EFFECT OF LIGHT CONDITIONS ON THE RESISTANCE OF CURRENT-YEAR *FAGUS CRENATA* SEEDLINGS AGAINST FUNGAL PATHOGENS CAUSING DAMPING-OFF IN A NATURAL BEECH FOREST: FUNGUS ISOLATION AND HISTOLOGICAL AND CHEMICAL RESISTANCE

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Abstract—Forest gap dynamics affects the light intensity on the forest floor, which in turn may influence defense and survival of tree seedlings. Current-year *Fagus crenata* seedlings show high mortality under canopy caused by damping-off, on the contrary, they survive pathogen attacks in gaps. However, defense mechanisms against damping-off have not been fully understood in *F. crenata*. In order to determine the resistance factors that affect mortality in current-year seedlings, we compared seedling survival and chemical and histological characteristics of the hypocotyls of seedlings from closed-stand and forest-edge plots. Damping-off occurred in the current-year seedlings mainly from the end of June to July; the survival rate of the seedlings was higher in the forest-edge plot than in the closed-stand plot. We identified *Colletotrichum dematium* and *Cylindrocarpon* sp. as the causative pathogens only under low illumination by performing an inoculation test on the seedling hypocotyls. In the beginning of July, only the seedling hypocotyls from the forest-edge plot exhibited periderm formation. From mid-June to July, seedling hypocotyls from the forest-edge plot accumulated approximately twice the amount of total phenols that was accumulated by the seedling hypocotyls from the closed-stand plot. The ethyl acetate phase of methanol extracts of hypocotyls showed antifungal activity. We conclude that seedlings from the forest-edge plot may resist pathogenic attack via periderm formation and increased phenol synthesis. Plant defense mechanisms that are controlled by light intensity may be important for promoting seedling regeneration in forest gap dynamics.

Key Words—*Fagus crenata*, damping-off, light intensity, periderm, antifungal substance.

INTRODUCTION

In forests, canopy gaps created by the death or damage of trees are often filled by new trees, shrubs, and herbaceous vegetation. Such gap dynamics are observed in tropical, temperate evergreen broadleaf, temperate deciduous broadleaf, temperate conifer, and boreal forests (Gray and Spies, 1996; Yamamoto, 2000; Whitmore, 1989). Gaps in the canopy are associated with environmental changes, especially better light conditions on the forest floor, and effects on the seedling survival (Augspurger, 1984; Gray and Spies, 1997). Improvement in the survival rate of seedlings growing in such gaps is mainly attributable to decrease in the incidence of damping-off. In contrast, the mortality of seedlings growing under shaded conditions is high (Augspurger, 1984; Hood et al., 2004; Jarosz and Davelos, 1995; Sahashi et al., 1994). Several soil-borne fungi have been reported to induce damping-off in seedling populations on the forest floor (Jarosz and Davelos, 1995; Packer and Clay, 2000; Sahashi et al., 1995). However, the factors that determine seedling survival or death under changed environments in gaps have not been fully understood.

Canopy gap dynamics has intensively been studied in the cool temperate forests of Japan where *Fagus crenata* is the main climax species (Nakashizuka, 1984; Yamamoto, 2000). The mortality rate of current-year *F. crenata* seedlings is high because of the occurrence of damping-off at several weeks after germination. Further, the mortality rate is lower for seedlings growing in the gaps than those growing under shaded conditions (Abe et al., 2005; Hashizume and Yamamoto, 1975; Maeda, 1988; Nakashizuka, 1988; Sahashi et al., 1994, 1995). However, the factors affecting resistance to fungal invasion are unclear.

Generally, tree seedlings resist fungal invasion by forming a specific tissue (Agrios, 2005; Eyles et al., 2003; Laflamme et al., 2006; Tippett and Hill, 1984) and by producing antifungal substances (Agrios, 2005; Bonello and Pearce, 1993; Ingham, 1973; Siegrist et al., 1994; Treutter, 2006). Therefore, differences in the resistance of seedlings grown under different light conditions to fungal invasion should be considered by comparing the histological structure (periderm formation) and chemical content (concentration of phenolic compounds) of the seedlings. Increase in the concentration of phenolic compounds has been reported in seedlings grown in gaps or increased light intensity (Nichols-Orians, 1991; Entry et al., 1991). From a phytopathological viewpoint, the confirmation of whether such differences in the defense mechanisms are attributable to light conditions in the field is essential for a better understanding of gap dynamics.

In order to determine the resistance factors that affect the mortality due to damping-off in current-year *F. crenata* seedlings, we identified the causative fungal species and compared the tissue structure of the periderm in the seedling hypocotyls and the change in the total phenol concentration between current-year seedlings grown under two different light conditions in a natural beech forest in northern Japan.

METHODS AND MATERIALS

Study Site. The field study was conducted in a secondary forest of *F. crenata* (39°59' N, 140°54' E) in Appi, Iwate Prefecture in northern Honshu, Japan. The canopy height in the forest was 25 m. An experimental plot was established along 75 m of the forest road (4 m

width, no pavement, and very rare traffic) and 25 m into the closed stand. An area less than 5 m from the forest road was designated as the forest-edge plot, and an area more than 10 m wide was designated as the closed-stand plot. The forest-edge plot was established under canopy facing north direction, and had leaf litter of *F. crenata*, which was not extremely dry. The relative illuminations in the closed-stand and forest-edge plots were evaluated using a light meter, model LI-250 (Li-COR Inc., Lincoln, NE, USA) and were 2.6% (SE, ±0.22) and 35.5% (SE, ±3.03), respectively.

Seedling Observation, Fungal Isolation, and Inoculation. From the forest-edge and the closed-stand plots, 100 and 101 current-year seedlings, respectively, were randomly selected. These seedlings were observed from May 28, 2004 (soon after germination) to September 28, 2004 (just before leaf coloring in autumn) to detect the occurrence of damping-off and determine the factors related to seedling death. Dead seedlings were collected when their leaves wilted.

Fungus Isolation from Seedlings That Had Died Because of Damping-off. Seedlings that had died because of damping-off were collected, and the lower part of their hypocotyls was sterilized in 70% ethanol solution and 1% sodium hypochlorite solution for 1 min each, rinsed twice with sterile distilled water, transferred onto 1% malt-extract agar (MEA), and incubated at 20 °C in darkness. Two wk after incubation, the fungi emerging from the tissues were isolated and maintained on MEA plates. The fungal species to which the isolates belonged

were identified on the basis of the morphological characteristics of the cells in the culture medium and on the hypocotyls, as observed under a light microscope.

Inoculation of Isolated Fungi into Seedling Hypocotyls. Four fungi, which were isolated at high frequencies from the dead seedlings, were grown on 1% MEA for 3 wk and used for an inoculation test. Seeds of *F. crenata* collected from Appi in October 2005 were peeled, and the cotyledons with the seed coat were sterilized with 1% sodium hypochlorite solution for 1 min. These were then potted in enameled iron dishes (33 cm × 38 cm; depth, 5 cm) containing Kanuma pumice (4 cm), which had been autoclaved at 121 °C for 30 min. The potted seeds were incubated at 4 °C in darkness for 1 month, according to a previously described method of germination (Katsuta et al., 1998). After germination, these dishes were transferred to a growth chamber (NK system, LP-1.5PH; Nihonika Co., Osaka, Japan), and the seedlings were grown at 15 °C under a cycle of 15 hr fluorescent light (3.0 μmol cm^{-2} s^{-1}) and 9 hr darkness for 1 month until the cotyledons had fully expanded and the first pair of true leaves had developed. Subsequently, several mycelial disks (each of diameter 5 mm) taken from the colonies of the four fungal species and non-mycelial 1% MEA disks were inoculated onto a wound sliced into the hypocotyls. We used 19–23 seedlings in each treatment. The development of disease symptoms and the number of dead seedlings were periodically recorded over 20 d.

Inoculation of Pathogenic Fungi under Two Different Illumination Conditions. Two

pathogenic fungi were used for another inoculation test that was performed under two different illumination conditions. Seeds were potted in the same dishes as described above and incubated at 4 °C in darkness for 1 month. After incubation, the seedlings were grown in the same growth chamber under either high illumination (36.1 μmol cm^{-2} s^{-1}) or low illumination (2.9 μmol cm^{-2} s^{-1}), achieved by using a shading net, at 15 °C under a cycle of 15 hr fluorescent light and 9 hr darkness for 1 month until the cotyledons were fully expanded and the first pair of true leaves developed. Two pathogenic fungi were inoculated into a wound sliced into the hypocotyls of these seedlings by using the same method as that used in the previous inoculation test. The test seedlings were observed for 2 wk and categorized as healthy, partially diseased, or wilted.

Tissue Structure of Hypocotyls. For histological analysis, 3 healthy seedlings were randomly collected from both the forest-edge and the closed-stand plots on June 1, June 15, July 6, and September 10. The lower part of the hypocotyls was cut and fixed in 5% glutaraldehyde in 0.1% sodium phosphate buffer, pH 7.2, and stored at 4 °C. The parts were then rinsed thrice in the phosphate buffer and post-fixed overnight at 4 °C in 2% osmium tetroxide prepared in the same buffer. The samples were then rinsed thrice with the buffer, dehydrated in an ethanol series, and embedded in Poly/Bed 812 (Polyscience Inc., Warrington, PA, USA). Thin cross-sections (2 μm) were cut with glass knives on an MT2-B ultramicrotome (Sorvall Instruments; DuPont Co., Wilmington, DE, USA) and gently heat-fixed to glass microscope slides. The sections were then stained with 0.1% toluidine blue O for 10 min, destained with

distilled water, air dried, mounted in Eukitt mounting reagent (O. Kindler, Breisgau, Germany) beneath a cover slip, and observed under an Optiphoto light microscope (Nikon Co., Tokyo, Japan). In addition, to localize the phenolic compounds in the tissue, an HM440E sliding microtome (Microm, Walldorf, Germany) was used to cut 30 μm-thick cross-sections of fixed hypocotyls of seedlings collected from the forest-edge and closed-stand plots on June 1, June 15, and July 6. The cross-sections were stained with 2% (w/v) $FeSO_4$ solution for about 1 hr to stain polyphenolic compounds with Fe (II) ions (Suzuki et al., 2003). The sections were then mounted in the staining solution beneath a cover slip and observed under an Optiphoto light microscope.

Analysis of Total Phenolic Compounds. Healthy seedlings were randomly collected from the forest-edge and closed-stand plots just after germination on May 28, June 15, July 6, and September 10 in 2004. From the collected seedlings, hypocotyls were taken and their FWs were measured. Two hypocotyls from 2 seedlings were cut into less than 1-mm pieces with scissors, and the phenolic compounds were extracted in 5 ml methanol in the dark over night at room temperature. The methanol extracts were filtered, adjusted at 5 ml, and stored at –20 °C. Total phenolic compounds were analyzed according to the Folin-Ciocalteau method (Waterman and Mole, 1994). The methanol extracts (100 or 200 μl) were added to distilled water (900 or 800 μl, respectively). A phenol reagent (500 μl) (Wako, Osaka, Japan) was added to the samples, and the samples were mixed by vortex. Na_2CO_3 solution (20% v/v, 2.5 ml) was continuously added and mixed, and distilled water (1 ml) was added to the sample.

After 20 min, the absorbance of the reactants was recorded at 700 nm (by using an ultraviolet-visible spectrophotometer, V-530; JASCO Co., Tokyo, Japan). A standard curve was prepared as follows: gallic acid (Wako, Osaka, Japan) (0.05, 0.1, 0.2, and 0.4 μg μl^{-1} of methanol) was subjected to the treatment described above for the methanol extracts, and each sample was analyzed thrice; the results were averaged. The amount of total phenolic compounds in the hypocotyls was calculated using the standard curve. Two seedlings were used for each analysis, and the results of five replicates were averaged. The concentrations of total phenolic compounds was expressed as mg gallic acid equivalents g^{-1} FW (±SE).

Antifungal Activity Test of the Ethyl Acetate Phase of the Methanol Extract of Hypocotyls. Seedling hypocotyls were randomly collected from the forest-edge (FW 67.9 g, equivalent to 487 seedlings) and closed-stand (FW 81.2 g, equivalent to 657 seedlings) plots on June 25. They were cut into pieces by using scissors into fragments less than 5 mm long and extracted in methanol (1 l) for 2 d in the dark at room temperature. The methanol extract was evaporated at 35 -40 °C, and the concentrated sample (approximately 100 ml) was extracted thrice with ethyl acetate (100 ml). The ethyl acetate phase (300 ml) was rinsed twice with saturated NaCl solution (150 ml) and dried overnight with Na_2SO_4. It was then evaporated and used for the antifungal activity test as follows. The ethyl acetate phase, equivalent to FW 5, 10, or 20 mg, was evaporated and dissolved in 50% methanol (5 μl). The samples were added to the wells (diameter, 10 mm) of a microplate (Asahi Techno Glass, Tokyo, Japan) and mixed with the spore solution (2.5×10^3 spores in 500 μl) of a pathogenic fungus. As a

control, 50% methanol (5 μl) was used instead of the sample. The plate was incubated in the dark at 20 °C for about 48 hr. Twenty-five spores were randomly selected from each well, and the percentage of germinated spores was calculated. Each sample was tested thrice, and the percentage of germinated spores was averaged.

Statistical Analysis. The Ekuseru-Toukei 2008 (Social Survey Research Information Co., Ltd.) software package was used for statistical analysis. The difference in survival rate between forest-edge and closed-stand plots was evaluated by Logrank test in survival analysis. Differences in the amount of total phenolic compounds between the hypocotyls of the seedlings from the forest-edge plot and those of the seedlings from the closed-stand plot were evaluated by Student's *t-test* for each sampling date. The square root of each percentage of spore germination in the antifungal activity test was converted to its arc sine (Sokal and Rohlf, 1995), and the differences between the control and the different concentrations of the ethyl acetate phases of the hypocotyls of the seedlings from the forest-edge and closed-stand plots were evaluated by two-way ANOVA and were followed by Tukey's test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Seedling Survival and Mortality in the Field. Between June and September, seedling death was observed mainly in late June and July after true leaves had fully expanded in both the forest-edge and closed-stand plots. On September 28, the mortality of seedlings was 7% and

50% in the forest-edge and closed-stand plots, respectively (Fig. 1), and was significantly different between the two plots (χ^2=42.57, *df*=1, *P*<0.01, logrank test in survival analysis). All seedlings had died due to damping-off, as indicated by their discolored hypocotyls and wilted leaves; no seedling had been damaged by rodents.

From June to July, the true leaves of most seedlings were damaged by insect herbivory. The damage by insect herbivory was classified as severe (complete loss of true leaves and stem above the cotyledons) or slight (partial loss of true leaves). The percentage of severely damaged, slightly damaged, and undamaged seedlings was 18.0, 48.0, and 34.0, respectively in the forest-edge plot and 2.0, 70.3, and 27.7, respectively in the closed-stand plot. Damping-off occurred only from the severely damaged seedlings in the forest-edge plot, however, it occurred in every insect-damage category in the closed-stand plot. A part of the severely damaged seedlings survived and produced new shoots from the base of the cotyledons in the forest-edge plot.

Fungi Isolated from Diseased Seedlings and Inoculation Test. From the lower part of the hypocotyls of the dead seedlings collected from both sites, four fungi, *Colletotrichum dematium*, *Cylindrocarpon* sp., *Phomopsis* sp., and *Discosia* sp. were mainly isolated (Table 1) throughout the experimental period.

Inoculation Test. The results of the inoculation test showed that *C. dematium* and *Cylindrocarpon* sp. induced necrosis in the hypocotyls resulting in the wilting of the seedlings

(Table 2). No disease symptoms were observed in the seedlings inoculated with *Phomopsis* sp. and *Discosia* sp. The results of the inoculation test performed under two different illumination conditions showed that *C. dematium* and *Cylindrocarpon* sp. induced necrosis in the hypocotyls and caused seedling death under low light intensity, but did not induce disease symptoms under high light intensity (Fig. 2).

Periderm Formation. Anatomical studies of the lower part of the hypocotyls of the healthy seedlings from each plot revealed histological differences in the epidermal and cortical tissue (Fig. 3). On June 1, live epidermal and cortical tissue was found in the seedlings from both plots (Fig. 3a, e). On June 15, it was observed that dead epidermal tissue surrounded live cortical tissue in the seedlings from both plots, but the layer of dead epidermal tissue was denser in the seedlings from the forest-edge plot than in those from the closed-stand plot (Fig. 3b, f). On July 6, periderm formation was observed in the seedlings from the forest-edge plot but not in those from the closed-stand plot (Fig. 3c, g). On September 10, periderm formation was also observed in the surviving seedlings from the closed-stand plot, but the periderm was thinner in these seedlings than in those from the forest-edge plot (Fig. 3d, h).

Distribution of Phenolic Compounds in the Tissue Layer. Light micrographs of sections of the seedlings from the forest-edge plot immersed in Fe (II) solution showed that epidermal cells were stained black to dark brown on June 1 (Fig. 4a). Positively stained cells were observed in the outer part of the layer of dead epidermal tissue and cortical tissue on June 15 (Fig. 4b)

and in the periderm on July 6 (Fig. 4c). The Fe (II)-stained cells were almost always localized with the cells containing black substances in resin-embedded sections (Fig. 3, 4). The sections of the seedlings from the closed-stand were stained similarly as those from the forest-edge, however, the Fe (II)-stained cells were rare in June 15 and July 6, when the black substance were also rare (Fig 3f, g).

Concentration of Total Phenolic Compounds in the Hypocotyls. The concentration of total phenolic compounds in the hypocotyls from the forest-edge plot increased after May 28 (Fig. 5), and that in hypocotyls from the closed-stand plot slightly increased after June 15 (Fig. 5). The concentration in the hypocotyls from the forest-edge plot on June 15 ($P < 0.001$), July 6 ($P < 0.001$), and September 10 ($P < 0.01$) was significantly higher (2.1, 2.2, and 3.7 times, respectively) than the corresponding concentrations in the hypocotyls from the closed-stand plot (Fig. 5).

Antifungal Activity of the Ethyl Acetate Phase of the Hypocotyls from the Forest-edge and Closed-stand Plots. Compared with the control, the ethyl acetate phases of the hypocotyls from the forest-edge plot, equivalent to FW 5, 10, and 20 mg significantly inhibited the spore germination of *C. dematium* ($P < 0.01$ in each treatment) (Fig. 6). Spore germination was also significantly inhibited by the ethyl acetate phases of the hypocotyls from the closed-stand plot, equivalent to FW 10 and 20 mg ($P < 0.01$ in each treatment) (Fig. 6). No difference was noted in the spore germination between the ethyl acetate phases of the hypocotyls from the

forest-edge and closed-stand plots ($P > 0.05$). The inhibitory activity on the spore germination of the hypocotyl extracts from the forest-edge plot was significantly higher than that of the extracts from the closed-stand plot (F=5.932, df=1, $P < 0.05$, Fig. 6). The values of the spore germination were significantly different among the concentration of the ethyl acetate phases (F=34.46, df=3, $P < 0.01$).

In natural *F. crenata* forests in Japan, damping-off in current-year seedlings has been reported to be caused by *C. dematium* (Sahashi et al., 1995). *Cylindrocarpon* sp. has also been isolated from current-year *F. crenata* seedlings that exhibit damping-off (Kobayashi et al., 1984), but its pathogenicity has not been confirmed. We isolated *C. dematium* and *Cylindrocarpon* sp. from diseased hypocotyls of current-year *F. crenata* seedlings that had died of damping-off in both the forest-edge and closed-stand plots (Table 1). We inoculated the four fungal species isolated in this study into the hypocotyls and found that *C. dematium* and *Cylindrocarpon sp.* induced hypocotyls necrosis and damping-off in the current-year seedlings (Table 2). This proved that *C. dematium* and *Cylindrocarpon* sp. are pathogenic to current-year *F. crenata* seedlings and that damping-off occurs in both the forest-edge and closed-stand plots. The results of another inoculation test conducted under two different illumination conditions showed that both fungi induced damping-off under low light intensity but not under high intensity (Fig. 2).

Field observations showed that the survival rate of *F. crenata* seedlings was substantially higher in the forest-edge plot than in the closed-stand plot (Fig. 1). Previous field studies have

also shown that *F. crenata* seedlings survive better under high illumination, which is observed mainly in gaps, and that under low light conditions, the incidence of damping-off is high (Nakashizuka, 1988; Sahashi et al., 1994). Therefore, light deficiency may result in high mortality due to damping-off. We also found that damping-off occurred under low light intensity but not under high light intensity from the inoculation test (Fig. 2), indicating that impaired carbon allocation due to low illumination decreases the resistance of hypocotyls to fungal invasion. Therefore, the survival of seedlings that grow under the forest-edge may be attributable, at least in part, to increase in the resistance to damping-off with increased illumination.

In the forest-edge plot, mortality due to damping-off occurred only in the severely damaged seedlings which had been induced by insect herbivory. This suggests that severe leaf loss that impairs carbon allocation decreases the resistance of the seedling hypocotyls to fungal invasion. The survival of a part of the severely damaged seedlings from the forest-edge plot might be attributable to the maintenance of photosynthesis in the cotyledons. However, few insect-damaged seedlings were included in this study, and the relationship between the severity of insect herbivory and seedling mortality should be studied using a greater number of seedlings.

The periderm contributes to the prevention of pathogen invasion in woody plants (Agrios, 2005; Eyles et al., 2003; Ichihara et al., 2000; Laflamme et al., 2006; Tippett and Hill, 1984). On July 6, we observed that periderm formation in hypocotyls was much more apparent in the healthy seedlings from the forest-edge plot than in those from the closed-stand plot (Fig. 3).

The periderm may have prevented fungal invasion of the seedlings from the forest-edge plot, which may account for the difference in the mortality between the two plots. Additionally, the layer of dead epidermal tissue, which formed before periderm formation may also have contributed to the prevention of fungal invasion, because this layer was denser in the seedlings from the forest-edge plot than in those from the closed-stand plot. Periderm degradation and poor development of the dead epidermal tissue layer under low light intensity may be caused by a decrease in the allocation of photosynthates to hypocotyls because photosynthesis would be decreased in the seedlings from the closed-stand plot. However, the relationship between the allocation of photosynthates and periderm formation in hypocotyls must be studied in more detail.

In general, plants resist fungal invasion by producing phenolic compounds with antifungal activity (Agrios, 2005; Grayer and Harborne, 1994). Phenolic compounds are involved in the resistance against pathogens in plant seedlings (Bonello and Pearce, 1993; Siegrist et al., 1994) and germinating seeds (Ceballos et al., 1998). The ethyl acetate phase of the methanol extract of seedling hypocotyls from both plots showed antifungal activity, and this activity was higher in the seedlings from the forest-edge plot (Fig. 6). Furthermore, the total phenol concentration on July 6, when damping-off occurred, was higher in the seedlings from the forest-edge plot than in those from the closed-stand plot (Fig. 5). Thus, seedlings in the forest-edge plot may survive by preventing pathogen invasion via phenol production. Additionally, because phenolic compounds were distributed in the outermost parts of the living tissue and in the outer part of the dead epidermal tissue layer, these compounds can

effectively impede fungi at invasion. Previous studies show that high light intensity results in an increase in the concentration of defense substances such as total phenolic compounds and tannins (Entry et al., 1991; Nichols-Orians, 1991).

We concluded that the difference in the mortality due to damping-off between conditions of high and low light intensity is influenced by both histological and chemical defense mechanisms. The degree of photosynthesis and allocation of photosynthates to hypocotyls could affect the effectiveness of the defense mechanisms against fungal invasion. Increased illumination associated with gap dynamics decreases the incidence of damping-off, which promotes seedling regeneration (Augspurger, 1984; Hood et al., 2004; Jarosz and Davelos, 1995; Sahashi et al., 1994).

Our results indicate that seedlings growing at improved light conditions associated with gap dynamics show increased resistance against fungal disease. Our study indicates that not only *C. dematium* but also *Cylindrocarpon* sp. are pathogenic and can cause damping-off in current-year *F. crenata* seedlings. Resistance against these fungi could be attributed to changes in production of phenolic compounds and in tissue structure. These changes may be very important to promote seedling regeneration. Moreover, because light conditions vary across forest floors, the relationship between light conditions to which each seedling is exposed and the mortality or resistance of the seedling should be studied associated with the resistant factors. This relationship is important for future research in order to understand one of the environmental threshold levels that determine survival of individual seedlings. In addition to light conditions, future studies need to address how other factors such as

temperature, humidity, light quality, and host genetic variation affect disease resistance of seedlings. Moreover, the antifungal substances associated with this chemical defense in hypocotyls of *F. crenata* seedlings must be identified.

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REFERENCES

ABE, M., MIGUCHI, H., HONDA, A., MAKITA, A., and NAKASHIZUKA, T. 2005. Short-term changes affecting regeneration of *Fagus crenata* after the simultaneous death of *Sasa kurilensis*. *J. Veg. Sci.* 16:49-56.

AGRIOS, G. N. 2005. Plant Pathology 5th edition. Elsevier, Burlington.

AUGSPURGER, C. K. 1984. Seedling survival of tropical tree species: Interactions of dispersal distance, light-gaps, and pathogens. *Ecology* 65:1075-1712.

BONELLO, P. and PEARCE, R. B. 1993. Biochemical defense responses in primary roots of Scots pine challenged in vitro with *Cylindrocarpon destructans*. *Plant Pathol.* 42:203-211.

CEBALLOS, L., HOSSAERT-MCKEY, M., MCKEY, D., and ANDARY, C. 1998. Rapid deployment of allelochemicals in exudates of germinating seeds of *Sesbania* (Fabaceae): roles of seed anatomy and histolocalization of polyphenolic compounds in anti-pathogen defense of seedlings. *Chemoecology* 8:141-151.

ENTRY, J. A., CORMACK JR., K., HANSEN, E. and WARING, R. 1991. Response of western coniferous seedlings to infection by *Armillaria ostoyae* under limited light and nitrogen. *Phytopathol.*. 81:89-94.

EYLES, A., DAVIES, N. W., YUAN, Z. Q. and MOHAMMED, C. 2003. Host responses to natural infection by *Cytonaema* sp. in the aerial bark of *Eucalyptus globules*. *For. Pathol.* 33:317-331.

GRAY, A. N. and SPIES, T. A. 1996. Gap size, within-gap position and canopy structure effects on conifer seedling establishment. *J. Ecol.* 84:635-645.

GRAY, A. N. and SPIES, T. A. 1997. Microsite controls on tree seedling establishment in conifer forest canopy gaps. *Ecology* 78:2458-2473.

GRAYER, R. J. and HARBORNE, J. B. 1994. A survey of antifungal compounds from higher plants, 1982-1993. *Phytochemistry.* 37:19-42.

HASHIZUME, H. and YAMAMOTO, S. 1975. Regeneration process of the Japanese beech forest (I). Seed fall and emergence and disappearance of seedlings. *Trans. Jpn. For. Soc.* 86:226-227 (in Japanese).

HOOD, L. A., SWAIDE, M. D. and MASON, P. A. 2004. The influence of spatial patterns of damping-off disease and arbuscular mycorrhizal colonization on tree seedling establishment in Ghanaian tropical forest soil. *J. Ecol.* 92:816-823.

ICHIHARA, Y., FUKUDA, K. and SUZUKI, K. 2000. The effect of periderm formation in the cortex of *Pinus thunbergii* on early invasion by the pinewood nematode. *For. Pathol.* 30: 141-148.

INGHAM, J. L. 1973. Disease resistance in higher plants, the concept of pre-infectional and post –infectional resistance. *Phytopathol. Z.* 78:314-335.

JAROSZ, A. M. and DAVELOS, A. L. 1995. Effects of disease in wild plant populations and the evolution of pathogen aggressiveness. *New Phytol.* 129:371-387.

KATSUTA, M., MORI, T. and YOKOYAMA, T. 1998. Seeds of woody plants in Japan (Angiospermae). Japan Forest Tree Breeding Association, Tokyo.

KOBAYASHI, T., SASAKI, K. and TANAKA, K. 1984. Disappearance of Japanese beech seedlings and fungi associated with the damping-off. *Trans. Jpn. For. Soc.* 95:439-440.

LAFLAMME, G., RIOUX, D., SIMARD, M., BUSSIERES, G. and MALLETT, K. 2006. Resistance of *Pinus contorta* to the European race of *Gremmeniella abietina*. *For. Pathol.* 36:83-96.

MAEDA, T. 1988. Studies on natural regeneration of Beech (*Fagus crenata* Blume). *Bull. Col. Agr., Utsunomiya Univ.* 46:1-79 (in Japanese).

NAKASHIZUKA, T. 1984. Regeneration process of climax beech (*Fagus crenata* Blum) forests Ⅳ. Gap formation. *Jpn. J. Ecol.* 3:21-35.

NAKASHIZUKA, T. 1988. Regeneration of beech (*Fagus crenata*) after the simultaneous death of undergrowing dwarf bamboo (*Sasa kurilensis*). *Ecol. Res.* 34:75-85.

NICHOLS-ORIANS, C. M. 1991. The effects of light on foliar chemistry, growth and susceptibility of seedlings of a canopy tree to an attine ant. *Oecologia* 86:552-560.

PACKER, A. and CLAY, K. 2000. Soil pathogens and spatial patterns of seedling mortality in a temperate tree. *Nature* 404:278-281.

SAHASHI, N., KUBONO, T. and SHOJI, T. 1994. Temporal occurrence of dead seedlings of Japanese beech and associated fungi. *J. Jpn For. Soc.* 76:338-345.

SAHASHI, N., KUBONO, T. and SHOJI, T. 1995. Pathogenicity of *Colletotrichum dematium* isolated from current-year beech seedlings exhibiting damping-off. *Eur. J. For. Pathol.* 25:145-151.

SIEGRIST, J., JEBLICK, W. and KAUSS, H. 1994. Defense responses in infected and elicited cucumber (*Cucumis sativus* L.) hypocotyl segments exhibiting acquired resistance. *Plant Physiol.* 105:1365-1374.

SOKAL, R. R. and ROHLF, F. J. 1995. Biometry. W. H. Freeman and Company, New York.

SUZUKI, T., YAMAZAKI, N., SADA, Y., OGUNI, I. and MORIYASU, Y. 2003. Tissue distribution and intercellular localization of catechins in tea leaves. *Biosci. Biotech. Biochem.* 67:2683-2686.

TIPPETT, J. T. and HILL, T. C. 1984. Role of periderm in resistance of *Eucalyptus marginata* roots against *Phythopthora cinnamomi*. *Eur. J. For. Pathol.* 14:431-439.

TREUTTER, D. 2006. Significance of flavonoids in plant resistance: a review. *Environ. Chem. Lett.* 4:147-157.

YAMADA, T. 2001. Defense mechanisms in the sapwood of living trees against microbial infection. *J. For. Res.* 6:127-137.

YAMAMOTO, S. 2000. Forest gap dynamics and tree regeneration. *J. For. Res.* 5:223-229.

WATERMAN, P. G. and MOLE, S. 1994. Analysis of phenolic plant metabolites. Blackwell, Oxford.

WHITMORE, T. C. 1989. Canopy gaps and the two major groups of forest trees. *Ecology* 70: 536-538.

FIGURE LEGENDS

Fig. 1 Survival rates (%) of current-year seedlings grown under two different light conditions in a natural *Fagus crenata* forest. The relative light intensities were 35.5% in forest-edge plot (open circle) and 2.6% in closed-stand plot (closed circle). *N* seedlings = 100 in forest-edge plots and *N* = 101 in closed-stand plots.

Fig. 1

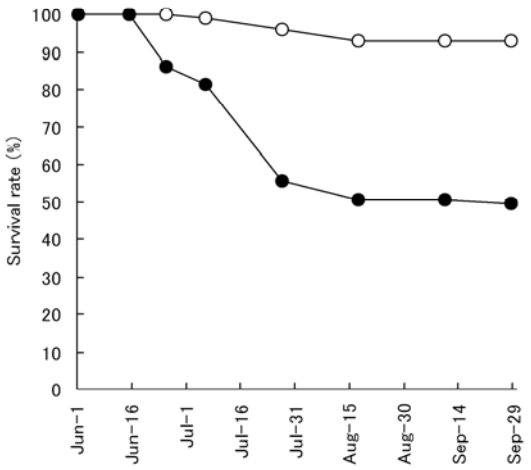

Fig. 2 Current-year *Fagus crenata* seedlings (%) with no disease symptoms (white bars), partial necrosis on hypocotyls (grey bars), and wilted ones (black bars) at different environmental conditions: high and low illumination; after inoculation with the pathogenic fungi, *Colletotrichum dematium* and *Cylindrocarpon* sp. The numbers above each bar indicates the number of seedlings in each treatment (N = 100%).

Fig. 2

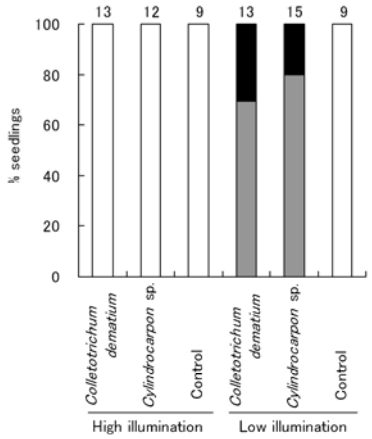

Fig. 3 Microphotographs showing cross sections of hypocotyls of current-year *Fagus crenata* seedlings collected from a natural beech forest. (a)-(d): seedlings were collected from the forest-edge plot; (e)-(h): seedlings were collected from the closed-stand plot. The seedlings were collected on June 1 (a, e), June 15 (b, f), July 6 (c, g) and September 10 (d, h), respectively. Arrows indicate periderm. Scale bars indicate 100 μm.

Fig.-3

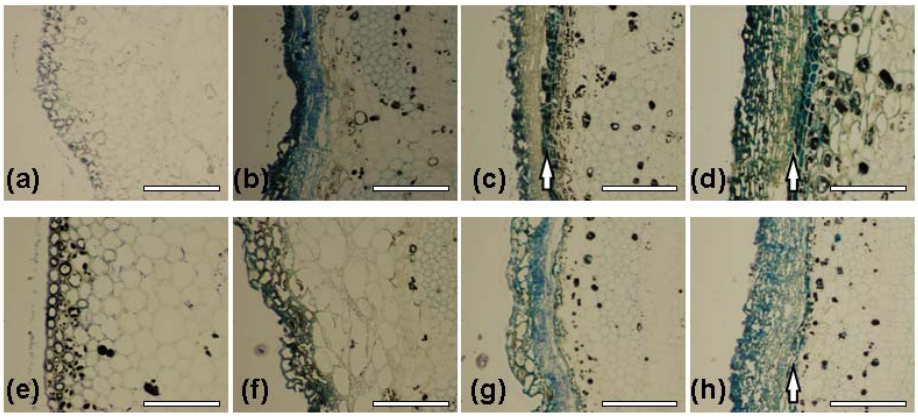

Fig. 4 Microphotographs showing Fe (II)-stained cross sections of hypocotyls of current-year *Fagus crenata* seedlings collected from the forest-edge plot in a natural beech forest. The seedlings were collected on June 1 (a), June 15 (b) and July 6 (c), respectively. Scale bars indicate 100 μm.

Fig.-4

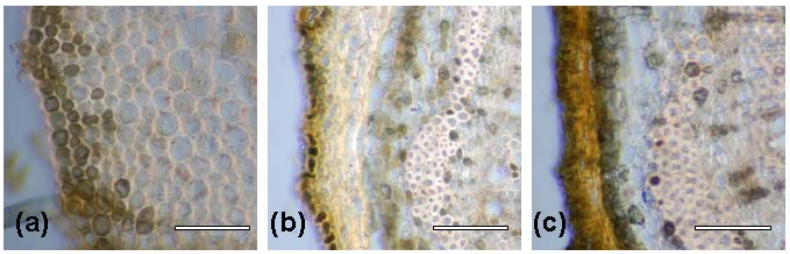

Fig. 5 Concentration of total phenolic compounds in the hypocotyls of current-year *Fagus crenata* seedlings collected from the forest-edge (open circles) and closed-stand (closed circles) plots in a natural beech forest. Data and error bars indicate means and standard errrors (N = 5). Hypocotyls were collected immediately after germination on May 28.

Fig. 5

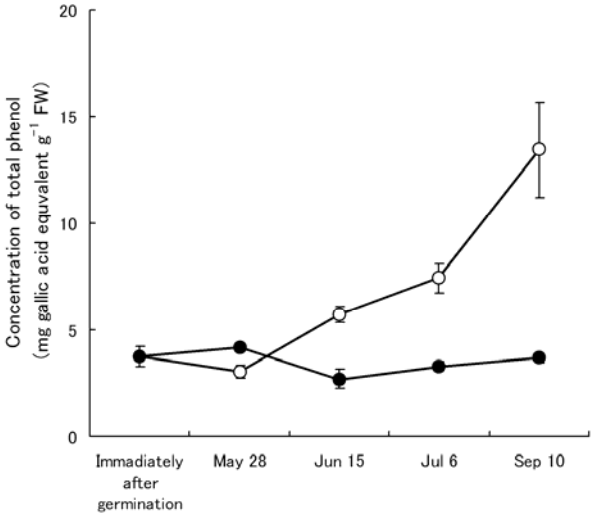

Fig. 6 Antifungal activity of the ethyl acetate phase of the methanol extracts of hypocotyls of seedlings collected from the forest-edge and closed-stand plots in a natural beech forest. The germination of *Colletotrichum dematium* spores with or without the ethyl acetate phases of hypocotyls of the seedlings from the forest-edge and closed-stand plots were observed. The bars and error bars indicate the means of the germination rate and standard errors ($N = 3$). The extracts of seedlings collected from the forest-edge and the closed-stand seedlings are represented with grey and black bars, respectively. Values marked with * are statistically different from the control (Tukey test; $P<0.01$).

Fig. 6

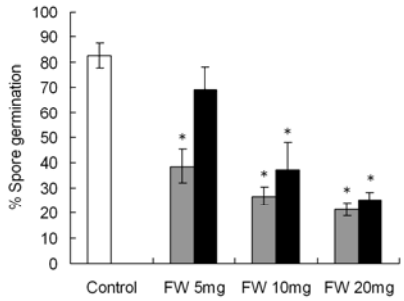

Table 1 ISOLATION RATE OF FUNGI FROM HYPOCOTYLS OF THE DEAD CURRENT-YEAR *FAGUS CRENATA* SEEDLINGS EXHIBITING DAMPING-OFF IN TWO DIFFERENT LIGHT CONDITIONS, THE FOREST-EDGE AND THE CLOSED-STAND PLOTS IN A NATURAL BEECH FOREST

Plot	Isolated fungi	25-Jun	6-Jul	27-Jul	17-Aug	28-Sept
Forest-edge	No. of seedlings	0	9	5	5	0
	Colletotrichum dematium	-	44.4	20.0	0.0	-
	Cylindrocarpon sp.	-	55.6	40.0	20.0	-
	Phomopsis sp.	-	44.4	40.0	80.0	-
	Discosia sp.	-	44.4	80.0	20.0	-
	Other fungi	-	22.2	40.0	100.0	-
Closed-stand	No. of seedlings	7	16	11	3	2
	Colletotrichum dematium	71.4	87.5	54.5	66.7	100.0
	Cylindrocarpon sp.	85.7	62.5	36.4	66.7	100.0
	Phomopsis sp.	0.0	25.0	81.8	33.3	0.0
	Discosia sp.	28.6	18.8	18.2	33.3	0.0
	Other fungi	0.0	6.3	18.2	0.0	0.0

Isolation rate (%) of each fungus was emergence rate from dead seedlings in each sampling date.

Table 2 THE PERCENTAGES OF EACH DISEASE SYMPTOM OBSERVED ON HYPOCOTYLS OF CURRENT-YEAR SEEDLINGS UNDER AN INOCULATION TEST WITH FOUR FUNGI ISOLATED FROM DEAD *FAGUS CRENATA* SEEDLINGS EXHIBITING DAMPING-OFF

Inoculated fungi	No. of seedlings	No symptom	Partial necrosis on hypocotyls	Wilted
Colletotrichum dematium	20	70.0	15.0*	15.0*
Cylindrocarpon sp.	19	31.6	63.2*	5.3*
Phomopsis sp.	23	100.0	0.0	0.0
Discosia sp.	23	100.0	0.0	0.0
Control	19	100.0	0.0	0.0

* *Inoculated fungi were re-isolated from the diseased seedlings*