disease development in a tree) to the epidemic phase (spread in a pine forest). The third-stage dispersal juvenile (J_{III}), which emerges from second-stage propagative juvenile and overwinters inside a dead pine tree, is the key point of the shift of disease cycle. Only J_{III} can molt to the fourth-stage dispersal juvenile (J_{IV}), which is loaded on the vector beetle.

Previous studies showed that phoresy, in which nematodes use other organisms as a transporting vehicle as *B. xylophilus* does, is the intermediate phase in the evolution from free-living to animal parasitism. The dauer-like stages (dispersal stages in *B. xylophilus*, and infective juvenile stage in parasitic nematodes) are indispensable for the parasitism because nematodes in that stage actually interact with other organisms. Therefore, J_{III}-inducing mechanism is an important and interesting theme to study for not only protecting the pine forest, but also revealing the parasitism evolution. In this study, physiological and molecular biological analyses were conducted in order to reveal J_{III}-inducing mechanism.

At first, nematode materials were screened to select the strain of the highest J_{III}-inducing ability, which can be used in the following analyses. A field isolate T4,

showed the highest J_{III} emergence rate among tested 4 isolates, and inbred lines were established from T4 by sister-brother mating for 12 generations. At last, ST2, one of the inbred lines, showed highest and stable J_{III} emergence rate. Therefore, I selected ST2 line to be used in the following analyses.

In the above-mentioned screening test, the inbred lines showed 3 types of J_{III} emergence rate (high, medium, and low). As T4 is known to have low genetic diversity in its population, it was suggested that J_{III} inducing ability is regulated by small number of key genes. In the experiment using the inbred lines, the total number of nematode was negatively correlated to J_{III} emergence rate. This is because the individuals that molted to J_{III} did not develop to the reproductive stage, and the number of adults that can make the next generation nematodes became small. To understand the evolutionally significance of J_{III} inducing ability in *B. xylophilus*, difference of the life cycle strategy among isolates should be investigated under the field condition.

The model organism, *Caenorhabditis elegans*, has "dauer" stage which is similar to dispersal stages in *B. xylophilus* in morphological and functional aspects. Therefore, *B. xylophilus* is supposed to have similar J_{III}-inducing mechanism to dauer-induction in *C. elegans*. As *C. elegans* is known to have dauer-inducing pheromone (Daumone) whose concentration reflects population density, the existence of J_{III}-inducing pheromone in *B. xylophilus* is hypothesized. Using the method which I newly established, I tested J_{III}-induction activity of materials secreted by nematodes. As a result, the hypothesis was proved correct, however, it was suggested that J_{III}-inducing pheromone was different kind of substance from Daumone in *C. elegans*.

Lipid droplets are one of the main characters of J_{III} identification because J_{III} has to survive during the winter under unfavorable condition inside the dead tree. However, J_{III} induced by the newly established method had small amount of lipids inside its body. In the field condition, nematodes feed on blue-stain fungi, and these fungi are known to degrade host pine lipids as nutrient. Therefore, nematodes grow under the lipid-rich

condition and can store lipids easily. However, in the new method, I used just plain agar and yeast as the food source. Hence, nematodes could not store lipids, but they could molt to J_{III}. Conclusively, it is shown that accumulation of lipid droplets is not necessary to induce J_{III}.

For investigating J_{III}-inducing mechanism, RNA-based analyses are useful. However, the conventional method of RNA extraction spends a lot of nematodes, time, and effort. Therefore, a new RNA extraction method with chemical digestion was established. Using this method, I could extract larger amount of RNA from smaller number of nematodes than the conventional method, and its quality was also high enough for RNA-seq analysis. In comparison of the quantity of extracted RNA between several nematode species, it was revealed that RNA is more easily extracted from nematode species living in the soil or decayed organisms, than from nematode species inhabiting in live plants. It suggested that the important factor to determine RNA extraction efficiency in this method is the nematode tolerance against the chemical digestion.

RNA-seq analysis was conducted for characterizing "J_{III}" in comparison with other stages. From the results, some genes in the main pathways of dauer induction (cGMP, IIS, TGF-β, and DA/DAF-12 signaling pathway) in *C. elegans* showed the similar expression pattern in J_{III} and J_{IV} to dauer stage in *C. elegans*. Therefore, it was suggested that the dauer mechanism and function in *C. elegans* was divided into two dispersal stages in *B. xylophilus*. Next, transcriptomic analysis of J_2 with/without treatment by J_{III}-inducing pheromone (nematode secretion filtrate) was also conducted to reveal the regulating system of J_{III} induction. The dauer-related genes identified in *C. elegans* showed different expression patterns in *B. xylophilus*. On the other hand, GO enrichment analysis showed that some chemosensory genes such as cGMP-related genes were up-regulated in J_2 during J_{III} induction. From these results, it was suggested that dauer-maintenance mechanism in *C. elegans* was divided and conserved in two

dispersal stages-maintenance mechanisms, J_{III} and J_{IV} in *B. xylophilus*. However, J_{III}-inducing mechanism in *B. xylophilus* was indicated to be different from dauer-inducing mechanism in *C. elegans*.

This study showed that J_{III}-inducing mechanism in *B. xylophilus* might be different from dauer-inducing mechanism in *C. elegans* from both physiological and molecular biological aspects, i.e., the different pheromone and the different signaling pathway to induce the dormant stage. In the adaptation to parasitism, induction of dispersal stage/infective juvenile might have needed different mechanism from dauer-induction in free-living nematodes like *C. elegans*. In order to understand the parasitism evolution in nematodes, further analyses of inducing mechanism of dauer-like stages using multi-layered omic and computational methods are needed.

Chapter 1.
General Introduction.

Section 1-1. History of Pine Wilt Disease.

Pine wilt disease is one of the most serious forest diseases in the world. In Japan, pine wilt disease was first reported in Nagasaki prefecture via imported pine logs from North America in 1905 (Yano 1913). Until 1960s, most scientists believed that some of bark-boring insects such as longhorn beetles, *Monochamus alternatus* and *Arhopalus rusticus*, weevils *Pissodes obscurus* and *Shirahoshizo* spp., and bark beetles *Cryphalus fulvus* and *Tomicus piniperda* were the causal agent of massive mortality of pine forests, because dead pine trees contained enormous number of the larvae under the bark (Sata 1942; Inoue 1949; Nitto et al. 1959a and 1959b; Oda 1964; Kishi 1988). However, these "secondary insects" do not attack healthy pine trees, but only weakened trees (Iwasaki and Morimoto 1971), thus other biotic and abiotic factors to weaken pine trees were surveyed. Around 1970, they have discovered that a nematode was the causal agent of pine wilt disease (Tokushige and Kiyohara 1969; Kiyohara and Tokushige 1971), and Mamiya and Kiyohara (1972) named the pathogen pine wood nematode, *Bursaphelenchus lignicolus* Mamiya et Kiyohara. Although this nematode was described as a new species, Nickle et al. (1981) showed that this nematode was the same species as a previously described nematode, *B. xylophilus* (Steiner et Buhrer) Nickle, which was first isolated in 1931 from blue-stained pine log in Louisiana, USA and named *Aphelenchoides xylophilus* Steiner et Buhrer (Steiner and Buhrer 1934), and transfered to *Bursaphelenchus xylophilus* by Nickle (1970).

The genus *Bursaphelenchus* which was established in 1937 (Fuchs 1937) contains 110 to 120 species (Futai 2013). Most *Bursaphelenchus* spp. inhabit inside weakened or dead trees, feed on fungi inside the tree, and transported to other trees on vector beetles

(Ryss et al. 2005). It was reported that *B. xylophilus* is also mycophagous and associated with the vector insects, *Monochamus alternatus* Hope (Mamiya and Kiyohara 1972; Morimoto and Iwasaki 1972) and *M. saltuarius* Gebler (Sato et al. 1987), pine sawyer beetles native to Japan. This mycophagous/plant parasitic nematode is native to North America (Steiner and Buhrer 1934; Nickle et al. 1981; de Guiran and Bruguier 1989; Jones et al. 2008) and vectored by *M. carolinensis* there (Kondo et al. 1982; Linit et al. 1983; Linit 1988). It was thought that pine wood nematode was introduced from United States to Nagasaki in 1905, and now spreads into all prefectures except Hokkaido during these 100 years (Takemoto and Futai 2007; Kimura et al. 2011). Moreover, this nematode has spread into East Asian countries including China, Taiwan and Korea (Cheng et al. 1986; Han et al. 2008), and now spreading even in West European countries including Portugal and Spain in spite of enormous efforts for quarantine activities (Mota et al. 1999; Abelleira et al. 2011; Jones et al. 2013).

Section 1-2. Disease Cycle and Life Cycle of *B. xylophilus*.

The disease cycle of pine wilt disease is divided to two phases, i.e., one is the pathogenetic phase (disease development in pine trees), and the other is the epidemic phase (spread of the pathogen in a pine forest). The first phase starts when *B. xylophilus* invades into healthy pine trees attracted by pine volatiles via wounds caused by maturation feeding or oviposition of the vector beetles (Mamiya and Enda 1972; Morimoto and Iwasaki 1972; Wingfield 1983; Linit 1990; Aikawa and Togashi 1998; Arakawa and Togashi 2002). After invasion, this nematode moves into the xylem resin canals, and eats epithelial cells (Mamiya 1972). As the result, cavitation occurs around xylem, and water potential gets decreased, leading the death of the host tree (Mamiya and Tamura 1977; Ikeda and Suzaki 1984; Kuroda et al. 1988; Fukuda 1997; Ichihara et al. 2000). Coinciding with chlorosis of old needles and wilting of current shoots, *B.*

xylophilus multiplies enormously, and it was suggested that the symptoms are caused by this nematode increase (Kiyohara and Suzuki 1975). *Monochamus* beetles oviposit on the weakened or newly dead pine tree, and the offspring larvae develop feeding on inner bark tissue. In the next early summer, adult beetles loading nematodes in their tracheal system emerge from the dead pine tree, and spread the disease to healthy pine tree (Figure 1 and 2) (Mamiya and Enda 1972; Morimoto and Iwasaki 1972).

In conjunction with this disease cycle, *B. xylophilus* has an interesting life cycle, which includes two different series of juvenile forms, i.e., propagative form and dispersal form (Figure 3) (Mamiya 1975). Under favorable condition in pine tissues, *B. xylophilus* increases its population through propagative form. The first stage propagative juvenile molts within the egg shell and the second stage propagative juvenile (J_2) hatches out. Then J_2 feeds on pine cells in living trees or feeds on fungal hyphae in dead trees, and it develops into the third stage propagative juvenile (J_3) and then the fourth stage propagative juvenile (J_4), to become a male or female adult to mate and lay eggs. On the other hand, under unfavorable condition, i.e., starvation and high population density, J_2 doesn't develop J_3 but the alternative third stage juvenile, i.e. third stage dispersal juvenile (J_{III}) which is morphologically distinguishable from propagative nematodes (Mamiya and Enda 1972; Kiyohara and Suzuki 1975; Ishibashi and Kondo 1977; Fukushige 1991; Maehara and Futai 2000). The J_{III} can overwinter and survive under the unfavorable condition until adult beetles emerge in pupal chambers under pine bark in the next early summer. When J_{III} received the chemical signals from an adult beetle, J_{III} molts to the fourth stage dispersal juvenile (J_{IV}) (Kiyohara and Suzuki 1975; Ishibashi and Kondo 1977; Warren and Linit 1993; Maehara and Futai 1996; Necibi and Linit 1998; Zhao et al. 2013). The J_{IV}s move into the tracheae of the vector beetle, and the beetle flies to healthy pine trees (Morimoto and Iwasaki 1973; Aikawa and Togashi 2000). When the beetle feeds on the branches, J_{IV} nematodes invade into the host pine tree via the wounds made by the beetle feeding,

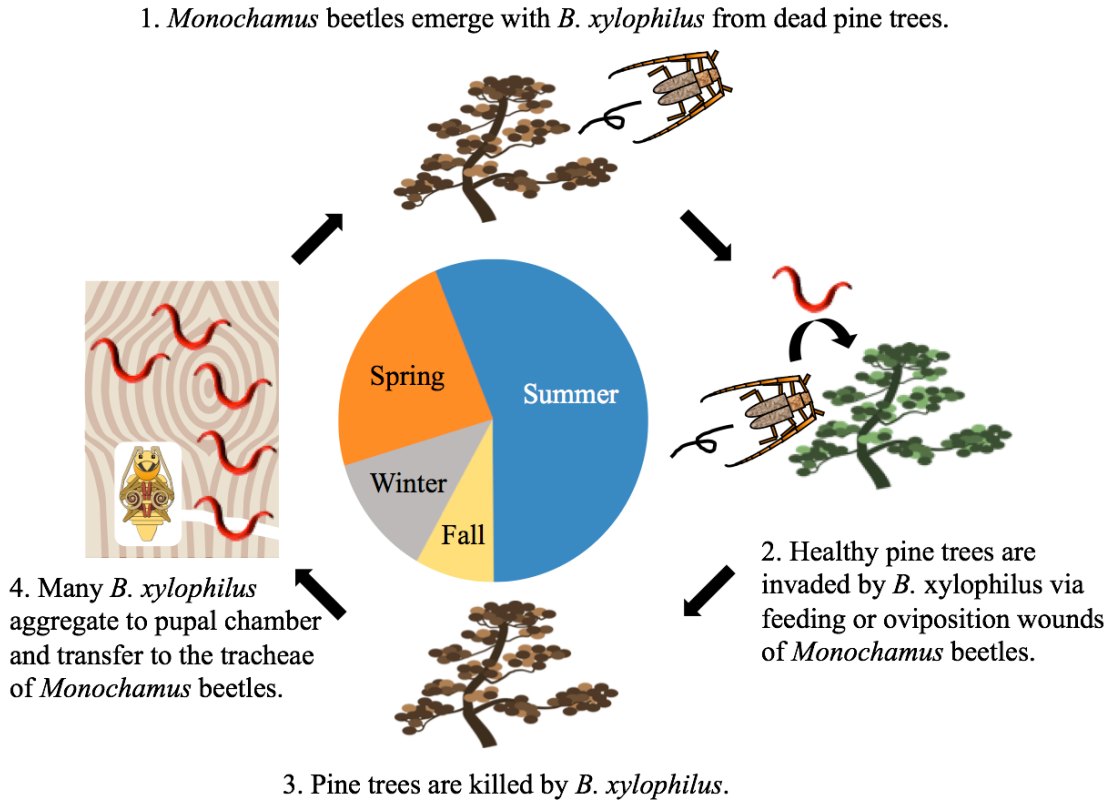

Figure 1. The disease cycle of pine wilt associated with pine trees, nematodes, and beetles. The center circle indicates seasons, although the length of the arc does not correspond to the actual span of the season in Japan.

and the J_{IV}s molt to adults which migrate into the pine stem, multiply, and kill the tree.

Section 1-3. Previous Studies about *B. xylophilus*.

In previous studies on *B. xylophilus*, many researches focused on pathogenicity to pine trees and to the interaction with vector beetles. Some researches showed that *B. xylophilus* feed on and propagate on some fungi such as blue-stain fungi, however, other fungi are unsuitable for this nematode propagation (Kobayashi et al. 1974, 1975; McGawley et al. 1985; Wingfield 1987; Fukushige 1991; Maehara and Futai 2000). Some other researchers suggested that catalase activity is an important factor in order to avoid the host immune system 'hypersensitive reaction (HR)' which is the plant

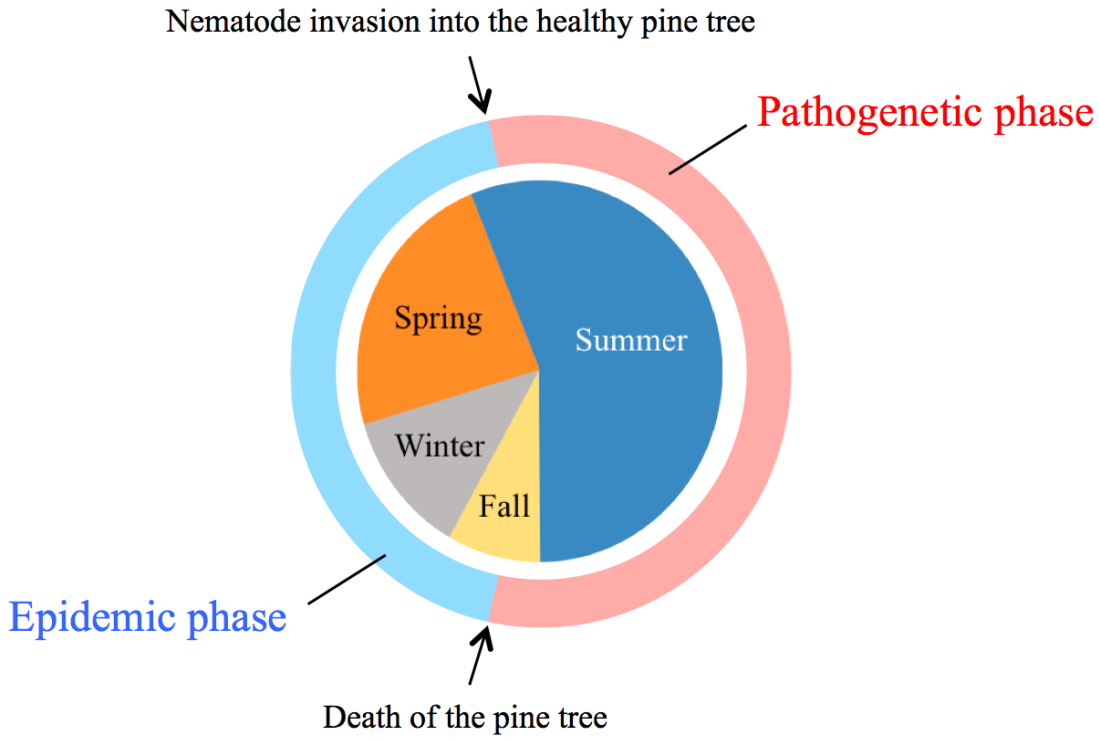

Figure 2. Two phases of pine wilt disease. The center circle indicates seasons, although the length of the arc does not correspond to the actual span of the season in Japan.

immune system accompanying generation of reactive oxygen species called "oxidative burst". (Klement and Goodman 1967; Iwahori and Futai 1993; Hammond-Kosak and Jones 1996; Vicente et al. 2013, 2015; Wu et al. 2013). Other researchers investigated the secretion of cellulase by this nematode which helps its feeding on pine cells and induces wilting symptoms (Odani et al. 1985; Yamamoto et al. 1986; Kojima et al. 1994) and a lytic enzyme to degrade microbe cell wall component in dying/dead pine stem (Kuroda 1987). From recent genomic studies, it was revealed that the cellulase- and the chitinase-associated genes of *B. xylophilus* were acquired by horizontal gene transfer from fungi and bacteria, respectively (Kikuchi et al. 2004, 2005, 2006, 2007, 2011). The adaptation of *B. xylophilus* to surrounding environment i.e. the conversion of phytophagous and mycophagous phase, was also investigated (Tsai et al. 2016). Some other researchers found that nematode surface is stained by fluorescent

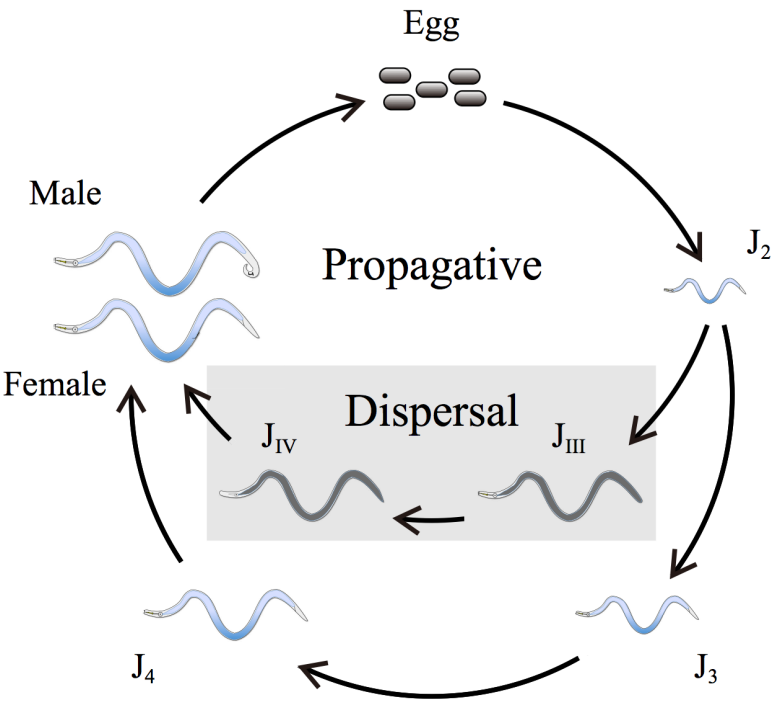

Figure 3. The life cycle of the pine wood nematode.

WGA in pathogenic strains, although the surface of non-pathogenic nematodes is not stained (Komatsu et al. 2008; Shinya et al. 2009a; Umebayashi et al. 2017), and some surface coat proteins which may act as reactive oxygen species scavengers in order to escape HR of the host tree were identified (Shinya et al. 2010, 2013). Morphological researches also exist that the tail tip form of an adult female is different between pathogenic strains and non-pathogenic ones; pathogenic strains have rounded tail tip, although non-pathogenic ones have a short mucron at the female tail tip (Kanzaki and Giblin-Davis 2016).

As described above, countless studies associated with pathogenicity of the nematode were conducted based on molecular biology, chemistry, and morphology. In the meanwhile, many researches were also conducted on nematode phoresis on beetles. These studies focused on the J_{IV}, such as the correlation between nematode density and number of nematodes loading to *Monochamus* beetles (Togashi 1985; Maehara and Futai 1996; Aikawa and Togashi 2000; Meahara et al. 2006), transmission pathways

from beetles to newly healthy pine trees (Linit 1990; Arakawa and Togashi 2002), and the induction of phoretic stage, J_{IV}, of *B. xylophilus* by the chemical signals from the beetle (Necibi and Linit 1998; Maehara and Futai 2001; Ogura and Nakashima 2002; Nakazato et al. 2012; Zhao et al. 2013). However, few studies associated with J_{III} have been conducted, in spite of its importance; J_{III} is the key stage associated with the transition of nematode survival strategy from propagative stages to dispersal stages. J_{III} nematodes tolerate for a long time inside the pine tree under unfavorable conditions such as dryness and starvation (Ishibashi and Kondo 1977). Moreover, J_{IV}, the loading stage to the beetle, can emerge only via J_{III} (Ishibashi and Kondo 1977). Therefore, I focused on J_{III} induction as the important tolerance stage for nematode survival system.

Section 1-4. Comparison of Induction System of Dispersal Stages Between *B. xylophilus* and Other Nematode Species.

Many nematode species have dispersal-like stage, called “dauer” (Ishibashi and Kondo 1974; Cassada and Russell 1975; Sommer et al. 1996), which can tolerate under unfavorable condition or live inside the host animals or insects. Phoretic and necromenic associations in which nematodes use the host insects or invertebrates as the transportation tool from the current environment to the next one are considered as the pre-adaptations towards parasitism (Hermann and Sommer 2011). In other words, parasitic nematodes are evolved from free-living nematodes via phoretic and necromenic nematodes. Phoretic nematodes may have the intermediate mechanisms for adaptation to utilize their hosts in their life cycle between those in free-living and in parasitic nematodes. When we conduct comparative analysis using free-living, phoretic, and parasitic nematodes, we can clarify one aspect of parasitism evolution. *Bursaphelenchus xylophilus* belongs to the phoretic nematodes, i.e., the intermediate species in the evolution of parasitism. Therefore, the induction mechanism of dispersal

form in this nematode species is important in revealing the evolution of nematodes.

The model organism, *Caenorhabditis elegans*, is the most important reference in studying the special/tolerance stage because *C. elegans* also has dauer stage and is investigated very well, especially from the point of view of molecular biology and chemistry (Fuchs 1915; Cassada and Russell 1975; Klass and Hirsh 1976; Golden and Riddle 1982, 1984a and 1984b; Jeong et al. 2005; Hu 2007; Kaplan 2011; Ludewig et al. 2013; Hussey et al. 2017). The primary cue to induce dauer stage in *C. elegans* is a dauer-inducing pheromone (Daumone), (-)-6-(3,5-dihydroxy-6-methyltetrahydropyran--2-yloxy) heptanoic acid, which was first identified in 2005 (Jeong et al. 2005). The food signal that reflects food supply and temperature is also important at intermediate Daumone concentration. It has been assumed that Daumone binds to a special cell surface receptor located in amphid neurons (Bargmann and Horvitz 1991; Schackwitz et al. 1996), and the signal is transmitted to Guanylyl cyclase (Thomas et al. 1993; Birnby et al. 2000), Insulin/IGF (Kimura et al. 1997; Lin et al. 1997; Ogg et al. 1997), and Transforming growth factor β (Thomas et al. 1993). Finally, this signal is arrived in DA/DAF-12 module, and the decision whether worms should become dauer stage or not is conducted (Antebi et al. 2000; Hu et al. 2007). From previous studies, *B. xylophilus* is known to change its life cycle to dispersal forms by low food supply and high nematode density (Ishibashi and Kondo 1977; Fukushige 1991; Maehara and Futai 2000). This phenomenon is similar to dauer induction in *C. elegans*. Other reports also supposed that *B. xylophilus* might have similar mechanism to dauer formation in *C. elegans* because J_{III} relative abundance is correlated with population density and *B. xylophilus* mostly conserved dauer-related genes of *C. elegans* (Forge and Sutherland 1996; Kikuchi et al 2011).

Section 1-5. Aim of This Study.

Based on the previous researches as above, I set the aim of this study to reveal the stage transition mechanism from propagative stages to dispersal stages, i.e. from J_2 to J_{III}, which is the key point in nematode survival strategy, from physiological and molecular biological approaches. After selecting the nematode material for experiments, I established artificial induction method of J_{III}. Using this method, I conducted transcriptomic analyses about the maintenance and induction of J_{III}.

In chapter 2, in order to select the nematode material appropriate to the analysis of J_{III} induction, I compared J_{III} emergence rate in nematode isolates collected from field. One field isolate showing the highest J_{III} emergence was selected, and newly 8 inbred lines were established from the isolate through brother-sister mating. After comparison of J_{III} emergence rate of inbred lines, one inbred line showing the highest J_{III} emergence and low genetic diversity was selected as the nematode material for J_{III} induction analysis. In chapter 3, by using the newly established inbred line, the method of artificially J_{III} induction was established, and I clarified the existence of J_{III}-inducing pheromone in the secretion of *B. xylophilus*. In chapter 4, I established simple and quick method for RNA extraction from *B. xylophilus* in order to conduct transcriptomic analysis more easily and effectively. In chapter 5, transcriptomic analysis of J_{III} was conducted compared with the other all stages in order to show the specifically expressed genes and the characters in J_{III}. I also conducted the RNA-seq analysis to reveal gene expression pattern during J_{III} induction in this chapter. In the last chapter, I will discuss the evolution of the life cycle of *B. xylophilus* including dispersal form by referencing the dauer form in *C. elegans* and other nematodes.

Chapter 2.
Establishment of New Inbred Lines Showing High J_{III} Inducing Ability of *Bursaphelenchus xylophilus*.

Section 2-1. Introduction.

As the first step, I have to select the nematode strain appropriate to analyses of J_{III} induction. There are more than 29 field isolates collected from different geographical sites in Japan (Takemoto and Futai, 2007). It is known that pathogenicity varies among these isolates, and also the propagation rate (Kiyohara and Bolla 1990; Kosaka et al. 2001). They were used differently for each experiment, especially in the studies on the pathogenicity for pine trees and the interaction with host beetles. However, the isolate appropriate to J_{III} induction research, i.e. showing the highest rate of J_{III} emergence, still remained unknown. In this chapter, in order to select appropriate nematode material to J_{III} induction study, I selected the nematode isolate with the highest J_{III} emergence rate among field isolates which varied in pathogenicity and propagation rate. After that, I established inbred lines from the selected field isolate and investigated the J_{III} emergence rate of them.

Section 2-2. Materials and Methods.

Nematode.

Four Japanese field isolates of *B. xylophilus* from different georgraphical origin, namely, virulent S10, T4, and Ka4, and avirulent C14-5 were used for this study, as candidates for the parental strains of inbred lines with high J_{III}-emergence. The virulent strains S10, T4, and Ka4 were originally collected from dead trees of the Japanese red pine, *Pinus densiflora*, in the Shimane Prefecture in 1982, the Iwate Prefecture in 1992,

and the Ibaraki Prefecture in 1994, respectively. C14-5 was obtained from an adult *M. alternatus* beetle in Chiba Prefecture in 1975 (Takemoto and Futai 2007), and is physiologically and biologically different from other strains, thus it has been widely used for experiments as a comparative strain for other highly virulent strains (Kiyohara and Bolla 1990; Kosaka et al. 2001). For S10, T4, and Ka4, nematodes were reared on a fungal lawn of *Botrytis cinerea* on potato dextrose agar (PDA) media in 9-cm-diameter Petri dishes, and kept at 25°C for 7 days, while C14-5 was kept at 25°C for 16 days because the propagation of C14-5 is slower.

Hereafter, I define "isolate" as a field-collected population without genetic purification, and "inbred line" as the genetically purified population derived from the "isolate".

Investigation of the Emergence Rate of J_{III}.

Propagated nematodes were collected using the Baermann funnel technique, and surface-sterilized using lactic acid following the method described in Mamiya et al. (2004). In short, nematodes were collected in a 10-mL glass tube, washed three times with distilled water, and the same volume of ca. 6% lactic acid solution was added to the nematode suspension. After 30 seconds of soaking the nematodes in the lactic acid solution, the suspension was centrifuged at 1,500 rpm for 30 seconds, and the supernatant was removed. Nematodes were then rinsed three times with sterilized water. Fifty surface-sterilized nematodes of each isolate were reared on the hyphae of *Cosmospora viridescens*, which is frequently used in experiments involving dispersal stage induction (Kanzaki et al. 2016; Maehara and Kanzaki 2016), growing on malt extract agar (2% malt extract, 1.5% agarose) in 4-cm-diameter Petri dishes, and kept at 25°C for 10, 20, and 30 days. Each treatment was repeated eight times.

Then, nematodes were collected using the Baermann funnel technique, and killed by heat (ca. 60°C for 1 min), and fixed in TAF (2% triethanolamine, 2.775%

formaldehyde) (Hooper 1986). Fixed nematodes were kept at room temperature until microscopic observation. The numbers of total nematodes and J_{III} were counted for each sample using a light microscope (Eclipse 80i, Nikon), and the rate of J_{III} (J_{III} emergence rate) was calculated.

Establishment of Inbred Lines and Evaluation of Their J_{III} Emergence Rate.

Field-collected isolates of *B. xylophilus* usually have high genomic diversity (Cheng et al. 2008); therefore, it is necessary to establish inbred lines for molecular and biochemical analyses. I selected and used the isolate T4, which showed the highest J_{III} formation rate in the above-mentioned test, as the parental isolate.

Botrytis cinerea was inoculated on 4% plain (water) agar in 48-well microplates, and kept at 25°C for 1 day. Distilled water (400 μL) was added on a fungal mat in the microplate, and one J_{III} nematode of T4 isolate was inoculated into the water in each well, and reared for 2 days at 25°C to obtain unmated adult females and males. *Botrytis cinerea* was cultured on 4% plain agar in 4-cm-diameter Petri dishes for 1 day. An unmated female and unmated male were then transferred to fungal hyphae on the dish. The culture plates were kept at room temperature and observed everyday, and a young gravid female of the next generation was transferred to a new dish. Offspring were isolated using a microplate and used for the next mating. This procedure was repeated for 12 generations, and eight inbred lines were thus established from T4, and coded as ST1 to ST8.

The J_{III} emergence rate of the eight newly established inbred lines were examined with the method described above. After that, I conducted the correlation test between total number and J_{III} emergence rate of nematodes.

Statistical Analysis.

Statistical analysis was performed using R version 3.2.4. I calculated the emergence

rate of J_{III} using the following formula:

$$J_{III} \text{ emergence rate (\%)} = [(\text{number of } J_{III}) / (\text{number of all worms})] \times 100$$

Data represent the mean ± standard error (SE). For the statistical analysis, I performed the normality test to reveal the normality of the data to decide whether to use parametric or non-parametric tests. For the J_{III} emergence rates of isolates which are non-parametric data, Kruskal–Wallis one-way analysis was performed to test between isolates, and subsequent Steel–Dwass multiple comparison tests were conducted. To analyze J_{III} emergence rates of inbred lines which are parametric data, one-way analysis of variance (ANOVA) and post hoc Tukey–Kramer multiple comparison tests were conducted. To analyze the correlation of all numbers and J_{III} rates, I conducted Spearman's rank correlation test.

Section 2-3. Results.

J_{III} Emergece Rate of Each Isolate.

The results are summarized in Figure 4 and Table 1. Two virulent isolates, S10 and Ka4, propagated rapidly, and maintained high population densities over the 30 days of the experimental period. Conversely, virulent T4 and avirulent C14-5 propagated slowly ($P < 0.01$). However, both number and rate of J_{III} were significantly higher in T4 than in the other three isolates ($P < 0.01$ for both).

J_{III} Emergence Rate of Each Inbred Line.

The results are summarized in Figure 5, and Table 2. The total number of nematodes in lines ST4 and ST5 was significantly higher than in the other six lines, while the J_{III} formation rate of ST1 and ST2 was significantly higher than in the other lines, and that

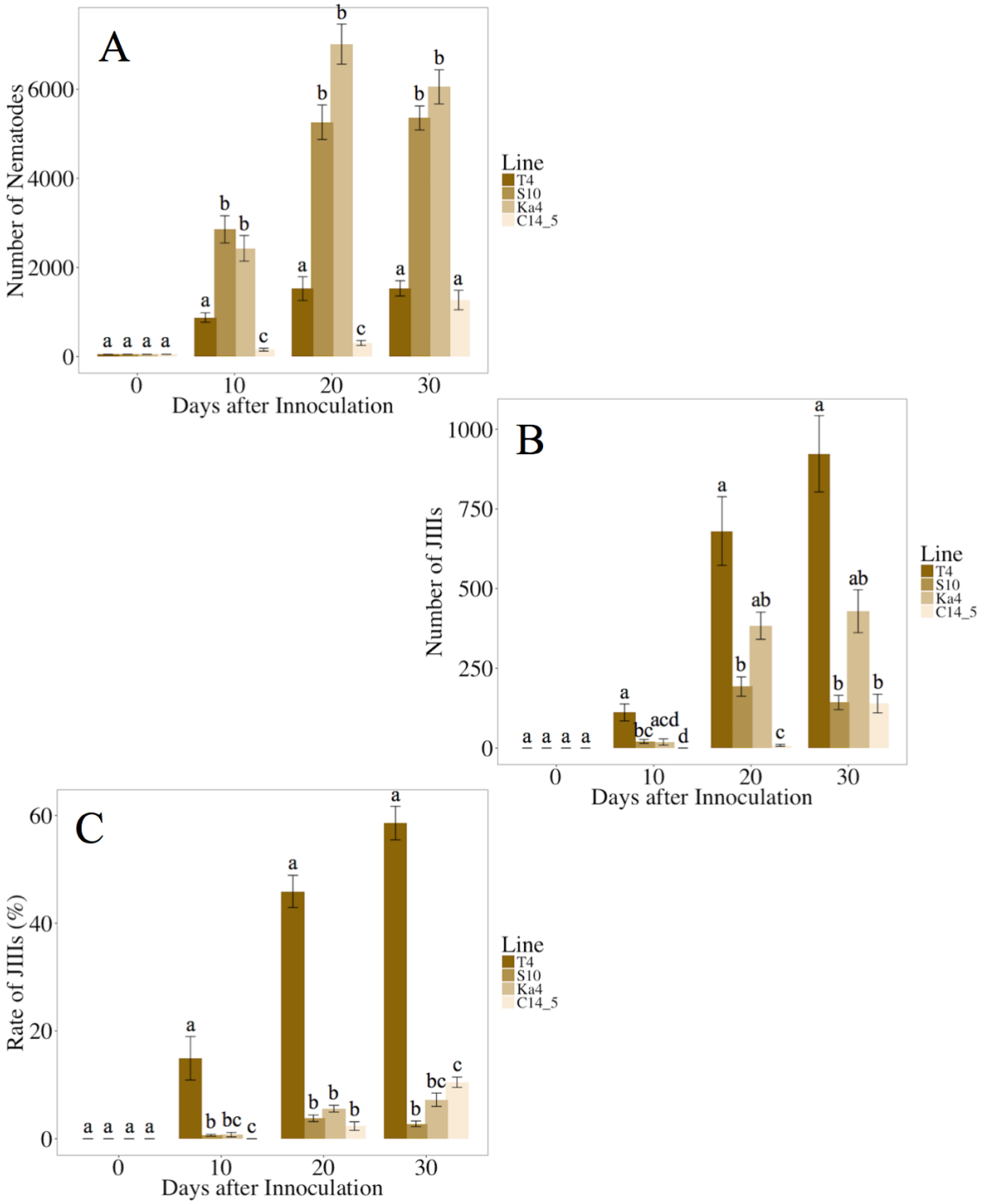

Figure 4. Propagation and J_{III} production of four isolates of *B. xylophilus* after 10, 20, and 30 days of inoculation. A: total number of nematodes; B: number of J_{III}; C: J_{III} emergence rate. The same letters indicate non-significant differences between the samples at the same time point. Bars and error bars represent averages and standard errors for ten replicates, respectively.

of ST4 and ST5 was significantly lower than the others. This tendency was supported by the significant negative correlation between the total number of nematodes and J_{III}

Table 1. Variation in the number of total and J_{III} nematodes and J_{III} emergence rate of each field isolate.

Isolate	Days	Number	J_{III} Number	J_{III} Rate (%)
T4	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	877.8±105.9	112.0±26.6	14.9±4.0
	20	1524.2±265.0	680.2±107.9	45.9±3.0
	30	1530.2±171.6	922.8±120.0	58.59±3.1
S10	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	2855.0±305.4	21.1±6.0	0.7±0.2
	20	5260.8±385.8	192.9±30.2	3.8±0.6
	30	5356.1±268.4	143.1±22.4	2.8±0.5
Ka4	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	2430.5±287.8	19.0±10.0	0.8±0.4
	20	7010.7±448.7	383.6±42.5	5.6±0.6
	30	6053.0±382.6	429.0±67.2	7.3±1.2
C14-5	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	154.5±31.8	0.0±0.0	0.0±0.0
	20	304.6±55.9	8.46±3.2	2.4±0.8
	30	1268.6±218.4	139.5±29.3	10.5±0.9

"Line" shows the nematode line provided to the experiment. "Days" shows the incubation days. "Number" shows the mean value and SE (standard error) from the total nematode number. "J_{III} Number" shows the mean value and SE of the number of J_{III}. "J_{III} Rate" shows the mean value and SE of the rate of J_{III} derived from the total number and J_{III} number. This experiment was repeated 10 times, and the mean value and SE was calculated from the results of repeats.

emergence rate ($r^2 = 0.50$, $P < 0.01$; Figure 6).

Section 2-4. Discussion.

Many isolates of *B. xylophilus* have been isolated and maintained in laboratories,

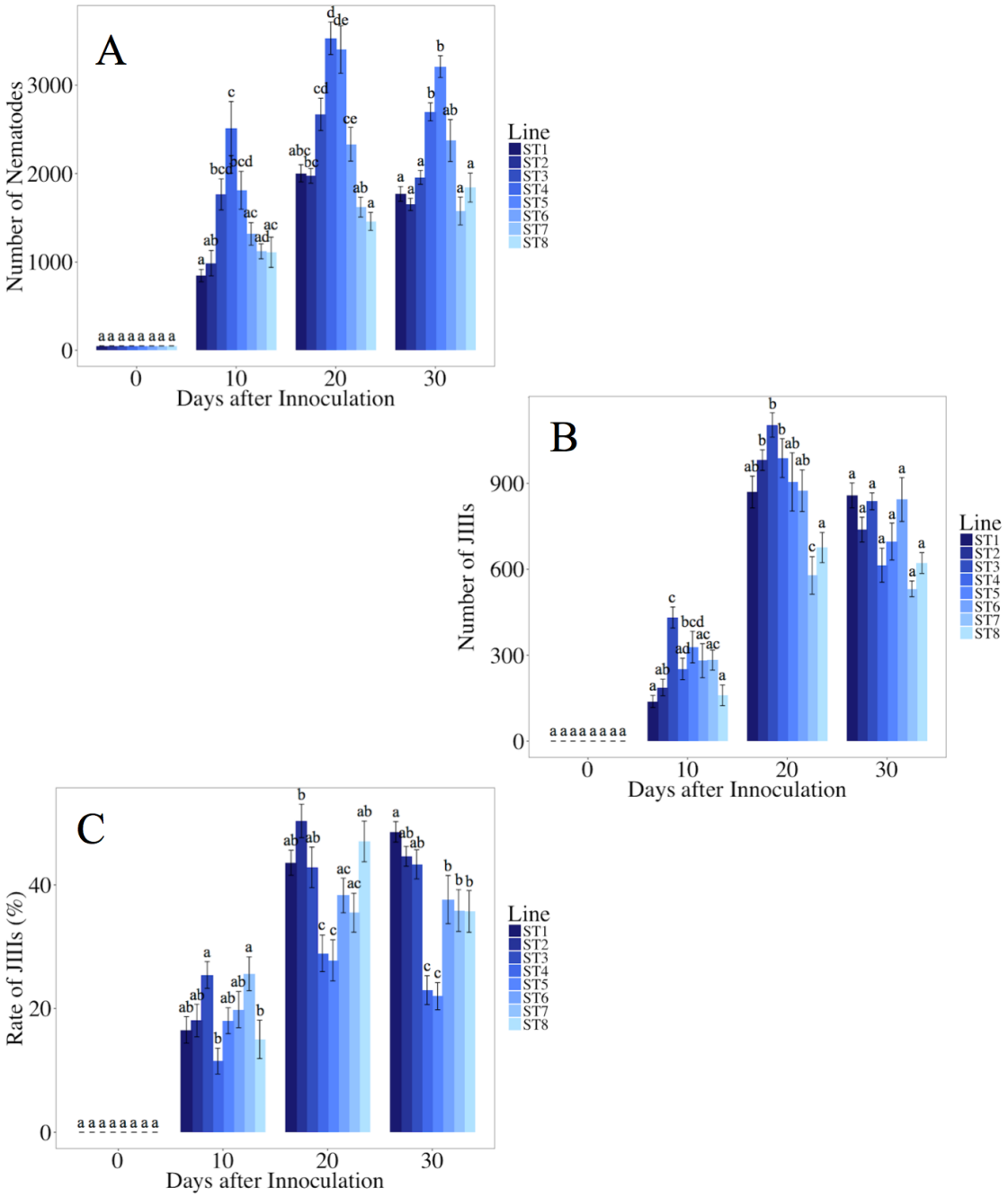

Figure 5. Propagation and J_{III} proportion of eight inbred lines of *B. xylophilus* derived from the T4 isolate after 10, 20, and 30 days of inoculation. A: total number of nematodes; B: number of J_{III}; C: J_{III} emergence rate. The same letters indicate non-significant differences between the samples at the same time point. Bars and error bars represent averages and standard errors for ten replicates, respectively.

and are quite variable in some biological characteristics, e.g., virulence, propagation, and efficiency of phoresy (Kiyohara and Bolla 1990). However, regardless of its importance, J_{III} formation rate has not been compared among isolates. In this study, T4

Table 2. Variation in the number of total and J_{III} nematodes and J_{III} emergence rate of each inbred line after 10, 20, and 30 days incubation.

Line	Days	Number	J_{III} Number	J_{III} Rate (%)
ST1	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	846.5±69.3	138.6±21.0	16.5±2.1
	20	2003.0±96.9	869.8±55.6	43.6±2.0
	30	1769.7±82.8	858.1±43.5	48.6±1.7
ST2	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	986.7±144.1	187.4±28.9	18.1±2.6
	20	1973.9±82.9	981.1±36.0	50.3±2.7
	30	1650.8±70.3	738.4±43.5	44.6±1.6
ST3	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	1764.0±175.7	431.6±36.5	25.4±2.2
	20	2668.8±183.4	1104.3±42.6	42.8±3.3
	30	1956.8±79.4	837.5±29.7	43.3±2.4
ST4	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	2509.3±304.9	252.2±37.3	11.5±2.1
	20	3530.0±184.7	988.1±67.8	28.9±3.0
	30	2697.6±101.9	614.1±59.3	23.0±2.3
ST5	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	1810.7±214.2	328.4±55.1	18.0±2.1
	20	3401.6±268.3	905.0±101.8	27.8±3.3
	30	3209.0±123.4	696.8±64.6	22.0±2.2
ST6	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	1316.7±126.8	280.9±59.3	19.8±2.9
	20	2331.6±191.5	874.2±72.4	38.3±2.8
	30	2373.4±237.3	843.6±76.5	37.6±3.9
ST7	0	50.0±0.0	0.0±0.0	0.0±0.0
	10	1120.2±84.8	283.0±35.0	25.6±2.7
	20	1620.7±113.2	578.3±65.6	35.5±3.1
	30	1577.0±157.9	531.6±27.5	35.8±3.4
ST8	0	50.0±0.0	0.0±0.0	0.0±0.0

10	1109.2±171.0	159.9±35.9	15.0±3.1
20	1457.5±102.3	675.7±52.7	47.0±3.3
30	1841.5±163.9	621.7±36.3	35.7±3.4

"Line" shows the nematode line provided to the experiment. "Days" shows the incubation days. "Number" shows the mean value and SE (standard error) from the total nematode number. "J_{III} Number" shows the mean value and SE of the number of J_{III}. "J_{III} Rate" shows the mean value and SE of the rate of J_{III} derived from the total number and J_{III} number. This experiment was repeated 10 times, and the mean value and SE was calculated from the results of repeats.

isolate showed significantly higher J_{III} emergence rate than the other three isolates (Figure 4, Table 1). Interestingly, although the T4 isolate is known to propagate quickly (Aikawa and Kosaka 1998), its population size was significantly smaller than the other two pathogenic isolates throughout the experiment. In previous studies, J_{III} began to appear after the nematode population reached the maximum level, and the rate and number of J_{III} increased as the population decreased (Mamiya 1972; Kiyohara and Suzuki 1975; Ishibashi and Kondo 1977). Considering the relationship between population size and J_{III} formation rate, J_2 nematodes of T4 are suspected to be more sensitive to J_{III} induction signals, such as unfavorable conditions, than in other isolates, and they shifted to the dispersal life cycle in the early phase of incubation.

In comparison, most individuals of the S10 and Ka4 isolates were J_2 after 30 days of the experimental period (Table 3). This suggests that the nematodes of these two isolates, S10 and Ka4, were starved and suspended their growth before turning to J_{III}. In the model organism nematode *C. elegans*, four distinct pathways that regulate dauer arrest have been confirmed by analyses of dauer-constitutive and dauer-defective mutants (guanylyl cyclase pathway, TGFβ-like pathway, insulin-like pathway, and steroid hormone pathway) (Riddle et al. 1981; Vowels and Thomas 1992; Thomas et al. 1993; Gottlieb and Ruvkun 1994; Gerisch et al. 2001; Jia et al. 2002; Viney et al. 2003). These analyses demonstrated that the ability to form dauer differs among mutants. Further, a satellite model species, *Pristionchus pacificus*, has natural variation

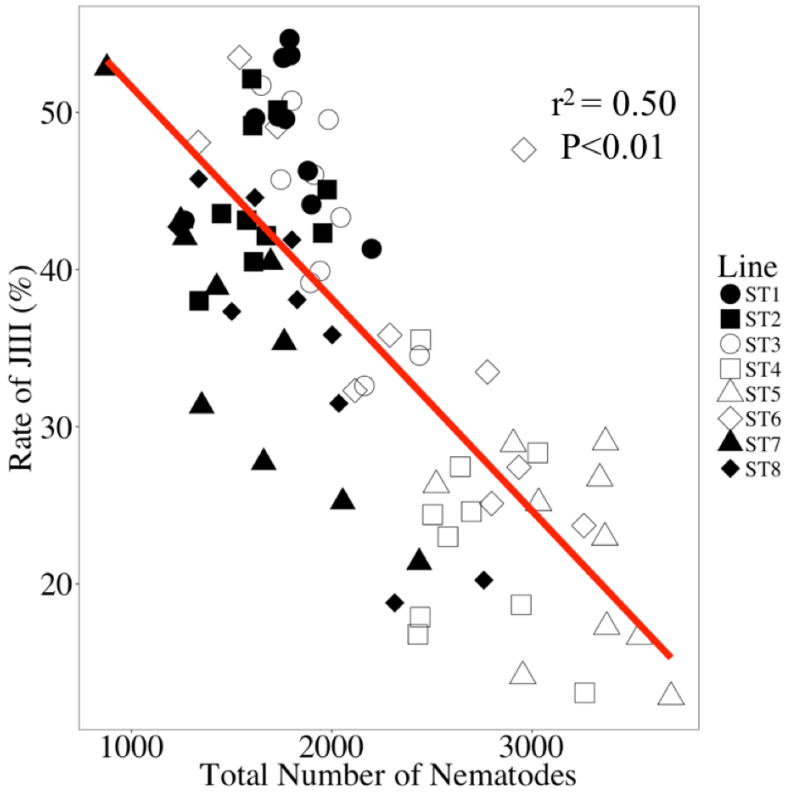

Figure 6. Correlation between total number of nematodes and J_{III} emergence rate. Each plot represents a culture plate.

Table 3. The number of each stage of two field isolates (S10 and Ka4) after 30days incubation.

Line	Number				
	L2	L3	L4	Adult	J_{III}
S10	110.5±14.5	23.0±3.0	17.5±2.5	13.0±2.0	6.5±1.5
Ka4	101.0±1.0	13.0±4.0	17.0±2.0	8.5±4.5	5.5±0.5

"Line" shows the nematode line provided to the experiment. "Number" shows the nematode number of each stage. Value shows "mean ± SE (standard error)". This experiment was repeated twice, and the mean value and SE was calculated from the results of repeats.

in pheromone production and sensitivity derived from genetic differentiation among several strains collected from different localities all over the world (Mayer and Sommer 2011; Bose et al. 2014). The variation in J_{III} emergence among PWN isolates seems similar to that of dauer formation among *C. elegans* and *P. pacificus* strains, and

therefore, nematodes of the isolate T4 may have some mutations associated with J_{III} formation, so they more readily form J_{III} compared to other isolates. In this study, I could not reveal whether production or sensitivity of pheromone resulted in the high J_{III} rate in T4; further physiological and chemical analyses are required to reveal the answer. It is interesting that T4 nematodes have maintained a high J_{III} production ability for a long sub-culturing history, despite extended sub-culturing of PWN isolates sometimes causing reductions in the pathogenicity against host pine trees and decreases in the rate of J_{III} formation (Kiyohara 1976; Ishibashi and Kondo 1977).

In a previous study, T4 showed a high degree of homozygosity (only 4.26% of variants in the T4 isolate genome) (Palomares-Rius et al. 2015), suggesting that the genetic diversity is rather low in this isolate. However, in the present study, the rate of J_{III} formation varied among inbred lines derived from the isolate; i.e., T4 retains genetic diversity associated with J_{III} formation within the highly homozygous isolate. The number of dauer-related (*daf*) genes varies among the nematode groups. For example, *C. elegans* has 1,259 *daf* genes in the latest version of gene association analysis (WS258) (http://www.wormbase.org), and only 52 *daf*-gene groups were identified in *Pristionchus pacificus* (http://www.wormbase.org). In the case of T4, although the isolate has low genetic diversity, the J_{III}-related genes seem variable among inbred lines because of their discontinuous J_{III} formation rates, i.e., the J_{III} emergence rates of inbred lines were separated statistically into high (ST1, ST2, and ST3), medium (ST6, ST7, and ST8), and low (ST4 and ST5) rate groups (Figure 5). Therefore, J_{III} formation of T4 is likely regulated by a relatively small number of key genes as in *P. pacificus*, and recombinant inbred lines derived from a line in the high J_{III} group and one in the low J_{III} group will be useful for identify key genes regulating J_{III} induction.

The total number of nematodes was negatively correlated with the J_{III} rate; i.e., the lines showing a high J_{III} formation rate did not propagate well during the experimental period. This negative correlation is probably because of the developmental arrest in J_{III}.

J_{III} is a kind of dauer (dormant) stage, and thus the individual that molted to J_{III} does not develop to the reproductive stage (= adult) under certain conditions. Therefore, the next generation of nematodes was produced only by the adults that had emerged at an earlier stage of culture (= early generation of adults). However, in the lines in which the J_{III} formation rate was lower, nematodes developed to adults and reproduced more effectively than the lines with higher J_{III} formation, and the total nematode population became larger in such lines.

Chapter 3.
Artificial J_{III} Induction of *Bursaphelenchus xylophilus*.

Section 3-1. Introduction.

As described in chapter 1, *B. xylophilus* might have similar dispersal inducing mechanism to that in *C. elegans* dauer formation (Ishibashi and Kondo 1977; Fukushige 1991; Forge and Sutherland 1996; Maehara and Futai 2000; Kikuchi et al 2011). In *C. elegans*, daumone which is the pheromone reflecting population density is the main signal substance for dauer formation (Jeong et al. 2005). Therefore, I hypothesized that *B. xylophilus* might have daumone-like signal substance ("DBX" in the following text) associated with dispersal form induction. In this chapter, I revealed the existence of dispersal form inducing pheromone in *B. xylophilus* (DBX) and investigated the chemical properties of it.

Section 3-2. Materilas and Methods.

Extraction of J_{III}-Inducing Pheromone.

I prepared crude DBX for artificial induction of J_{III}. In this experiment, I used the ST2 line, which had the highest J_{III} production rate in the J_{III} induction test in chapter 2. ST2 nematodes were reared for four days on *B. cinerea* grown on 10-mL barley grains in a 50-mL Erlenmeyer flask. Nematodes were collected using the Baermann funnel technique for three hours, rinsed with distilled water three times, and a sterilized water suspension of the nematodes (10 worms/μL) was prepared. The suspension was shaken using a Triple Shaker NR-80 (Taitec, Japan) at 95 rpm for 48 hours at 25°C (Figure 7), and the suspension was filtrated using filter paper (Kiriyama Filter paper No. 5A, 7 μm; Kiriyama Glass, Tokyo) twice to remove worms. The filtered solution which is

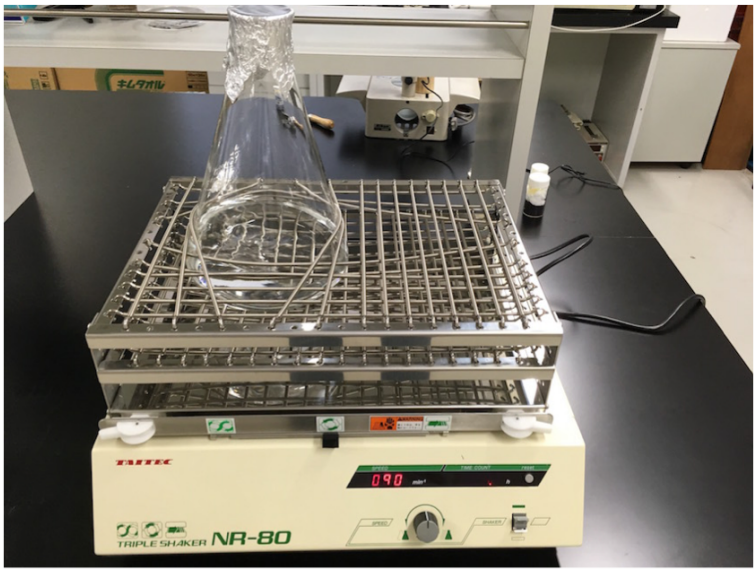

Figure 7. Nematode suspension was shaken in the water

supposed to contain DBX was concentrated by freeze-drying, and the dried materials were dissolved in sterilized water, adjusting to 2,000,000 nematodes' secretions per 3 mL. The solution was tentatively called CDBX for crude DBX.

Artificial Induction of J_{III}.

Filtrated CDBX (200 µL) using 0.20 µm pore-disposal filter unit (ADVANTEC) for sterilization was inoculated on the surface of 4% plain agar in 4-cm Petri dishes (J_{III} inducing media), and 20 µL of yeast (*Saccharomyces cerevisiae*; 0.25 mg/mL or 1 mg/mL) suspension was added as food for the nematodes. In the control group, the same volume of sterilized distilled water was added instead of CDBX, along with the same amount of yeast. The plates were kept for two days at 25°C. Thereafter, ten females and three males of ST2 were reared on each plate, and cultured for 4 days. Each treatment was repeated three times. After confirming the propagation (presence of offspring), I extracted the nematodes using the Baermann funnel technique overnight and counted the number of total and J_{III} nematodes to calculate the J_{III} rate.

In addition to the above experiment, I examined J_{III} formation under different conditions: with higher food concentrations (4 mg/mL suspension of *S. cerevisiae*), and

with a longer incubation (5 days) for all treatments. After four or five days of incubation, all juvenile nematodes were first generation progenies because the life cycle of *B. xylophilus* on yeast is longer than five days at 25°C (Mamiya 1972).

本部分の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定。

Chemical Analysis of the Pheromone

本項の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定。

Statistical analysis

The statistical analysis was performed using R ver. 3.2.4. I calculated the emergence rate of J_{III} using the following formula:

$$\text{Rate (\%)} = [(\text{number of } J_{III}) / (\text{total number of worms})] \times 100$$

Data represent the mean ± standard error (SE). For the statistical analysis, I performed the normality test to reveal the normality of the data to decide whether to use parametric or non-parametric tests. When analyzing artificial induction of J_{III} tests, one-way ANOVA and post hoc Tukey–Kramer multiple comparison tests were performed.

Section 3-3. Results.

Artificial induction of J_{III}

Results are summarized in Figure 9 and Table 4. Total number of nematodes was higher in the treatment (CDBX-added) group than in the corresponding control group (*P*

= 0.023). The rate of J_{III} was also higher in the treatment group than in the control group (P = 0.011). When observing the plates, the nematodes that remained were mostly J_2 (data not shown).

To confirm the reproducibility of this phenomenon, we added another treatment group, a well-fed group with 4 mg/mL food and five days of incubation. The results showed a similar tendency; i.e., the number of total nematodes, number of J_{III}, and the rate of J_{III} were higher in the treatment group than in the control group (Figure 10, Table 5). However, the rate of J_{III} was much higher than in the previous experiment (five-day incubation, food 1 mg/mL, mean ± SE = 85.21% ± 1.248; four-day incubation, food 1 mg/mL, mean ± SE = 28.75% ± 8.088) (Figures 9 and 10; Tables 4 and 5).

本部分の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定。

Chemical Analysis of the Pheromone

本項の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定。

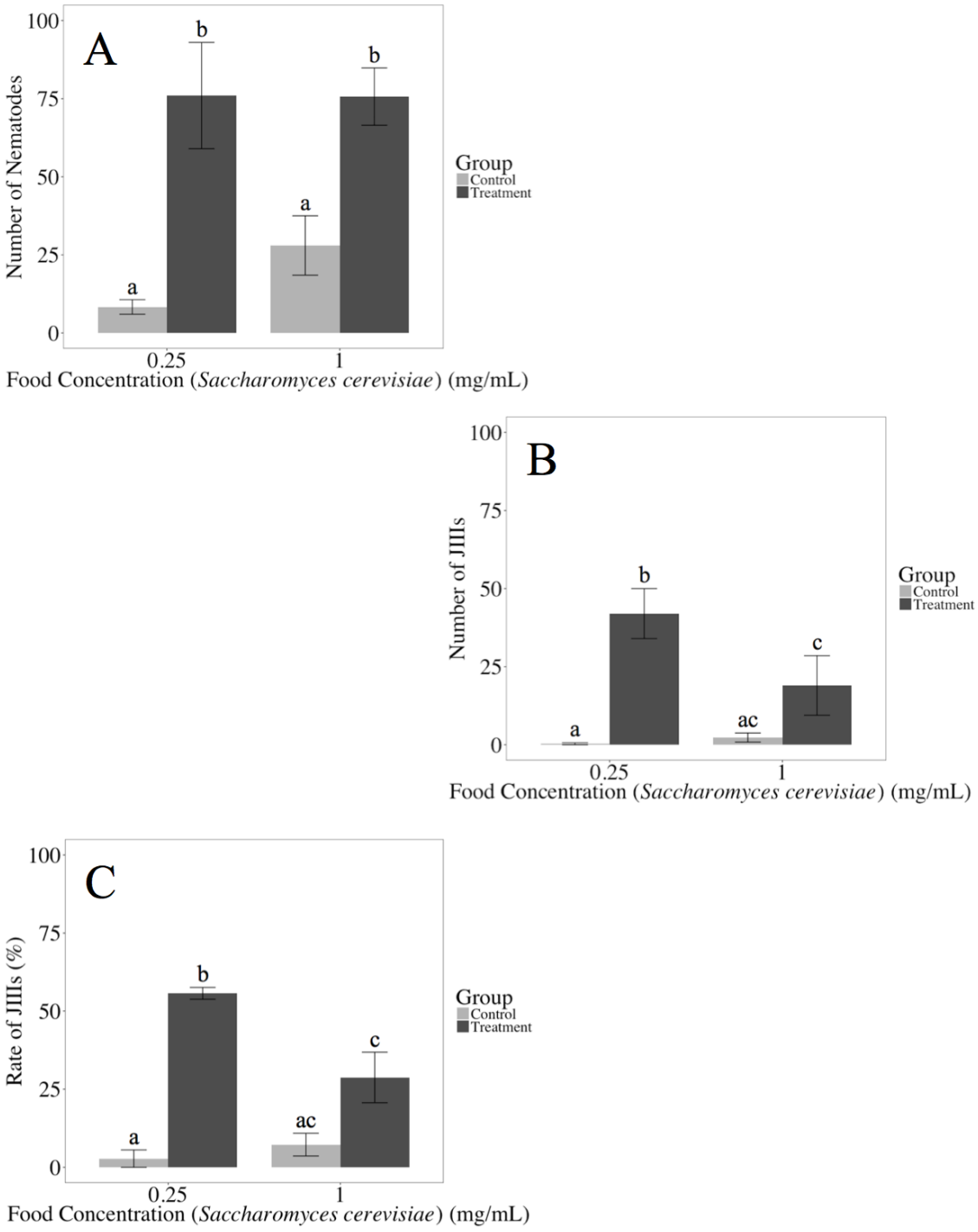

Figure 9. Propagation and J_{III} production of the ST2 line of *B. xylophilus* by adding CDBX and various concentrations of yeast (*S. cerevisiae*) after 4 days of incubation. A: total number of nematodes; B: number of J_{III}; C: J_{III} emergence rate. The same letters indicate non-significant differences between the samples. Bars and error bars represent averages and standard errors for three replicates, respectively.

Table 4. Response of nematodes by adding DBX and various food concentration after 4 days incubation.

	Food source (*Saccharomyces cerevisiae*) (mg/mL)	Number	J_{III} Number	J_{III} Rate (%)
Treatment	0.25	76.0±17.0	42.0±8.0	55.7±0.0
	1	75.7±9.2	19.0±9.5	2.8±0.0
Control	0.25	8.3±2.3	0.3±0.3	28.8±0.1
	1	28±9.5	2.3±1.5	7.2±0.0

"Number" shows the mean value and SE (standard error) of the total nematode number. "J_{III} Number" shows the mean value and SE of J_{III} nematodes. "J_{III} Rate" shows the mean value and SE of J_{III} rate derived from the total and J_{III} number. This experiment was repeated 3 times, and the mean value and SE was calculated from the results of repeats.

Section 3-4. Discussion

In spite of its importance of the mechanism of J_{III} induction in the disease cycle, an artificial J_{III} induction method had not been established. In this research, artificial J_{III} inducing assay method using nematode secreted materials (CDBX) was established. After the first-generation adults were exposed to CDBX, most offspring of the next generation (J_2) molted to J_{III}, suggesting the presence of water-soluble material(s) that induce J_{III} formation. In *C. elegans*, daumone was first identified as ascaroside (-)-6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy) heptanoic acid (Jeoung et al. 2005); i.e., a component of ascarylose and fatty acid side chain. Ascarosides are associated with various behaviors such as gender-specific attraction, repulsion, aggregation, olfactory plasticity, and dauer induction (Golden and Riddle 1984a, 1984b; Butcher et al. 2007, 2009; Srinivasan et al. 2008, 2012; Pungaliya et al. 2009; Macosko et al. 2009; Yamada et al. 2011), and are often used as signaling molecules in various nematode species including free-living and parasitic species (*C. elegans*, *C. afra*,

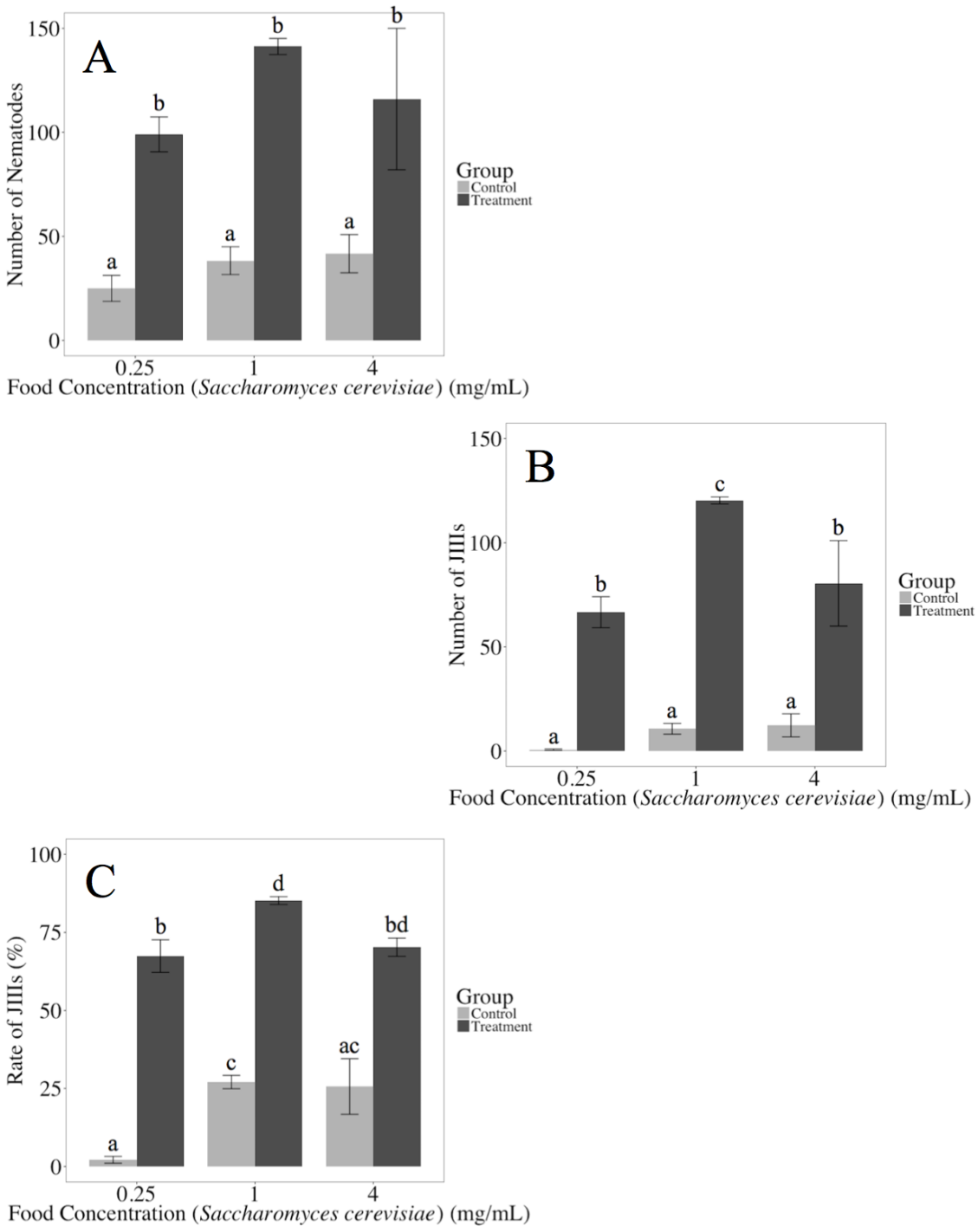

Figure 10. Propagation and J_{III} production of ST2 line of *B. xylophilus* by adding CDBX and various concentrations of yeast (*S. cerevisiae*) after five days of incubation. A: total number of nematodes; B: number of J_{III}s; C: J_{III} emergence rate. The same letters indicate non-significant differences between the samples. Bars and error bars represent averages and standard errors for three replicates, respectively.

Table 5. Reponse of nematodes by adding DBX and various food concentration, and added one higher food concentration and incubated much longer than that shown in Table 4, i.e. 5 days.

	Food source (*Saccharomyces cerevisiae*) (mg/mL)	Number	J_{III} Number	J_{III} Rate (%)
Treatment	0.25	99±8.4	66.7±7.4	67.4±0.1
	1	141.3±3.8	120.3±1.7	85.2±0.0
	4	116.0±34.0	80.5±20.5	70.3±0.0
Control	0.25	25.0±6.2	0.7±0.3	2.2±0.0
	1	38.3±6.7	10.7±2.6	27.1±0.0
	4	41.7±9.2	12.3±5.5	25.6±0.1

"Number" shows the mean value and SE (standard error) of the total nematode number. "J_{III} Number" shows the mean value and SE of J_{III} nematodes. "J_{III} Rate" shows the mean value and SE of J_{III} rate derived from the total and J_{III} number. This experiment was repeated 3 times, and the mean value and SE was calculated from the results of repeats.

Heterorhabditis bacteriophora, *Allodiplogaster seani*, *Nippostrongylus brasiliensis*, *Oscheius carolinensis*, *O. tipulae*, *Parastrongyloides trichosuri*, *Pelodera strongyloides*, *P. pacificus*, *Rhabditis* sp. AF5, *Steinernema carpocapsae*, *S. glaseri*, and *S. riobrave*) (Choe et al. 2012; Noguez et al. 2012; Stasiuk et al. 2012; Felix et al. 2014; Kanzaki et al. 2015). It was also reported that the dauer-inducing pheromones of *C. elegans* and *P. pacificus* did not induce dauer formation of *P. pacificus* and *C. elegans*, respectively, although the physical properties of those two pheromones were similar to one another (Ogawa et al. 2009). This suggests that dauer-inducing pheromones are species-specific or affect only closely related (narrow-ranged) species.

However in this study, some components in the fraction (f) induced J_{III}, although the fraction (a) which induced dauer juveniles in *C. elegans* (Jeong et al. 2005) did not show J_{III} inducing activity in *B. xylophilus*. This phenomenon means that DBX has different chemical properties from daumone in *C. elegans*. Yamaji et al. (2005) showed

that the fraction (f) contains organic acids, and therefore, DBX might be some substances elaborated by organic acids. Most organic acids are soluble in organic solvent such as ethyl acetate, and organic acids have many functions such as sex, alarm, recruiting, and aggregating pheromones in many organisms (Law and Regnier 1971; Happ 1973). J_{IV} of *B. xylophilus* is induced by the fatty acid ethyl ester of which is constructed by organic acid (Zhao et al. 2013), and dauer production of *B. seani* was supposed to be correlated with the presence of glycerides or fatty acids which are one of organic acids (Giblin and Kaya 1984). Organic acids are also used as a nematicide (McBride et al. 2000). However, components in fraction (a), which are soluble in organic solvent, did not show J_{III} inducing ability. This suggested that DBX contains some polar substituent and is completely different substance from well-known dauer-inducing pheromones, i.e. ascaroside, in *C. elegans* and *P. pacificus*.

Chapter 4.
Simple and Effective Method of RNA Extraction of *Bursaphelenchus xylophilus*.

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定。

Chapter 5.
Transcriptomic Analysis of Third Stage Dispersal Juvenile of *Bursaphelenchus xylophilus*.

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定。

Chapter 6.
General Discussion.

Section 6-1. Points of This Study.

Pine wood nematode is one of the most serious pathogens to forest ecosystem and industry in East Asia and Europe. In the life cycle of pine wood nematode *Bursaphenchus xylophilus*, J_{III} induction is the key event of the life strategy shift from killing a pine tree and multiplying in it to moving from dead tree to another living one. For controlling this disease, several efforts have been taken; for example killing the vector beetle by the spray of insecticide, injecting nematicide into healthy trees, and cutting and fumigating infested or dead trees. However, these methods are sometimes difficult because of economical and ecological reasons. A new control method is needed for more effective regulation of this disease. In order to resolve this problem, J_{III}-inducing mechanism has some potential, because J_{III} induction ability is directly associated with J_{IV} induction ability, and hence influence the ability of loading to *Monochamus* beetle. Therefore, investigating of J_{III}-inducing mechanism is useful for finding a new way to control pine wilt disease via preventing nematodes from loading to the beetle.

The present study aimed to reveal J_{III} inducing mechanism from the viewpoint of physiology and molecular biology. Chapter 2 showed J_{III} inducing ability of 4 field isolates and 8 inbred lines derived from T4, which showed the highest J_{III} emergence rate in the field isolates. Using the inbred line ST2, showing the highest J_{III} emergence rate, the existence of J_{III} inducing pheromone was revealed in chapter 3. In chapter 4, a new RNA extraction method without homogenizing nematodes was established. Using this method, comparative transcriptomic analyses associated with J_{III} induction were conducted, and key genes were successfully detected in chapter 5.

Section 6-2. Physiological Aspects of J_{III} Induction.

From the physiological investigation of J_{III} induction, it was revealed that J_{III} induction ability varied between isolates. Kiyohara and Bolla (1990) showed that the pathogenicity of *B. xylophilus* was different between populations, and they showed that T4 field isolate had high J_{III}-inducing ability, while the other 3 isolates (highly virulent S10, Ka4 and avirulent C14-5) did not. Thus, pathogenicity of the nematode isolates is not correlated to J_{III} inducting ability. Despite their low J_{III} induction ability, S10 and Ka4 field isolates had been isolated from serious damaged stands by pine wilt disease (Mori and Inoue 1986; Bolla and Tamura 1989; Kosaka et al. 2001). Therefore, they should have enough ability of dispersal stages induction, J_{III} and J_{IV}, so that they could move to a healthy pine tree via the host beetle. Once T4 encounter bad condition, they shift the stages to J_{III}s soon. On the other hand, nematodes of S10 and Ka4 isolate may remain increasing their population even in the early stage of bad condition. However, because the total population of S10 and Ka4 become much larger than T4, J_{III} number tends to be larger, at least for Ka4, although J_{III} emergence rate is low (Figure 4). Namely, S10 and Ka4 might get enough J_{III} number for epidemic because of its enormous population. As the experiments in chapter 2 was conducted under a specific temperature and culture condition, further studies are needed to evaluate the life cycle strategy of each nematode isolates under field conditions.

As J_{III} is a dormant stage to survive during winter, it accumulates lipids as energy source. Lipid droplets are one of the main characters of J_{III} identification (Kondo and Ishibashi 1978; Futai 2013). However, during the J_{III}-inducing analysis in chapter 2, J_{III} seemed to have lower amount of lipid droplets inside its body than in other reports (Figure 20). They were confirmed as J_{III} by the morphological and physiological observations, namely, body length (572 ~ 776 mm; J_3 is 320 ~ 475 mm), roundness of

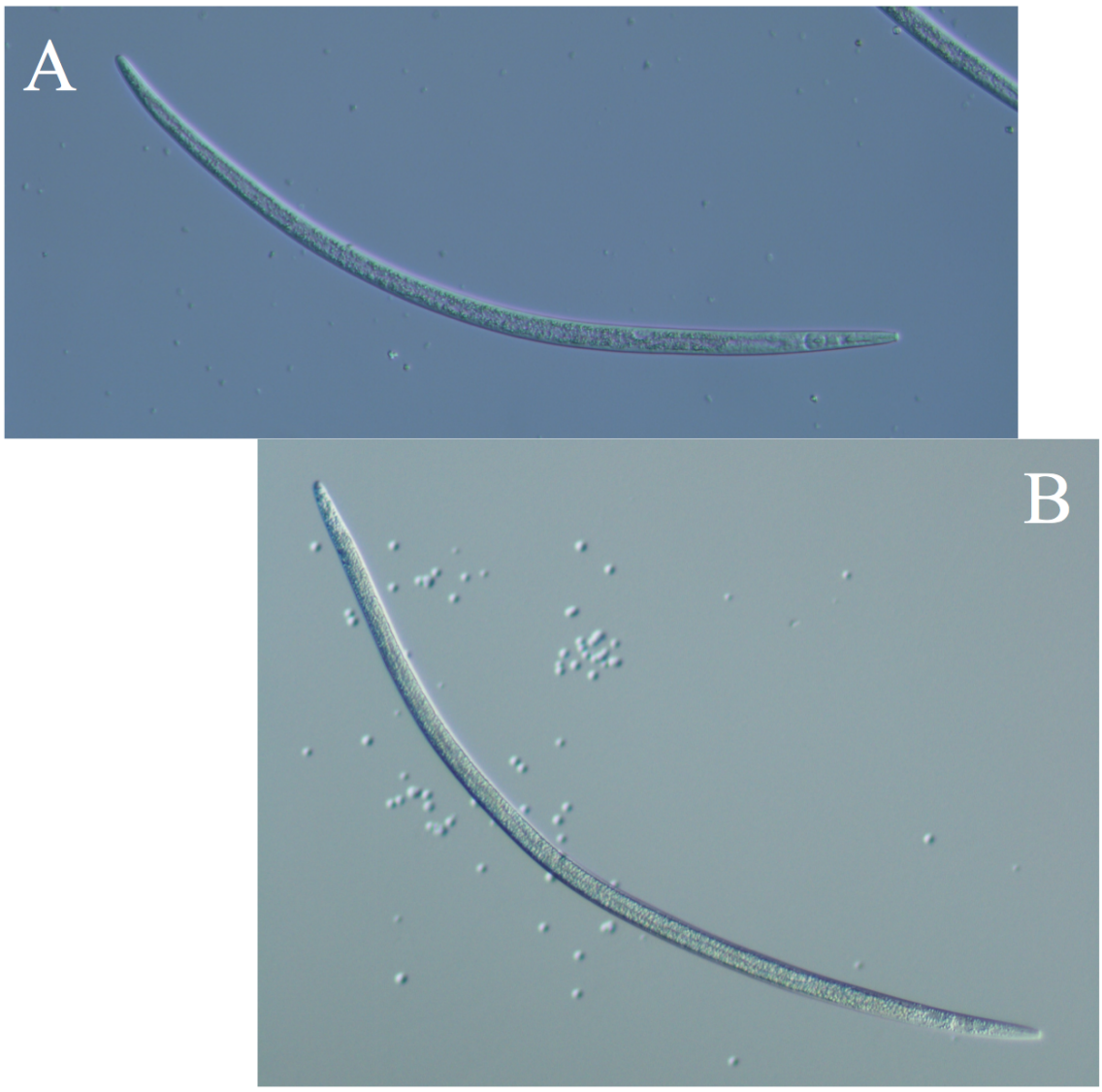

Figure 20. J_{III} of *B. xylophilus* induced by different method. A: J_{III} induced by the newly established J_{III}-inducing method in chapter 3. B: J_{III} induced by the previously established method by Nakazato et al. (2012).

the tail tip (tail of J_3 is sharper than J_{III}), and molting to J_4 in the favorable condition (Mamiya 1975). Then, why did not this J_{III} have amount of lipid droplets? After pine trees death by pine wilt disease, blue stain fungi, e.g. *Ophiostoma picea*, grow in the sapwood, and these blue-stain fungi are known to degrade host pine lipids as the nutrient (Abraham et al. 1998). Therefore, when nematodes eat the blue-stain fungi as the food source, large amount of lipids are taken into the nematode bodies. In addition,

grown on glycerol-added media, a number of nematodes were induced to J_{III} (Nakazato et al. 2012). From these previous studies in the field or in the experimental condition, it is suggested that J_{III} was induced in the lipid-abundant conditions, and J_{III} could store a lot of lipid droplets inside the body. However in this study, J_{III} was induced in the low amount of lipid condition, because there are only yeasts for the food source grown on the plain agar without any lipid. Therefore, nematodes couldn't store enough lipid droplets. In other words, the result of chapter 2 suggests that lipids are not necessary for J_{III} induction.

J_{III} is supposed to be induced in the condition of high DBX concentration. It was reported that J_{III} was induced after the nematode population was peaked (Ishibashi and Kondo 1977). It means that as the nematode population increase, DBX concentration also increases because DBX is the pheromone secreted by the nematodes themselves. Namely, DBX is the pheromone showing the nematode density (Chapter 3). In other nematodes, the population density pheromones were identified as ascaroside as mentioned in the chapter 3. However, this study suggested that another substance, not ascaroside, was the identity of DBX.

Some previous studies showed that *B. xylophilus* has some favorite food fungal species (Kobayashi et al. 1974 and 1975; Fukushige 1991; Sriwati et al. 2007), and also showed that J_{III} emergence rate is different between the food fungi (Maehara and Futai 2000). In that paper, number of J_{III} nematodes was tended to be larger on the fungi on which nematodes multiply faster. This fact agrees to our hypothesis that the density of DBX which reflects nematode population density is the key factor of J_{III} induction.

Section 6-3. Molecular Biological Aspects of J_{III} Induction.

本項の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定

Section 6-4. Ecological Aspects of J_{III} Induction.

本項の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定

Section 6-5. Conclusion.

本項の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定

Acknowledgements.

I would like to express full of my gratitude to Dr. Kenji Fukuda, Professor of The University of Tokyo, for his invaluable advice and critical reading of this manuscript. I wish to appreciate to Dr. Norihisa Matsushita, Associate Professor of The University of Tokyo, for his insightful advice to this thesis. I also have to give my appreciation to Dr. Katsumi Togashi, Professor, and Dr. Chunlan Lian, Associate Professor, of The University of Tokyo, for reviewing this manuscript.

I sincerely thank Dr. Natsumi Kanzaki, Research Fellow of Kansai Research Center of Forestry and Forest Products Research Institute (FFPRI) for his correct advice and support of whole this study and for critical reading of this manuscript. I also give my gratitude to Dr. Yuko Takeuchi-Kaneko, Assistant Professor of Kyoto University, for her great advice. I also have to thank her husband, Mr. Akira Kaneko for teaching me the first step of my research life. I also appreciate Dr. Kazuyoshi Futai, former Professor of Kyoto University, for his providing direction of the study. I would like to thank Dr. Ryoji Shinya, Assistant Professor of Meiji University, for giving me the opportunity of studying J_{III} of *B. xylophilus*. I have to appreciate Dr. Chihiro Tanaka, Professor of Kyoto University, and Dr. Kosuke Izumitsu, Assistant Professor of The University of Shiga Prefecture, for giving advices of my research. I also thank Dr. Etsuko Okumura, Postdoctoral Fellow of Saga University, for giving me some suggestions.

I will thank Dr. Takuya Aikawa, Research Fellow of Tohoku Research Center of FFPRI, for giving me the nematode and fungal materials and advices of J_{III} inducing assay (Chapter 2 and 3). I also give my gratitude to Dr. Toshihiro Yokoi, Research Fellow of FFPRI, for his suggestion of the newly established RNA extraction method in Chapter 4.

I have to give my gratitude to Dr. Taisei Kikuchi, Associate Professor, Mr. Yasunobu Maeda, PhD student, of Miyazaki University, and Dr. Mehmet Dayi,

Postdoctoral Fellow of Duzce University, for their supports of RNA-seq analysis in Chapter 5. I also thank Dr. Ryusei Tanaka, Assistant Professor of Miyazaki University, for giving me amount of advice in whole this study. I would like to thank Dr. Kazuko Tsuchihara, Associate Professor of Tohoku Gakuin University, and Dr. Masahiko Tokoro, Director of FFPRI for their support of my first step of chemical research. I also thank Dr. Yu Ichihara, Group Leader of Kansai Research Center of FFPRI for his support of my chemical analysis.

I will give my appreciation to Dr. Atsushi J. Nagano, Assistant Professor, Dr. Ayumi Tezuka and Dr. Makoto Kashima, Postdoctoral Fellows of Ryukoku University, for their suggestions of RNA extraction method in chapter 4.

I wish to thank all members of Laboratory of Forest Botany of The University of Tokyo, Laboratory of Forest Pathology of FFPRI, Kansai Research Center of FFPRI, Laboratory of Terrestrial Microbial Ecology of Kyoto University, and Laboratory of Parasitology of Miyazaki University.

Finally, the deep gratitude with all of my heart should be taken to my parents, Mr. Takeru Tanaka and Mrs. Kumiko Nishio-Tanaka, my brother, Dr. Yu Tanaka, who is Assistant Professor of Laboratory of Crop Science of Kyoto University, and my fiance, Ms. Michiko Nemoto, for their great support and encouragement.

Suguru Eifu Tanaka

References.

1. **Abelleira, A., Picoaga, A., Mansilla, J.P., and Aguin, O.** (2011) Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in Northwestern Spain. *Plant disease.* 95:6:776.
2. **Abraham, L., Hoffman, B., Gao, Y., and Breuil, C.** (1998) Action of *Ophiostoma piceae* proteinase and lipase on wood nutrients. *Canadian Journal of Microbiology.* 44:7:698-701.
3. **Adachi, H., Fujiwara, Y., and Ishii, N.** (1997) Effects of oxygen on protein carbonyl and aging in *Caenorhabditis elegans* mutants with long (*age-1*) and short (*mev-1*) life spans. *The journal of Gerontology Series A: Biological Sciences and Medical Sciences.* 53:4:B240-B244.
4. **Aguirre, J., Ríos-Momberg, M., Hewitt, D., and Hansberg W.** (2005) Reactive oxygen species and development in microbial eukaryotes. *Trends in Microbiology.* 13:3:111-118.
5. **Aikawa, T., and Kosaka, H.** (1998) Difference in multiplication between the virulent and avirulent isolates of *Bursaphelenchus xylophilus*. *Proceedings, Symposium: 24th International Nematology. Nematologica.* 44:451-452.
6. **Aikawa, T., and Togashi, K.** (1997) An effect of inoculum quantity of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) on the nematode load of *Monochamus alternatus* (Coleoptera: Cerambycidae) in laboratory. *Nematological Research.* 27:1:14-21.
7. **Aikawa, T., and Togashi, K.** (1998) An effect of pine volatiles on departure of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) from *Monochamus alternatus* (Coleoptera: Cerambycidae). *Applied Entomology and Zoology.* 33:2:231-237.
8. **Aikawa, T., and Togashi, K.** (2000) Movement of *Bursaphelenchus xylophilus*

(Nematoda: Aphelenchoididae) in tracheal system of adult *Monochamus alternatus* (Coleoptera: Cerambycidae). *Nematology*. 2:5:495-500.

9. **Albarqi, M.M.Y., Stoltzfus, J.D., Pilgrim, A.A., Nolan, T.J., Wang, Z., Kliewer, S.A., Mangelsdolf, D.J., and Lok, J.B.** (2016) Regulation of life cycle checkpoints and developmental activation of infective larvae in *Strongyloides stercoralis* by dafachronic acid. *PLoS Pathogens*. 12:1: e1005358.
10. **Alexa, A., Rahnenführer, J., and Lengauer, T.** (2006) Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. *Bioinformatics*. 22:13:1600-1607.
11. **Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., and Riddle, D.L.** (2000) *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. *Genes and Development*. 14:1512-1527.
12. **Apel, K., and Hirt, H.** (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Plant Biology*. 55:373-399.
13. **Apfeld, J., and Kenyon, C.** (1999) Regulation of lifespan by sensory perception in *Caenorhabditis elegans*. *Nature*. 402:6763:804-809.
14. **Arakawa, Y., and Togashi, K.** (2002) Newly discovered transmission pathway of *Bursaphelenchus xylophilus* from males of the beetle *Monochamus alternatus* to *Pinus densiflora* trees via oviposition wounds. *Journal of Nematology*. 34:4:396-404.
15. **Arantes-Oliveira, N., Apfeld, J., Dillin, A., and Kenyon, C.** (2002) Regulation of life-span by germ-line stem cells in *Caenorhabditis elegans*. *Science*. 295:5554:502-505.
16. **Avery, L.** (2014) A model of the effect of uncertainty on the *C. elegans* L2/L2d decision. *PLoS ONE*. 9:7:e100580.
17. **Ayyadevara, S., Alla, R., Thaden, J.J., and Shmookler Reis, R.J.** (2008) Remarkable longevity and stress resistance of nematode PI3K-null mutants. *Aging*

Cell. 7:1:13-22.

18. **Bai, X., Adams, B.J., Ciche, T.A., Clifton, S., Gaugler, R., Kim, K., Spieth, J., Sternberg, P.W., Wilson, R.K., and Grewal, P.S.** (2013) A lover and a fighter: the genome sequence of an entomopathogenic nematode *Heterorhabditis bacteriophora*. *PLoS ONE*. 8:7:e69618.
19. **Bandyopadhyay, U., Das, D., and Banerjee, R.K.** (1999) Reactive oxygen species: Oxidative damage and pathogenesis. *Current Science*. 77:5:658-666.
20. **Bargmann, C.I., and Horvitz, H.R.** (1991) Control of larval development by chemosensory neurons in *Caenorhabditis elegans*. *Science*. 251:4998:1243-1246.
21. **Baugh, L.R.** (2013) To grow or not to grow: Nutritional control of development during *Caenorhabditis elegans* L1 arrest. *Genetics*. 194:3:539-555.
22. **Bento, G., Ogawa, A., and Sommer, R.J.** (2010) Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. *Nature*. 466:494-498.
23. **Birnby, D.A., Link, E.M., Vowels, J.J., Tian, H., Colacurcio, P.L., and Thomas, J.H.** (2000) A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in *Caenorhabditis elegans*. *Genetics*. 155:85-104.
24. **Bolla, R.I., and Tamura, H.** (1989) Mating potential and chromosome number of some Japanese and U.S. populations of the pine wood nematode, *Bursaphelenchus xylophilus*. *Japanese Journals of Nematology*. 19:7-12.
25. **Bose, N., Meyer, J.M., Yim, J.J., Mayer, M.G., Markov, G.V., Ogawa, A., Schroeder, F.C., and Sommer, R.J.** (2014) Natural variation in dauer pheromone production and sensing supports intraspecific competition in nematodes. 24:13:1536-1541.
26. **Bottomly, D., Walter, N.A.R., Hunter, J.E., Darakjian, P., Kawane, S., Buck, K.J., Searles, R.P., Mooney, M., Mcweeney, S.K., and Hitzemann, R.** (2011)

Evaluating gene expression in C57BL/6J and DBA/2J mouse striatum using RNA-seq and microarrays. *PLoS ONE*. 6:3:e17820.

27. **Braasch, H.** (2001) *Bursaphelenchus* species in conifers in Europe: distribution and morphological relationships. *EPPO bulletin*. 31:2:127-142.
28. **Braasch, H., and Schönfeld, U.** (2015) Improved morphological key to the species of the *xylophilus* group of the genus *Bursaphelenchus* Fuchs, 1937. *EPPO bulletin*. 45:1:73-80.
29. **Brenner, S.** (1974) The genetics of *Caenorhabditis elegans*. *Genetics*. 77:71-94.
30. **Butcher, R.A., Fujita, M., Schroeder, F.C., and Clardy, J.** (2007) Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. *Nature Chemical Biology*. 3:7:420-422.
31. **Butcher, R.A., Ragains, J.R., and Clardy, J.** (2009) An indole-containing dauer pheromone component with unusual dauer inhibitory activity at higher concentrations. *Organic Letters*. 11:14:3100-3103.
32. **Cabiscol, E., Tamarit, J., and Ros, J.** (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology*. 3:3-8.
33. **Cassada, R.C., and Russel, R.L.** (1975) The dauerlarava, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Developmental Biology*. 46:2:326-342.
34. **Chen, Z., Ling, J., and Gallie, D.R.** (2004) RNase activity requires formation of disulfide bonds and is regulated by the redox state. *Plant Molecular Biology*. 55:83-96.
35. **Cheng, H.R., Lin, M.S., and Qian, R.J.** (1986) A study on morphological diagnosis and pathogenicity of the pine wood nematode. *Journal of Nanjing Agricultural University*. 2:55-59.
36. **Cheng, X.Y., Cheng, F.X., Xu, R.M., and Xie, B.Y.** (2008) Genetic variation in the invasive process of *Bursaphelenchus xylophilus* (Aphelenchida:

Aphelenchoididae) and its possible spread routes in China. *Heredity*. 100:4:356-365.

37. **Choe, A., von Reuss, S.H., Kogan, D., Gasser, R.B., Platzer, E.G., Schroeder, F.C., Sternberg, P.W.** (2012) Ascaroside signaling is widely conserved among nematodes. *Current Biology*. 22:9:772-780.
38. **Coburn, C.M., and Bargmann, C.I.** (1996) A putative cyclic nucleotide-gated channel is required for sensory development and function in *C. elegans*. *Neuron*. 17:4:695-706.
39. **Cui, H., Kong, Y., and Zhang, H.** (2012) Oxidative stress, mitochondrial dysfunction, and aging. *Journal of Signal Transduction*. 2012:e646354.
40. **de Castro E., de Castro, S.H., and Johnson, T.E.** (2004) Isolation of long-lived mutants in *Caenorhabditis elegans* using selection for resistance to juglone. *Free Radical Biology and Medecine*. 37:2:139-145.
41. **de Guiran, G., and Bruguier, N.** (1989) Hybridization and phylogeny of the pine wood nematode (*Bursaphelenchus* spp.). *Nematologica*. 35:321-330.
42. **Espada, M., Silva, A.C., Eves van den Akker, S., Cock, P.J.A., Mota, M., and Jones, J. T.** (2015) Identification and characterization of parasitism genes from the pinewood nematode *Bursaphelenchus xylophilus* reveals a multilayered detoxification strategy. *Molecular Plant Pathology*. 17:2:286-295.
43. **Estevez, M., Attlsano, L., Wrana, J.L., Albert, P.S., Massague, J., and Riddle, D.L.** (1993) The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. *Nature*. 365:6447:644-649.
44. **Félix, M.A., and Braendle, C.** (2010) The natural history of *Caenorhabditis elegans*. *Current Biology*. 20:22:R965-R969.
45. **Félix, M.A., Braendle, C., and Cutter, A.D.** (2014) A streamlined system for species diagnosis in *Caenorhabditis* (Nematoda: Rhabditidae) with name designations for 15 distinct biological species. *PLoS ONE*. 9:4:e94723.

46. **Finkel, T.** (2011) Signal transduction by reactive oxygen species. *The Journal of Cell Biology*. 194:1:7-15.

47. **Forge, T.A., and Sutherland, J.R.** (1996) Population dynamics of the pine wood nematode, *Bursaphelenchus xylophilus*, in excised branch segments of western North American conifers. *Fundamental and Applied Nematology*. 19:4:349-356.

48. **Fuchs, G.** (1915) Die Naturgeschichte der Nematoden und einiger anderer Parasiten. *Zoologische Jahrbücher*. 38:109-222.

49. **Fuchs, G.** (1937) Neue Parasitische und halbparasitische Nematoden bei Borkenkäfern und einige andere Nematoden. I. Teil. Die Parasiten der Waldgärtner, *Myelophilus piniperda* L. und *minor* Hartig und die Genera *Rhabditis* Dujardin 1845, und *Aphelenchus* Bastian, 1865. *Zoologische Jahrbücher*. 70:291-380.

50. **Fukuda, K.** (1997) Physiological process of the symptom development and resistance mechanism in pine wilt disease. *Journal of Forest Research*. 2:3:171-181.

51. **Fukushige, H.** (1991) Population of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) on fungi growing in pine-shoot segments. *Applied Entomology and Zoology*. 26:3:371-376.

52. **Futai, K.** (2013) Pine wood nematode, *Bursaphelenchus xylophilus*. *Annual Review of Phytopathology*. 51:61-83.

53. **Gerisch, B., and Weitzel, C.** (2001) A hormonal signaling pathway influencing *C. elegans* metabolism, reproductive development, and life span. *Developmental Cell*. 1:6:841-851.

54. **Giblin, R.M., and Kaya, H.K.** (1984) Host, temperature and media additive effects on the growth of *Bursaphelenchus seani*. *Revue de Nématologie*. 7:1:13-17.

55. **Gilabert, A., Curran, D.M., Harvey, S.C., and Wasmuth, J.D.** (2016) Expanding the view on the evolution of the nematode dauer signaling pathways: refinement through gene gain and pathway co-option. *BMC Genomics*. 17:476.

56. **Golden, J.W., and Riddle, D.L.** (1982) A pheromone influences larval development in the nematode *Caenorhabditis elegans*. *Science*. 218:578-580.

57. **Golden, J.W., and Riddle, D.L.** (1984a) The *Caenorhabditis elegans* dauer larva: Developmental effects of pheromone, food, and temperature. *Developmental Biology*. 102:2:368-378.

58. **Golden, J.W., and Riddle, D.L.** (1984b) A *Caenorhabditis elegans* dauer-inducing pheromone and an antagonistic component of the food supply. *Journal of Chemical Ecology*. 10:8:1265-1280.

59. **Gottlieb, S., and Ruvkun, G.** (1994) *daf-2*, *daf-16* and *daf-23*: Genetically interacting genes controlling dauer formation in *Caenorhabditis elegans*. *Genetics*. 137:107-120.

60. **Grün, D., Kirchner, M., Thierfelder, N., Stoeckius, M., Selbach, M., and Rajewsky, N.** (2014) Conservation of mRNA and protein expression during development of *C. elegans*. 6:3:565-577.

61. **Hammond-Kosak, K.E., and Jones, J.D.G.** (1996) Resistance gene-development plant defence responses. *The Plant Cell*. 8.1773-1791.

62. **Han, H., Chung, Y.J., and Shin, S.C.** (2008) First report of pine wilt disease on *Pinus koraiensis* in Korea. *Plant Disease*. 92:8:1251.

63. **Happ, G.M.** (1973) Chemical signals between animals: Allomones and pheromones. In: *Humoral control of growth and differentiation 2*. 149-190.

64. **Herman, D.** (1956) Aging: A theory based on free radical and radiation chemistry. *Journal of Gerontology*. 11:3:298-300.

65. **Herrmann, M., Mayer, W.E., and Sommer, R.J.** (2006) Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. *Zoology*. 109:2:96-108.

66. **Herrmann, M., and Sommer, R.J.** (2011) The Genome of *Pristionchus pacificus* and implications for survival attributes. In: *Molecular and Physiological Basis of*

Nematode Survival. 86-98.

67. **Hooper, D.J.** (1986) Handling, fixing, staining and mounting nematodes. In: *Laboratory methods for work with plant and soil nematodes*. 59-80.
68. **Houthoofd, K., Johnson, T.E., and Vanfleteren, J.R.** (2005) Dietary restriction in the nematode *Caenorhabditis elegans*. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences.* 60:9:1125-1131.
69. **Hsin, H., and Kenyon, C.** (1999) Signals from the reproductive system regulate the lifespan of *C. elegans*. *Nature*. 399:362-366.
70. **Hu, P.J.** (2007) Dauer. In: *WormBook*. 1-19.
71. **Hussey, R., Stieglitz, J., Mesgarzadeh, J., Locke, T.T., Zhang, Y.K., Schroeder, F.C., and Srinivasan, S.** (2017) Pheromone-sensing neurons regulate peripheral lipid metabolism in *Caenorhabditis elegans*. *PLoS Genetics*. 13:5:e1006806.
72. **Ichihara, Y., Fukuda, K., and Suzuki, K.** (2000) Early symptom development and histological changes associated with migration of *Bursaphelenchus xylophilus* in seedling tissues of *Pinus thunbergii*. *Plant Disease*. 84:6:675-680.
73. **Ikeda, T., and Suzaki, T.** (1984) Influence of pine-wood nematode on hydraulic conductivity and water status in *Pinus thunbergii*. *Journal of Japanese Forest Society*. 66:10:412-420.
74. **Inoue, M.** (1949) Damage of pine sawyer beetle and its control. *Bulletin of the Hokkaido Prefectural Forest Experimental Station*. 62:77. In Japanese.
75. **Ishibashi, N., and Kondo, E.** (1974) The developmental stages of nematodes for survival, with some discussions on the term “infective” and “dauer”. *Japanese Journal of Nematology*. 4:1-10. In Japanese.
76. **Ishibashi, N., and Kondo, E.** (1977) Occurrence and survival of the dispersal forms of pine wood nematode, *Bursaphelenchus lignicolus* Mamiya and Kiyohara. *Applied Entomology and Zoology*. 12:4:293-302.
77. **Iwahori, H., and Futai, K.** (1985) A simple method for disinfection of pine wood

nematode eggs using their adhesiveness. *Japanese Journal of Nematology*. 15:64. In Japanese.

78. **Iwahori, H., and Futai, K.** (1993) Lipid peroxidation tissues and ion exudation of pine callus tissues inoculated with pinewood nematodes. *Japanese Journal of Nematology*. 23:2:79-89.
79. **Iwasaki, A., and Morimoto, K.** (1971) Host conditions suitable for oviposition by pine beetles. *Transaction of the 25th Annual Meeting of Kyushu Branch of the Japanese Forestry Society*. 168-169. In Japanese.
80. **Jeong, P.Y., Jung, M., Yim, Y.H., Kim, H., Park, M., Hong, E., Lee, W., Kim, Y.H., Kim, K., and Paik, Y.K.** (2005) Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. *Nature*. 433:7025:541-545.
81. **Jia, K., Albert, P.S., and Riddle, D.L.** (2002) DAF-9, a cytochrome P450 regulating *C. elegans* larval development and adult longevity. *Development*. 129:221-231.
82. **Jones, J.T., Moens, M., Mota, M., Li, H., and Kikuchi, T.** (2008) *Bursaphelenchus xylophilus*: opportunities in comparative genomics and molecular host-parasite interactions. *Molecular Plant Pathology*. 9:3:357-368.
83. **Jones, J.T., Haegeman, A., Danchin, E.G.J., Gaur, H.S., Helder, J., Jones, M.G.K., Kikuchi, T., Manzanilla-López, R., Palomares-Rius, J.E., Wesemael, W.M.L., and Perry, R.N.** (2013) Top 10 plant-parasitic nematodes in molecular plant pathology. 14:9:946-961.
84. **Kanzaki, N., Tsuda, K., and Futai, K.** (2000) Description of *Bursaphelenchus conicaudatus* n. sp. (Nematoda: Aphelenchoididae), isolated from the yellow-spotted longicorn beetle, *Psacothea hilaris* (Coleoptera: Cerambycidae) and fig trees, *Ficus carica*. *Nematology*. 2:2:165-168.
85. **Kanzaki, N., and Futai, K.** (2003) Description and phylogeny of

Bursaphelenchus luxuriosae n. sp. (Nematoda: Aphelenchoididae) isolated from *Acalolepta luxuriosa* (Coleoptera: Cerambycidae). *Nematology*. 5:4:565-572.

86. **Kanzaki, N.** (2006) Description of *Aphelenchoides xylocopae* n. sp. (Nematoda: Aphelenchoididae), the first observed association between nematodes and carpenter bees. *Nematology*. 8:4:555-562.
87. **Kanzaki, N.** (2008) Taxonomy and Systematics of the nematode genus *Bursaphelenchus* (Nematoda: Parasitaphelenchidae). In: *Pine Wilt Disease*. 44-66.
88. **Kanzaki, N., Ragsdale, E.J., Herrmann, M., Mayer, W.E., and Sommer, R.J.** (2012) Description of three *Pristionchus* species (Nematoda: Diplogastridae) from Japan that form a cryptic species complex with the model organism *P. pacificus*. *Zoological Science*. 29:6:403-417.
89. **Kanzaki, N., Tanaka, R., Giblin-Davis, R.M., and Davies, K.A.** (2014) New plant-parasitic nematode from the mostly mycophagous genus *Bursaphelenchus* discovered inside figs in Japan. *PLoS ONE*. 9:6:e99241.
90. **Kanzaki, N., Giblin-Davis, R.M., and Ragsdale, E.J.** (2015) *Allodiplogaster josephi* n. sp. and *A. seani* n. sp. (Nematoda: Diplogastridae), associates of soil-dwelling bees in the eastern USA. *Nematology*. 17:7:831-863.
91. **Kanzaki, N., and Giblin-Davis, R.M.** (2016) Pine wilt and red ring, lethal plant diseases caused by insect-mediated *Bursaphelenchus* nematodes. In: *Vector-Mediated Transmission of Plant Pathogens*. 87-107.
92. **Kanzaki, N., Maehara, N., Akiba, M., Tanaka, S.E., and Ide, T.** (2016) Morphological characters of dauer juveniles of three species of *Bursaphelenchus* Fuchs, 1937. *Nematology*. 18:2:209-220.
93. **Kaplan, F., Srinivasan, J., Mahanti, P., Ajredini, R., Durak, O., Nimalendran, R., Sternberg, P.W., Teal, P.E.A., Schroeder, F.C., Edison, A.S., and Alborn, H.T.** (2011) Ascaroside expression in *Caenorhabditis elegans* is strongly dependent on diet and developmental stage. *PLoS ONE*. 6:3:e17804.

94. **Kato, T., Kaneko, A., Honjo, M., Yasugi, M., Nagano, A.J., Kudo, H., Mori, K., Hisahara, T., Takeuchi, Y.** (2014) Genomic approaches for understanding important traits in recombinant inbred lines of pine wood nematode by RAD-seq. *Transaction of the 125th Annual Meeting of the Japanese Forest Society*. 160. In Japanese.

95. **Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtlang, R.** (1993) A *C. elegans* mutant that lives twice as long as wild type. *Nature*. 366:6454:461-464.

96. **Kikuchi, T., Jones, J.T., Aikawa, T., Kosaka, H., and Ogura, N.** (2004) A family of glycosyl hydrolase family 45 cellulase from the pine wood nematode, *Bursaphelenchus xylophilus*. *FEBS letters*. 572:201-205.

97. **Kikuchi, T., Shibuya, H., and Jones, J.T.** (2005) Molecular and biochemical characterization of an endo-β-1,3-glucanase from the pinewood nematode *Bursaphelenchus xylophilus* acquired by horizontal gene transfer from bacteria. *Biochemical Journal*. 389:117-125.

98. **Kikuchi, T., Shibuya, H., Aikawa, T., and Jones, J.T.** (2006) Cloning and characterization of pectase lyases expressed in the esophageal gland of the pine wood nematode *Bursaphelenchus xylophilus*. *Molecular Plant-Microbe Interactions*. 19:3:280-287.

99. **Kikuchi, T., Aikawa, T., Kosaka, H., Pritchard, L., Ogura, N., and Jones, J.T.** (2007) Expressed sequence tag (EST) analysis of the pine wood nematode *Bursaphelenchus xylophilus* and *B. mucronatus*. *Molecular and Biochemical Parasitology*. 155:9-17.

100. **Kikuchi, T., Aikawa, T., Oeda, Y., Karim, N., and Kanzaki, N.** (2009) A rapid and precise diagnostic method for detecting the pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. *Phytopathology*. 99:12:1365-1369.

101. **Kikuchi, T., Cotton, J.A., Dalzell, J.J., Hasegawa, K., Kanzaki, N., McVeigh,**

P., Takanashi, T., Tsai, I.J., Assefa, S.A., Cock, P.J.A., Otto, T.D., Hunt, M., Reid, A.J., Sanchez-Flores, A., Tsuchihara, K., Yokoi, T., Larsson, M.C., Miwa, J., Maule, A.G., Sahashi, N., Jones, J.T., and Berriman, M. (2011) Genomic insights into the origin of parasitism in the emerging plant pathogen *Bursaphelenchus xylophilus*. *PLoS Pathogens*. 7:9:e1002219.

102. **Kikuchi, T., Eves-van den Akker, S., and Jones, J.T.** (2017) Genome evolution of plant-parasitic nematodes. *Annual Review of Phytopathology*. 55:333-354.
103. **Kim, K., Sato, K., Shibuya, M., Zeiger, D.M., Butcher, R.A., Ragains, J.R., Clardy, J., Touhara, K., and Sengupta, P.** (2009) Two chemoreceptors mediate developmental effects of dauer pheromone in *C. elegans*. *Science*. 326:994-998.
104. **Kimura, K.D., Tissenbaum, H.A., Liu, Y., and Ruvkun, G.** (1997) *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science*. 277:942-946.
105. **Kimura, K., Aikawa, T., Yamamoto, K., Maehara, N., Ichihara, Y., Kon, J., and Nakamura, K.** (2011) Detection of *Bursaphelenchus xylophilus* and searching for marks of feeding and oviposition by vector insects in a pine wilt-damaged tree occurred at Yomogita, Aomori Prefecture. *Tohoku Journal of Forest Science*. 16:1:7-11. In Japanese.
106. **Kishi, Y.** (1988) Pine wilt disease. *Thomas Company*. In Japanese.
107. **Kiyohara, T., and Tokushige, Y.** (1971) Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. *Journal of Japanese Forest Society*. 53:7:210-218. In Japanese.
108. **Kiyohara, T., and Suzuki, K.** (1975) Seasonal change in population of *Bursaphelenchus lignicolus* in the wood of Japanese black pine. *Transactions of the 85th Annual Meeting of the Japanese Forestry Society*. 296-298. In Japanese.
109. **Kiyohara, T.** (1976) The decrease of pathogenicity of pine wood nematode, *Bursaphelenchus lignicolus*, induced by the extended subculturing on the fungal

mat of *Botrytis cinerea*. *Japanese Journal of Nematology*. 6:2:56-59. In Japanese.

110. **Kiyohara, T., and Bolla, R.I.** (1990) Pathogenic variability among populations of the pinewood nematode, *Bursaphelenchus xylophilus*. *Forest Science*. 36:4:1061-1076.
111. **Klass, M., Hirsh, D.** (1976) Non-aging developmental variant of *Caenorhabditis elegans*. *Nature*. 260:523-525.
112. **Klement, Z., and Goodman, R.N.** (1967) The hypersensitive reaction to infection by bacterial plant pathogens. *Annual Review of Phytopathology*. 5:1:17-44.
113. **Kobayashi, T., Sasaki, K., and Mamiya, Y.** (1974) Fungi associated with *Bursaphelenchus lignicolus*, the pine wood nematode (I). *Journal of Japanese Forest Society*. 56:4:136-145. In Japanese.
114. **Kobayashi, T., Sasaki, K., and Mamiya, Y.** (1975) Fungi associated with *Bursaphelenchus lignicolus*, the pine wood nematode (II). *Journal of Japanese Forest Society*. 57:6:184-193. In Japanese.
115. **Kojima, K., Kamijo, A., Masumori, M., and Sasaki, S.** (1994) Cellulase activities of pine-wood nematode isolates with different virulences. *Journal of Japanese Forest Society*. 76:3:258-262.
116. **Komatsu, M., Son, J., Matsushita, N., and Hogetsu, T.** (2008) Fluorescein-labeled wheat germ agglutinin stains the pine wood nematode, *Bursaphelenchus xylophilus*. *Journal of Forest Research*. 13:2:132-136.
117. **Kondo, E., and Ishibashi, N.** (1978) Ultrastructural differences between the propagative and dispersal forms in pine wood nematode, *Bursaphelenchus lignicolus*, with reference to the survival. *Applied Entomology and Zoology*. 13:1:1-11.
118. **Kondo, E. Foudin, A., Linit, M., Smith, M., Bolla, R., Winter, R., and Dropkin, V.** (1982) Pine wilt disease - nematological, entomological, and biochemical investigations. *Agricultural Experiment Station University of Missouri-Columbia*.

119. **Kosaka, H., Aikawa, T., Ogura, N., Tabata, K., and Kiyohara, T.** (2001) Pine wilt disease caused by the pine wood nematode: the induced resistance of pine trees by the avirulent isolates of nematode. *European Journal of Plant Pathology*. 107:7:667-675.

120. **Kuroda, K.** (1987) Enzymes produced by pine wood nematode and its chemical characters. *Transactions of the 98th Annual Meeting of Japanese Forest Society*. 128.

121. **Kuroda, K., Yamada, T., Mineo, K., and Tamura, H.** (1988) Effects of cavitation on the development of pine wilt disease caused by *Bursaphelenchus xylophilus*. *Annals of the Phytopathological Society of Japan*. 54:606-615.

122. **Law, J.H., Regnier, F.E.** (1971) Pheromones. *Annual Review of Biochemistry*. 40:533-548.

123. **Lee, J., Koo, N., and Min, D.B.** (2004) Reactive oxygen species, aging, and antioxidative nutracueticals. *Comprehensive Reviews in Food Science and Food Safety*. 3:21-33.

124. **Li, L., Stoeckert Jr., C.J., and Roos, D.S.** (2003) OrthoMCL: Identification of ortholog groups for eukaryotic genomes. *Genome Research*. 13:9:2178-2189.

125. **Li, Z., Liu, X., Chu, Y., Wang, Y., Zhang, Q., and Zhou, X.** (2011) Cloning and characterization of a 2-cys peroxiredoxin in the pine wood nematode, *Bursaphelenchus xylophilus*, a putative genetic factor facilitating the infestation. *International Journal of Biological Sciences*. 7:6:823-836.

126. **Li, J.J., Huang, H., Bickel, P.J., and Brenner, S.E.** (2014) Comparison of *D. melanogaster* and *C. elegans* developmental stages, tissues, and cells by modENCODE RNA-seq data. *Genome Research*. 24:1086-1101.

127. **Lin, K., Dorman, J.B., Rodan, A., and Kenyon, C.** (1997) *daf-16*: An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science*. 278:5841:1319-1322.

128. **Linit, M.J., Kondo, E., and Smith, M.T.** (1983) Insects associated with the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), in Missouri. *Environmental Entomology*. 12:2:467-470.

129. **Linit, M.J.** (1988) Nematode-vector relationships in the pine wilt disease system. *Journal of Nematology*. 20:2:227-235.

130. **Linit, M.J.** (1990) Transmission of pinewood nematode through feeding wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). *Journal of Nematology*. 22:2:231-236.

131. **Ludewig, A.H., and Schroeder, F.C.** (2013) Ascaroside signaling in *C. elegans*. *WormBook*. 1-22.

132. **Ly, K., Reid, S.J., and Snell. R.G.** (2015) Rapid RNA analysis of individual *Caenorhabditis elegans*. *MethodsX*. 2:59-63.

133. **Macosko, E.Z., Pokala, N., Feinberg, E.H., Chalasani, S.H., Butcher, R.A., Clardy, J., and Bargmann, C.I.** (2009) A hub-and-spoke circuit drives pheromone attraction and social behavior in *C. elegans*. *Nature*. 458:7242:1171-1175.

134. **Maehara, N., and Futai, K.** (1996) Factors affecting both the numbers of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), carried by the Japanese pine sawyer, *Monochamus alternatus* (Coleoptera: Cerambycidae), and the nematode's life history. *Applied Entomology and Zoology*. 31:3:443-452.

135. **Maehara, N., and Futai, K.** (2000) Population changes of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), on fungi growing in pine-branch segments. *Applied Entomology and Zoology*. 35:3:413-417.

136. **Maehara, N., and Futai, K.** (2001) Presence of the cerambycid beetles *Psacothea hilaris* and *Monochamus alternatus* affecting the life cycle strategy of *Bursaphelenchus xylophilus*. *Nematology*. 3:5:455-461.

137. **Maehara, N., and Futai, K.** (2006) Effect of fungus inoculation on the number of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) carried by *Monochamus alternatus* (Coleoptera: Cerambycidae). *Nematology*. 8:1:59-67.

138. **Maehara, N., and Kanzaki, N.** (2016) Transfer of *Bursaphelenchus okinawaensis* (Nematoda: Aphelenchoididae) associated with *Monochamus maruokai* (Coleoptera: Cerambycidae) into *M. alternatus* (Coleoptera: Cerambycidae) and *Psacothea hilaris* (Coleoptera: Cerambycidae). *Nematology*. 18:6:679-685.

139. **Malagón, D., Benítez, R., Kašny, M., and Adroher, F.J.** (2013) Peptidases in parasitic nematodes. In: *Parasites*. 61-102.

140. **Mamiya, Y.** (1972) Pine wood nematode, *Bursaphelenchus lignicolus* Mamiya and Kiyohara, as a causal agent of pine wilting disease. *Review of Plant Protection Research*. 5:46-60.

141. **Mamiya, Y., and Enda, N.** (1972) Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). *Nematologica*. 18:2:159-162.

142. **Mamiya, Y., and Kiyohara, T.** (1972) Description of *Bursaphelenchus lignicolus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and histopathology of nematode-infested trees. *Nematologica*. 18:1:120-124.

143. **Mamiya, Y.** (1975) The life history of the pine wood nematode, *Bursaphelenchus lignicolus*. *Japanese Journal of Nematology*. 5:16-25. In Japanese.

144. **Mamiya, Y., and Tamura, H.** (1977) Transpiration reduction of pine seedlings inoculated with the pine wood nematode, *Bursaphelenchus lignicolus*. *Journal of Japanese Forest Society*. 59:2:59-63.

145. **Mamiya, Y., Futai, K., and Kosaka, H.** (2004) Culture method of pine wood nematode. In *Experimental Procedure of Nematology*. 141-143.

146. **Mayer, M.G., and Sommer, R.J.** (2011) Natural variation in *Pristionchus pacificus* dauer formation reveals cross-preference rather than self-preference of

nematode dauer pheromones. *Proceedings of the Royal Society: Biological Sciences*. 278:1719:2784-2790.

147. **McBride, R.G., Mikkelsen, R.L., and Barker, K.R.** (2000) The role of low molecular weight organic acids from decomposing rye in inhabiting root-knot nematode populations in soil. *Applied Soil Ecology*. 15:3:243-251.
148. **McGawley, E.C., Winchell, K.L., Jones, J.P., Birchfield, W., and Berggren, G.T.** (1985) Population development and influence of *Bursaphelenchus xylophilus* on *Gliocladium virens*. 17:1:69-76.
149. **Møller, I.M.** (2001) Plant mitochondria and oxidative stress: Electron Transport, NADPH Turnover, and metabolism of reactive oxygen species. *Annual Review of Plant Physiology and Plant Molecular Biology*. 52:561-591.
150. **Mori, T., and Inoue, T.** (1986) Pine-wood nematode-induced ethylene production in pine stems and cellulase as an inducer. *Journal of Japanese Forest Society*. 68:43-50.
151. **Morimoto, K., and Iwasaki, A.** (1972) Role of *Monochamus alternatus* (Coleoptera: Cerambycidae) as a vector of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae). 54:6:177-183.
152. **Morimoto, K., and Iwasaki, A.** (1973) Studies on *Monochamus alternatus* (IV). The pine wood nematode around the pupal chamber. *Transactions of the 26th Annual Meeting of Kyushu Branch of Japanese Forest Society*. 199-200.
153. **Mota, M.M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K., and Sousa, E.** (1999) First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. 1:727-734.
154. **Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A., and Mangelsdorf, D.J.** (2006) Identification of ligands for DAF-12 that govern dauer formation and reproduction in *C. elegans*. 124:6:1209-1223.

155. **Nakazato, G., Hayashi, K., and Ogura, N.** (2012) *In vitro* occurrence of dispersal third-stage juveniles in *Bursaphelenchus xylophilus*. *Nematological Research*. 42:1:9-14. In Japanese.

156. **Necibi, S., and Linit, M.J.** (1998) Effect of *Monochamus carolinensis* on *Bursaphelenchus xylophilus* dispersal stage formation. *Journal of Nematology*. 30:2:246-254.

157. **Nickle, W.R.** (1970) A taxonomic review of the Genera of the Aphelenchoidea (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). *Journal of Nematology*. 2:4:375-392.

158. **Nickle, W.R., Golden, A.M., Mamiya, Y., and Wergin, W.P.** (1981) On the taxonomy and morphology of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934) Nickle 1970. 13:3:385-392.

159. **Nitto, M., Oda, K., and Kato, Y.** (1959a) Study on the borers habiting in the pine trees: developmental process of most borers. *Transactions of the 69th Annual Meeting of the Japanese Forest Society*. 407-408.

160. **Nitto, M., Oda, K., and Kato, Y.** (1959b) Study on the borers habiting in the pine trees: Mortality types of trees and the process of the disease. *Transactions of the 69th Annual Meeting of the Japanese Forest Society*. 408-409.

161. **Noguez, J.H., Conner, E.S., Zhou, Y., Ciche, T.A., Ragains, J.R., and Butcher, R.A.** (2012) A novel ascaroside controls the parasitic life cycle of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *ACS Chemical Biology*. 7:6:961-966.

162. **Oda, K.** (1964) Pine sawyer beetle and its damage against the pine tree. *Forest Pests*. 13:100-102.

163. **Odani, K., Sasaki, S., Yamamoto, N., Nishiyama, Y., and Tamura, H.** (1985) Differences in dispersal and multiplication of two associated nematodes, *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* in pine seedlings in

relation to the pine wilt disease development. *Journal of Japanese Forest Society*. 67:10:398-403.

164. **Ogawa, A., Streit, A., Antebi, A., and Sommer, R.J.** (2009) A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. *Current Biology*. 19:1:67-71.
165. **Ogawa, A., Bento, G., Bartelmes, G., Dieterich, C., and Sommer, R.J.** (2011) *Pristionchus pacificus daf-16* is essential for dauer formation but dispensable for mouth form dimorphism. *Development*. 138:7:1281-1284.
166. **Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., and Ruvkun, G.** (1997) The fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature*. 389:6654:994-999.
167. **Ogura, N., and Nakashima, T.** (2002) *In vitro* occurrence of dispersal fourth stage juveniles in *Bursaphelenchus xylophilus* co-incubated with *Monochamus alternatus*. 32:2:53-59.
168. **Palomares-Rius, J.E., Tsai, I.J., Karim, N., Akiba, M., Kato, T., Maruyama, H., Takeuchi, Y., and Kikuchi, T.** (2015) Genome-wide variation in the pinewood nematode *Bursaphelenchus xylophilus* and its relationship with pathogenic traits. *BMC Genomics*. 16:845.
169. **Park, J.E., Lee, K.Y., Lee, S.J., Oh, W.S., Jeong, P.Y., Woo, T., Kim, C.B., Paik, Y.K., Koo, H.S.** (2008) The efficiency of RNA interference in *Bursaphelenchus xylophilus*. *Molecules and Cells*. 26:81-86.
170. **Peckol, E.L., Troemel, E.R., and Bargmann, C.I.** (2001) Sensory experience and sensory activity regulate chemosensory receptor gene expression in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*. 98:20:11032-11038.
171. **Pungaliya, C., Srinivasan, J., Fox, B.W., Malik, R.U., Ludewig, A.H.,**

Sternberg, P.W., Schroeder, F.C. (2009) A shortcut to identifying small molecule signals that regulate behavior and development in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*. 106:19:7708-7713.

172. **Reddigari, S.R., Jansma, P.L., Premachandran, D., and Hussey, R.S.** (1986) Cuticular collagenous proteins of second-stage juveniles and adult females of *Meloidogyne incognita*: Isolation and partial characterization. *Journal of Nematology*. 18:3:294-302.
173. **Riddle, D.L., Swanson, M.M., and Albert, P.S.** (1981) Interacting genes in nematode dauer larva formation. *Nature*. 290:668-671.
174. **Ryss, A., Vieira, P., Mota, M., and Kulinich, O.** (2005) A synopsis of the genus *Bursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species. *Nematology*. 7:3:393-458.
175. **Sata, K.** (1942) Discussion of borers seriously damaging pine trees In Hyogo Prefecture, and extermination and prevention of the borers. *Bulletin of the Hyogo Prefectural Forest Experimental Station*. 53. In Japanese.
176. **Sato, H., Sakuyama, T., and Kobayashi, M.** (1987) Transmission of *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle (Nematoda, Aphelenchoididae) by *Monochamus saltuarius* (Gebler) (Coleoptera, Cerambycidae). *Journal of Japanese Forest Society*. 69:12:492-496.
177. **Schackwitz, W.S., Inoue, T., and Thomas, J.H.** (1996) Chemosensory neurons function in parallel to mediate a pheromone response in *C. elegans*. *Neuron*. 17:4:719-728.
178. **Shinya, R., Takeuchi, Y., Miura, N., Kuroda, K., Ueda, M., and Futai, K.** (2009a) Surface coat proteins of the pine wood nematode, *Bursaphelenchus xylophilus*: profiles of stage- and isolate-specific characters. *Nematology*. 11:3:429-438.

179. **Shinya, R., Takeuchi, Y., and Futai, K.** (2009b) A technique for separating the developmental stages of the propagative form of the pine wood nematode, *Bursaphelenchus xylophilus*. *Nematology*. 11:2:305-307.

180. **Shinya, R., Morisaka, H., Takeuchi, Y., Ueda, M., and Futai, K.** (2010) Comparison of the surface coat proteins of the pine wood nematode appeared during host pine infection and *in vitro* culture by a proteomic approach. *Phytopathology*. 100:12:1289-1297.

181. **Shinya, R., Morisaka, H., Kikuchi, T., Takeuchi, Y., Ueda, M., and Futai, K.** (2013) Secretome analysis of the pine wood nematode *Bursaphelenchus xylophilus* reveals the tangled roots of parasitism and its potential for molecular mimicry. *PLoS ONE*. 8:6:e67377.

182. **Simms, D., Cizdziel, P.E., and Chomozynski, P.** (1993) TRIzol: A new reagent for optimal single-step isolation of RNA. *Focus*. 15:4:99-102.

183. **Sinha, A., Sommer, R.J., and Dieterich, C.** (2012) Divergent gene expression in the conserved dauer stage of the nematodes *Pristionchus pacificus* and *Caenorhabditis elegans*. *BMC Genomics*. 13:254.

184. **Sommer, R.J., Carta, L.K., Kim, S.Y., and Sternberg, P.W.** (1996) Morphological, genetic and molecular description of *Pristionchus pacificus* sp. n. (Nematoda: Neodiplogastridae). *Fundamental Applied Nematology*. 19:6:511-521.

185. **Srinivasan, J., Kaplan, F., Ajredini, R., Zachariah, C., Alborn, H.T., Teal, P.E.A., Malik, R.U., Edison, A.S., Sternberg, P.W., and Schroeder, F.C.** (2008) A blend of small molecules regulates both mating and development in *Caenorhabditis elegans*. *Nature*. 454:7208:1115-1118.

186. **Srinivasan, J., von Reuss, S.H., Bose, N., Zaslaver, A., Mahanti, P., Ho, M.C., O'Doherty, O.G., Edison, A.S., Sternberg, P.W., and Schroeder, F.C.** (2012) A modular library of small molecule signals regulates social behaviors in *Caenorhabditis elegans*. *PLoS Biology*. 10:1:e1001237.

187. **Sriwati, R., Takemoto, S., and Futai, K.** (2007) Cohabitation of the pine wood nematode, *Bursaphelenchus xylophilus*, and fungal species in pine trees inoculated with *B. xylophilus*. *Nematology*. 9:1:77-86.

188. **Stasiuk, S.J., Scott, M.J., and Grant, W.N.** (2012) Developmental plasticity and the evolution of parasitism in an unusual nematode, *Parastrongyloides trichosuri*. *EvoDevo*. 3:1.

189. **Steiner, G., and Buhrer, E.M.** (1934) *Aphelenchoides xylophilus*, n. sp. A nematode associated with blue-stain and other fungi in timber. *Journal of Agricultural Research*. 48:10:949-951.

190. **Stoltzfus, J.D., Minot, S., Berriman, M., Nolan, T.J., and Lok, J.B.** (2012) RNAseq analysis of the parasitic nematode *Strongyloides stercoralis* reveals divergent regulation of canonical dauer pathway. *PLoS neglected topical diseases*. 6:10:e1854.

191. **Sun, J., Nishiyama, T., Shimizu, K., and Kadota, K.** (2013) TCC: an R package for comparing tag count data with robust normalization strategies. *BMC Bioinformatics*. 14:219.

192. **Takemoto, S., and Futai, K.** (2007) Polymorphism of Japanese isolates of the pinewood nematode, *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae), at heat-shock protein 70A locus and the field detection of polymorphic populations. *Applied Entomology and Zoology*. 42:2:247-253.

193. **Takeuchi, Y., and Futai, K.** (2007) Asymptomatic carrier trees in pine stands naturally infected with *Bursaphelenchus xylophilus*. *Nematology*. 9:2:243-250.

194. **Tanaka, S.E., Tanaka, R., Akiba, M., Aikawa, T., Maehara, N., Takeuchi, Y., and Kanzaki, N.** (2014) *Bursaphelenchus niphades* n. sp. (Tylenchina: Aphelenchoididae) amensally associated with *Niphades variegatus* (Roelofs) (Coleoptera: Curculionidae). *Nematology*. 16:259-281.

195. **Thomas, J.H., Birnby, D.A., and Vowels, J.J.** (1993) Evidence for parallel

processing of sensory information controlling dauer formation in *Caenorhabditis elegans*. *Genetics*. 134:1105-1117.

196. **Togashi, K.** (1985) Transmission curves of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) from its vector, *Monochamus alternatus* (Coleoptera: Cerambycidae), to pine trees with reference to population performance. *Applied Entomology and Zoology*. 20:3:246-251.
197. **Tokushige, Y., and Kiyohara, T.** (1969) *Bursaphelenchus* sp. in the wood of dead pine trees. *Journal of Japanese Forest Society*. 51:7:193-195.
198. **Trapnell, C., Pachter, L., and Salzberg, S.L.** (2009) TopHat: discovering splice junctions with RNA-seq. *Bioinformatics*. 25:9:1105-1111.
199. **Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L.** (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols*. 7:3:562-578.
200. **Tsai, I.J., Tanaka, R., Kanzaki, N., Akiba, M., Yokoi, T., Espada, M., Jones, J.T., and Kikuchi, T.** (2016) Transcriptional and morphological changes in the transition from mycetophagous to phytophagous phase in the plant-parasitic nematode *Bursaphelenchus xylophilus*. *Molecular Plant Pathology*. 17:1:77-83.
201. **Umebayashi, T., Tamada, T., Fukuhara, K., Endo, R., Kusumoto, D., and Fukuda, K.** (2017) In situ observation of pinewood nematode in wood. *European Journal of Plant Pathology*. 147:2:463-467.
202. **Vanfleteren, J.R.** (1993) Oxidative stress and aging in *Caenorhabditis elegans*. *Biochemical Journal.* 292:605-608.
203. **van Megen, H., van den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers, T., Holovachov, O., Bakker, J., and Helder, J.** (2009) A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. *Nematology*. 11:6:927-950.

204. **Vicente, C.S.L., Ikuyo, Y., Mota, M., and Hasegawa, K.** (2013) Pinewood nematode-associated bacteria contribute to oxidative stress resistance of *Bursaphelenchus xylophilus*. *BMC Microbiology*. 13:299.

205. **Vicente, C.S.L., Ikuyo, Y., Shinya, R., Mota, M., and Hasegawa, K.** (2015) Catalases induction in high virulence pinewood nematode *Bursaphelenchus xylophilus* under hydrogen peroxide-induced stress. *PLoS ONE*. 10:4:e0123839.

206. **Viney, M.E., Gardner, M.P., and Jackson, J.A.** (2003) Variation in *Caenorhabditis elegans* dauer larva formation. *Development, Growth and Differentiation*. 45:389-396.

207. **von Heijne, G.** (1998) Life and death of a signal peptide. *Nature*. 396:111-113.

208. **Voss, M., Schröder, B., and Fluhrer, R.** (2013) Mechanism, specificity, and physiology of signal peptidase (SPP) and SPP-like proteases. *Biochimica et Biophysica Acta*. 1828:12:2828-2839.

209. **Vowels, J.J., and Thomas, J.H.** (1992) Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans*. *Genetics*. 130:105-123.

210. **Walker, G.E.** (1984) Ecology of the mycophagous nematode *Aphelenchus avenae* in wheat-field and pine-forest soils. *Plant and Soil*. 78:417-428.

211. **Warren, J.E., and Linit, M.J.** (1993) Effect of *Monochamus carolinensis* on the life history of the pinewood nematode, *Bursaphelenchus xylophilus*. *Journal of Nematology*. 25:4:703-709.

212. **Wingfield, M.J.** (1983) Transmission of pine wood nematode to cut timber and girdled trees. *Plant Disease*. 67:1:35-37.

213. **Wingfield, M.J.** (1987) Fungi associated with the pine wood nematode, *Bursaphelenchus xylophilus*, and Cerambycid beetles in Wisconsin. *Mycologia*. 79:2:325-328.

214. **Wu, X.Q., Yuan, W.M., Tian, X.J., Fan, B., Fang, X., Ye, J.R., and Ding, X.L.** (2013) Specific and functional diversity of endophytic bacteria from pine wood

nematode *Bursaphelenchus xylophilus* with different virulence. *International Journal of Biological Sciences*. 9:34-44.

215. **Yamada, K., Hirotsu, T., Matsuki, M., Butcher, R.A., Tomioka, M., Ishihara, T., Clardy, J., Kunitomo, H., and Iino, Y.** (2011) Olfactory plasticity is regulated by pheromonal signaling in *Caenorhabditis elegans*. 329:5999:1647-1650.
216. **Yamaji, K., Ishimoto, H., Usui, N., and Mori, S.** (2005) Organic acids and water-soluble phenolics produced by *Paxillus* sp. 60/92 together show antifungal activity against *Pythium vexans* under acidic culture conditions. *Mycorrhiza*. 15:17-23.
217. **Yamamoto, N., Odani, K., Sasaki, S., and Nishiyama, Y.** (1986) Cellulase exudation by the pine wood nematode – Detection of activity in its crawling track. *Journal of Japanese Forest Society*. 68:6:237-240.
218. **Yano, S.** (1913) Investigation on pine death in Nagasaki prefecture. *Sanrin-Kouhou*. 4:1-14. In Japanese.
219. **Yugi, K., Kubota, H., Hatano, A., and Kuroda, S.** (2016) Trans-Omics: How to reconstruct biochemical networks across multiple 'Omic' layers. *Trends in Biotechnology*. 34:4:276-290.
220. **Zhao, L., Zhang, S., Wei, W., Hao, H., Zhang, B., Butcher, R.A., and Sun, J.** (2013) Chemical signals synchronize the life cycle of a plant-parasitic nematode and its vector beetle. *Current Biology*. 23:20:1-6.
221. **Zhou, K.I., Pincus, Z., and Slack, F.J.** (2011) Longevity and stress in *Caenorhabditis elegans*. *Aging*. 3:8:1-21.

List of Publication.

Tanaka, S.E., Aikawa, T., Takeuchi-Kaneko, Y., Fukuda, K., and Kanzaki, N. (2017) Artificial induction of third-stage dispersal juveniles of *Bursaphelenchus xylophilus* using newly established inbred lines. *PLoS ONE*. 12:10:e0187127.

Related Publications.

Tanaka, S.E., Tanaka, R., Akiba, M., Aikawa, T., Maehara, N., Takeuchi, Y., and Kanzaki, N. (2014) *Bursaphelenchus niphades* n. sp. (Tylenchina: Aphelenchoididae) amensally associated with *Niphades variegatus* (Roelofs) (Coleoptera: Curclionidae). *Nematology*. 16:259-281.

Kanzaki, N., Maehara, N., Akiba, M., Tanaka, S.E., and Ide, T. (2016) Morphological characters of dauer juveniles of three species of *Bursaphelenchus* Fuchs, 1937. *Nematology*. 18:209-220.

Kanzaki, N., Tanaka, S.E., Fitza, K., Kosaka, H., Slippers, B., Kimura, K., Tsuchiya, S., and Tabata, M. (2016) *Deladenus nitobei* n. sp. (Tylenchomorpha: Allantonematidae) isolated from *Sirex nitobei* (Hymenoptera: Siricidae) from Aomori, Japan, a new member of the *siricidicola* superspecies. *Nematology*. 18:1199-1217.

Ekino, T., Tanaka, S.E., Kanzaki, N., and Takeuchi-Kaneko, Y. Tolerance to oxidative stress of inbred strains of the pine wood nematode, *Bursaphelenchus xylophilus*, differing in terms of virulence. *Nematology*. Under submission.