名大演報 No. 15 : 141-150 (1996)

Inoculation with Blue Stain Fungi Associated with *Ips cembrae* (Coleoptera: Scolytidae) on Seedlings of Japanese Larch and Japanese Red Pine

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Abstract

The pathogenicity of blue stain fungi, two strains of *Ophiostoma piceae* (MÜNCH) H. & P. SYDOW and one strain of *Leptographium* sp., associated with the bark beetle, *Ips cembrae* HEER (Coleoptera : Scolytidae), to Japanese larch, *Lalix leptolepis* GORDON, and Japanese red pine, *Pinus densiflora* SIEB. et ZUCC., was investigated by fungal inoculation experiments. During four months of the experimental period, none of the pine seedlings inoculated with all strains of blue stain fungi died, but the mortality of the larch seedlings inoculated with one strain of *O. piceae* was 4.2% and that with *Leptographium* sp. was 4.2%. In the seedlings of both tree species inoculated with blue stain fungi, the mean length of the necrotic lesion of the sapwood was significantly larger than that of the controls, and the mean water pressure potential of the xylem decreased in all fungal inoculations. The inoculated fungi were reisolated from the wood pieces near the inoculation points on the inoculated seedlings, but not from the controls. These results suggest that blue stain fungi associated with *I. cembrae* may have a negative effect on the attacked Japanese larch and Japanese red pine trees and might create a potential crisis in coniferous forests in Japan, if combined with intensive attacks of the beetles.

Keywords : blue stain fungi, *Ips cembrae*, Japanese larch, Japanese red pine, pathogenicity

Introduction

The larch ips, *Ips cembrae* HEER (Coleoptera : Scolytidae), is one of the most destructive insect pests of Japanese larch, *Larix leptolepis* GORDON, forests in the northern part of Honshu and Hokkaido, Japan (KOIZUMI, 1994). In Aichi Prefecture, central Japan, we observed that *I. cembrae* beetles attack living trees and fresh logs of Japanese larch and Japanese red pine, *Pinus densiflora* SIEB. et ZUCC. (PENG *et al.*, 1996b). It is well-known that many species of bark beetles attack coniferous and/or broad-leaved trees and are closely associated with blue stain fungi (CRAIGHEAD, 1928; AOSHIMA and HAYASHI, 1964; BASHAM, 1970; RANE and TATTAR, 1987). From the body surface of *I. cembrae* adults and their parent galleries in Aichi Prefecture, blue stain fungi, *Ophiostoma piceae* (MÜNCH) H. & P. SYDOW and *Leptographium* sp., were isolated in higher proportions

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(Accepted : Nov. 22, 1996)

(PENG *et al.*, 1996b). Among blue stain fungi associated with bark beetles attacking Japanese larch and Japanese red pine in other areas of Japan, *Ceratocystis piceae* (MÜNCH) BAKSHI (= *O. piceae*) is the dominant species (NISHIKADO and YAMAUTI, 1935; AOSHIMA and HAYASHI, 1964; YAMAGUCHI *et al.*, 1989).

Invasions of blue stain fungi in trees attacked by bark beetles might interfere with water transport in the trees, contributing to the death of the trees (BALLARD *et al.*, 1982, 1984; BASHAM, 1970; PARMETER *et al.*, 1989, 1992; YAMAOKA *et al.*, 1990; PENG *et al.*, 1996a). In Scotland, *I. cembrae* acts as a vector for *Ceratocystis laricicola* REDFERN & MINTER which kills the bark and cambium of European larch (*Larix decidua* MILLER) and invades the sapwood causing the formation of blue-stained and dry wood (REDFERN *et al.*, 1987). REDFERN *et al.* (1987) also suggested that multiple inoculations resulting from numerous beetle attacks may totally disrupt conduction causing dieback and death of the larch trees. However, inoculation experiments with blue stain fungi, *C. piceae*, on Japanese larch have shown low mortalities of inoculated trees (MAETO *et al.*, 1991; SASAKI *et al.*, 1990; YAMAGUCHI *et al.*, 1989, 1991; YAMAGUCHI, 1993). Experiments by KANEKO *et al.* (1993) also showed that *C. piceae* has weak pathogenicity forward the seedlings of the Japanese red pine.

The objective of this study was to investigate the pathogenicity of *O. piceae* and *Leptographium* sp., isolated from *I. cembrae* which attacks Japanese larch in Aichi Prefecture, to Japanese larch and Japanese red pine by inoculation experiments.

Materials and Methods

1. Inoculations

An experimental seedling stand is located near Inabu Town in the northeast area of Nagoya City, Aichi Prefecture (about 700 m above sea level), central Japan. The annual mean air temperature and annual precipitation at this location are 11.1℃ and 1,951.3 mm, respectively. This seedling stand consists of 4-year-old Japanese larch (mean diameter at ground height, 1.25 cm ; mean height, 105 cm) and Japanese red pine (mean diameter at ground height, 2.35 cm ; mean height, 104 cm).

A strain of *O. piceae* (hereafter referred to as *O. piceae* A) and a strain of *Leptographium* sp., both of which were isolated from the body surfaces of *I. cembrae* attacking Japanese larch in Aichi Prefecture (PENG *et al.*, 1996b), were used for inoculation. To compare the pathogenicity between fungal strains, another strain of *O. piceae* (hereafter referred to as *O. piceae* B) isolated from *I. cembrae* in Hokkaido (YAMAGUCHI *et al.*, 1989) was also used for inoculation. Before inoculations, each fungus was cultured on mixed media of pine sawdust and rice bran and incubated at 25℃ for 4 weeks.

On May 17, 1995, at the beginning of the *I. cembrae* attacks (PENG *et al.*, 1996b), a total of 72 Japanese larch and 60 Japanese red pine seedlings were inoculated with the three strains of blue stain fungi, *O. piceae* A, *O. piceae* B and *Leptographium* sp. For each fungal treatment, 24 Japanese larch and 20 Japanese red pine seedlings were prepared (Table 1). On the stem of each seedling, a half-circumferential strip, 1 cm wide, of the bark was peeled off with a sterilized knife around the circumference, approximately 20 cm above the ground, and another half-circumferential strip of the bark was peeled off about 2 cm above the first strip (Fig. 1). These bark strips were cut to depths reaching the cambium, and the upper strips were vertically alternated with the lower strips to avoid complete blockage of the transportation of water (Fig. 1).

Each wound was plugged with the pine sawdust-rice bran inoculum in which the fungal culture had grown. The fungal inoculation area was immediately covered with the bark strips which had been peeled from the stems and then sealed using a parafilm to avoid contamination and desiccation.

Other 24 Japanese larch and 20 Japanese red pine seedlings were also inoculated with fungus-free medium as control treatments. Moreover, 8 non-inoculated larch and 11 non-inoculated pine seedlings were used for comparison with other treatments (Table 1).

2. Observations and measurements

1) External appearances of inoculated trees

After the inoculations, symptomatic development of all seedlings was carefully examined up to mid-September, 1995. When a seedling showed discoloration of foliage throughout the crown and all its needles were wilted, it was considered to be dead.

2) Lesion formation

Once every 4 weeks after inoculation, in each treatment, 3 inoculated seedlings of

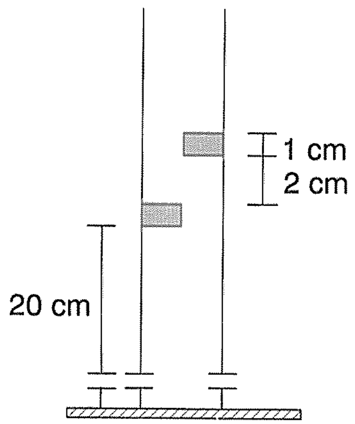

Fig. 1. Inoculation sites.

Table 1. Tree mortalities of Japanese larch and Japanese red pine after inoculation.

Fungal inoculum	Japanese larch			Japanese red pine		
	No. of dead seedlings	Total no. of seedlings	% Mortalities	No. of dead seedlings	Total no. of seedlings	% Mortalities
O. piceae A	1	24	4.2	0	20	0.0
O. piceae B	0	24	0.0	0	20	0.0
Leptographium sp.	1	24	4.2	0	20	0.0
Control	0	24	0.0	0	20	0.0
Non-treated	0	8	0.0	0	11	0.0

both Japanese larch and Japanese red pine were cut down for measurement of lesion length. After stripping the bark, the longest longitudinal length of necrotic lesions of the sapwood around each inoculation point was measured. The lesion lengths of control seedlings were also measured by the same method.

3) Xylem pressure potential (XPP)

It is known that host trees infected by blue stain fungi respond to changes in the water pressure potential of the xylem (XPP) (RANE and TATTAR, 1987). Hence, XPP was measured every 2 weeks after inoculations (for successive 2 days : on the first day, pine ; on the second day, larch) using a portable pressure chamber (Daiki Rika Kogyo Co., Ltd.). In each treatment, 8 larch and 10 pine seedlings were chosen for continous measurments over the study period. Among these seedlings, there were no significant differences in XPP before the fungal inoculations. Within 3 hours after sunrise, 3 larch twigs and 3 pine needles were sampled from each seedling and measured.

4) Fungal reisolation

Four, 8 and 12 weeks after inoculation, fungal reisolations were made from the lesions of sapwood near the inoculated points. In each treatment, about 15 small wood pieces of the lesions, $0.5 \times 0.5 \times 0.2$ cm in size, were cut from 3 seedlings used for measurement of lesion length. The pieces were surface-sterilized with ethanol (70%) and antiformine (5-fold dilution) for 3 seconds and 3 minutes, respectively, and then washed with sterilized water. Subsequently, the sterilized pieces were placed on PDA media and incubated at 25℃ for seven days. Isolates from the lesions were identified based on the morphological features of the conidia and the characteristics of the fungal colony. In control trees, the reisolation was done in the same manner.

Results

1. External appearances of inoculated trees

The mortalities of inoculated larch and pine seedlings are summarized in Table 1. During about four months after inoculation, discolorations and wilting of needles were observed on one larch seedling inoculated with *O. piceae* A and one with *Leptographium* sp. on the 60th and 80th day after inoculation, respectively. In contrast, neither the control nor non-treated larch seedlings were dead. However, Japanese red pine showed no mortality and no symptoms in all treatments during the study period (Table 1).

2. Xylem pressure potential (XPP)

Figure 2 shows the changes in average XPP of larch and pine seedlings after fungal inoculations. The means of the XPPs of larch seedlings inoculated with *O. piceae* A, *O. piceae* B and *Leptographium* sp. were significantly less than those of the control and non-treated seedlings throughout the experimental period, except at 8 weeks after inoculations when it rained before measuremant (DUNCAN's multiple range test, $P < 0.05$) (Fig. 2, above). From 2 to 16 weeks after inoculation, the mean XPPs of pine seedlings inoculated with the fungi were also significantly less than those observed in the control and non-treated seedlings (DUNCAN's multiple range test, $P < 0.05$) (Fig. 2, below). In both cases of larch and pine, however, differences among the mean XPPs of 3 fungal strains

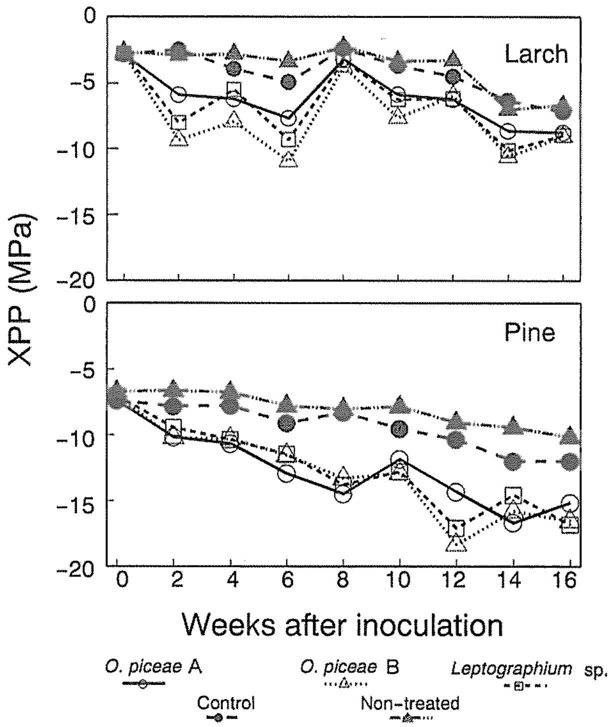

Fig. 2. Changes in average pressure potentials in the xylem (XPP) of Japanese larch (top) and Japanese red pine (bottom) seedlings after inoculation with *O. piceae* A, *O. piceae* B and *Leptographium* sp.

were not significant (DUNCAN's multiple range test, $P > 0.05$), except for 2 weeks (larch) and 12 weeks (pine) after inoculations.

3. Lesion formation in inoculated trees

Necrotic lesions were formed in the phloem and sapwood near the inoculated parts of all inoculated seedlings including the controls. Lesions in seedlings of both larch and pine inoculated with *O. piceae* A, *O. piceae* B and *Leptographium* sp. were significantly larger than those of the controls throughout the experimental period (DUNCAN's multiple range test, $P < 0.05$), reaching a plateau 8 or 12 weeks after inoculation (Fig. 3). Among the three fungal treatments in Japanese larch, the mean length of lesions caused by *O. piceae* B was significantly less than those of *O. piceae* A and *Leptographium* sp. 12 weeks after inoculation (DUNCAN's multiple range test, $P < 0.01$). In the case of Japanese red pine, however, the length in the *Leptographium* sp. treatment was significantly smaller than those in other treatments of the two fungal strains at the end of the experiments (DUNCAN's multiple range test, $P < 0.01$) (Fig. 3).

4. Fungal reisolations

From the seedlings inoculated with blue stain fungi, the fungi were reisolated 4, 8 and

12 weeks after inoculation. The reisolation rate of each inoculated fungus was determined by dividing the sum of the total number of wood pieces from which the fungi were reisolated by that of the pieces tested for the reisolation experiment (Table 2). In Japanese larch and Japanese red pine, the percentages of reisolation in the inoculated seedlings ranged from 41.5~60.0% and 42.6~52.1%, respectively. In larch, the reisolation rate of *O. piceae* B was the highest, but *Leptographium* sp. was most frequently reisolated from pine. No blue stain fungi were detected from all the wood pieces in the control trees (Table 2).

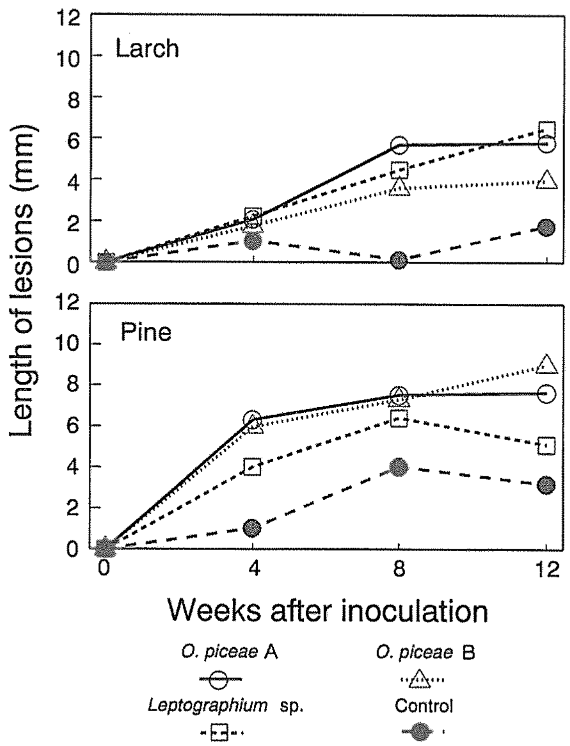

Fig. 3. Changes in average lesion length of Japanese larch (top) and Japanese red pine (bottom) seedlings after inoculation with *O. piceae* A, *O. piceae* B and *Leptographium* sp.

Table 2. Reisolation of inoculated blue stain fungi from sapwood near inoculation sites.

Fungal inoculum	Japanese larch		Japanese red pine	
	No. of pieces*	Reisolation rates of inoculated fungus(%)	No. of pieces*	Reisolation rates of inoculated fungus(%)
O. piceae A	41	41.5	45	48.9
O. piceae B	45	60.0	48	42.6
Leptographium sp.	44	43.2	47	52.1
Control	45	0.0	40	0.0

*Numbers of lesion pieces tested for reisolation.

Discussion

In this study, we found that seedlings in both Japanese larch and Japanese red pine were less likely to die if they were healthy seedlings without any stress such as girdling on the stem. In our experiments in 1992, 12-year-old Japanese red pine trees were girdled around the stem and inoculated with the blue stain fungus, *O. piceae* B, so that 4 of 14 inoculated trees died and some showed discoloration on the crown (PENG *et al*., 1996a). Studies by YAMAGUCHI *et al*. (1989) showed that in Hokkaido, girdled Japanese larch trees were also killed by *C. piceae*, the same fungal strain which is called *O. piceae* B in this study. Thus, girdling, as a source of stress in trees, would be capable of intensifying the decline and ultimate death of trees inoculated with *O. piceae*.

XPPs in seedlings inoculated with *O. piceae* A, *O. piceae* B and *Leptographium* sp. were significantly less than those of the controls and untreated seedlings in both the cases of Japanese larch and Japanese red pine, demonstrating that inoculated blue stain fungi have a negative effect on the transportation system of host trees. Thus, water flow may be blocked by the damage to xylem tracheids caused by invasion of the fungal mycelia. YAMAGUCHI (1993) observed that the XPP of Japanese larch seedlings decreased one week after inoculations of *C. piceae* (= *C. piceae* B), and then the seedlings died. In our experiment in 1992, non-girdled Japanese red pines also showed the same tendency in the XPP after inoculation with *O. piceae* B. In the girdling treatment, furthermore, the XPP observed in the inoculated pine trees decreased more severely than that in the control trees, which resulted in higher mortality of the trees (PENG *et al*., 1996a).

Variation in susceptibility of the host trees to isolates of blue stain fungi was noted in healthy seedlings, as confirmed by other studies (BASHAM, 1970; OWEN *et al*., 1987). BASHAM (1970) pointed out that young loblolly pine, *Pinus taeda* L., seedlings were susceptible to the inoculations of some *Ceratocystis* fungi. In the present inoculations, the tree species-fungal treatment interaction was obvious for lesion length, suggesting that the smaller lesions may reflect a more rapid and effective defensive reaction of trees to the fungus. In Japanese larch, the length of the lesions induced by inoculation with *O. piceae* B was shorter those that with *O. piceae* A and *Leptographium* sp., but inoculation with *Leptographium* sp. on Japanese red pine resulted in a smallest·lesion (Fig. 3). However, a few larch seedlings inoculated with the three strains of blue stain fungi died, and none of pine seedlings died (Table 1). This result suggests that blue stain fungi associated with *I. cembrae* might not induce severe damage on all healthy host trees, Japanese larch and Japanese red pine, although the fungi might interfere with the water transport of the host trees (Fig. 2).

All strains of inoculated fungi were also reisolated from lesions of inoculated seedlings but were not detected from those of the controls (Table 2), which demonstrates that inoculated blue stain fungi could invade, colonize and grow in the xylem of host trees. In seedlings of larch and pine inoculated with *O. piceae* A, *O. piceae* B and *Leptographium* sp., the lesion lengths of the sapwood were significantly larger than those of the controls. Eight or 12 weeks after inoculation, the extension rate of the lesions slowed down, suggesting that the growth of the inoculated fungi, *O. piceae* and *Leptographium* sp., is likely to be limited to the local tissues of the phloem and xylem.

It is known that in coniferous trees, the formation of necrotic lesions around the points of bark-beetle attacks and the decrease in XPP of host trees are strongly associated with the invasion of fungi vectored by the beetles into the trees (MOLNAR, 1965; REID *et al.*, 1967; REDFERN *et al.*, 1987). The bark beetle, *I. cembrae*, carries some blue stain fungi, including *O. piceae*, and attacks both Japanese larch and Japanese red pine over wide areas in Japan (KOIZUMI, 1994). In Aichi Prefecture, *I. cembrae* beetles which attack Japanese larch are also closely associated with the blue stain fungi, *O. piceae* and *Leptographium* sp. (PENG *et al.*, 1996b), both of which have a negative physiological effect on host trees, such as a decline in XPP, as clarified by the present study. Furthermore, the inoculation experiment with the *O. piceae* in stressed trees of young Japanese red pine led to higher mortality of the trees (PENG *et al.*, 1996a). In general, *I. cembrea* is considered to be a secondary insect that invades more stressed or weakened trees in which the water transport in the stems has decreased (ENDA, 1994), although it can also attack living Japanese larch trees and cause severe damage such as wilting in Hokkaido (TERASAKI *et al.*, 1987). All these results may imply that in Aichi Prefecture, the blue stain fungi associated with *I. cembrae* would not be capable of directly killing healthy host trees in large areas. However, if combined with an intense population level of the bark beetles and/or reduction of tree vigor by abiotic and biotic predisposing factors (PAINE and BAKER, 1993), the fungi may create a potential crisis in coniferous forests.

Acknowledgements

We thank Dr. S. ITO (Tohoku Res. Ctr., For. and Forest Prod. Res. Inst.) and Dr. N. HIJII (Nagoya University) for their critical comments on the manuscript. Thanks are also due to Dr. Y. YAMAOKA (Tsukuba University) and Dr. T. YAMAGUCHI (Hokkaido Res. Ctr., For. and Forest Prod. Res. Inst.) for identifying and providing the fungi used. We also thank all the members of the Laboratory of Forest Protection, Nagoya University, for their help with the experiments. This study was supported in part by a grant-in-Aid for Scientific Research (No. 06454090) from the Ministry of Education, Science and Culture, Japan.

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カラマツヤツバキクイムシに随伴する青変菌の カラマツとアカマツ苗木に対する接種

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カラマツヤツバキクイムシが伝播していると思われる青変菌2属3株のカラマツおよびアカマツ苗木に対する病原性を人工接種実験によって調べた。アカマツはいずれの青変菌の接種によっても枯れなかったが，カラマツは *Ophiostoma piceae* と *Leptographium* 属菌によってそれぞれ4.2%が枯死した。青変菌の接種木では，カラマツ・アカマツともに木部壊死斑長が対照木より大きくなり，また木部水ポテンシャルは大きく低下した。接種した菌は，接種部周辺の材片から再分離された。これらの結果から，カラマツヤツバキクイムシに随伴している青変菌のカラマツ・アカマツ樹体内への侵入は，これらの寄生木の生長・生存に悪影響を及ぼすものと考えられ，またキクイムシが大発生した場合には，林地において寄生木が枯死する危険性も示唆された。

キーワード：カラマツヤツバキクイムシ，青変菌，人工接種，カラマツ，アカマツ