Plant Biotechnology 25, 183–189 (2008)

Short Communication

Anatomical and histochemical characteristics of Japanese birch (Tohoku) plantlets infected with the *Inonotus obliquus* IO-U1 strain

Md. Mahabubur Rahman[1,2], Futoshi Ishiguri[2], Yuya Takashima[2], Mustafa Abul Kalam Azad[2], Kazuya Iizuka[2], Nobuo Yoshizawa[2], Shinso Yokota[2,*]

[1] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan; [2] Faculty of Agriculture, Utsunomiya University, Utsunomiya 321-8505, Japan
*E-mail: yokotas@cc.utsunomiya-u.ac.jp Tel: +81-28-649-5539 Fax: +81-28-649-5545

Received August 3, 2007; accepted December 20, 2007 (Edited by Y. Watanabe)

Abstract Host-pathogen interactions were investigated on a Japanese birch (*Betula platyphylla var. japonica*, Tohoku) plantlet after infection with a canker-rot fungus, the *Inonotus obliquus* IO-U1 strain. For a time-course study, intact, wounded, and infected plantlets were collected from 2 h to 30 days after treatment. Notable changes were observed morphologically in the treated portion of wounded and infected plantlets. Phenolics first deposited at the cut margin and subsequently in vessels after 4 h of infection. Their deposition extended to other xylem elements, the cortex, and the pith with an increase in the infection period. Phenolics deposition was extensive at 10 days post-inoculation (dpi), when most of the cells were entirely filled with phenolics. A necrophylactic periderm (NP) was formed at the junction of the original periderm with a layer of 2–4 new phellem cells at 30 dpi. Based on the results obtained, phenolics deposition and NP formation are considered to occur as infection-induced responses in Tohoku birch plantlets infected with *I. obliquus* IO-U1.

Key words: *Inonotus obliquus*, Japanese birch, necrophylactic periderm, phenolics, tree pathology.

Japanese white birch (*Betula platyphylla var. japonica*), which belongs to Betulaceae, makes a part of the wood biomass resource in Japan. The sap of this tree is slightly sweet and widely considered to be a healthy drink (Anetia et al. 2000). Bark extracts of birch contain some valuable compounds, namely, betulin and betulinic acid. Betulin exists in largest quantities in the bark of Japanese white birch, and it can be easily isolated from bark extracts. Betulin is used as a raw material for chemical production (Ohara et al. 1986). A white-rot fungus, *Inonotus obliquus*, the casual microorganism of canker disease of Japanese birch, is found in Honshu and northern Japan, especially in Hokkaido (Yamaguchi 1989).

Once infection with *I. obliquus* takes place, hyphae are established in the stem of the birch tree, resulting in wood decay and the formation of stem cankers when the fungus enters the tree through wounds; the wood is first altered as a result of the host response and is then infected with other microorganisms. Being well established in the wood, *I. obliquus* begins to form sterile conks around the infection court (Shigo 1969).

Phenolics are well known as defense-related compounds, and their products are induced in many types of plants after fungal infection. A clear correlation between fungal pathogenicity and phenolics deposition in plants has been reported in many studies (De Vecchi and Matta 1988; Siegrist et al. 1994; Moran 1998). Soluble and cell-bound phenolics were increased in tomato cells as infection-induced responses depending on the increase in the post-infection period (Beimen et al. 1992). The rapid deposition of phenolics as a defense reaction occurred around an infection site in soybean cotyledon tissues after infection (Graham and Graham 1991), and these phenolics led to varied levels of lignin deposition and resistance (Lagrimini 1991).

Lignification of cell walls is regarded as a key event for the induction of resistance against a pathogen attack (Vance et al. 1980; Biggs 1984). Suberized necrophylactic periderm (NP) is also directly involved in resistance to fungal infection. Several researchers have reported the formation of NP in response to pathogen infection (Biggs 1992; Simard et al. 2001; Robinson et al. 2004). The development of a lignified barrier is essential to subsequent defense responses, such as suberin deposition and reformation of a necrophylactic periderm (Biggs

Abbreviations: dpi, days post-inoculation; NP, necrophylactic periderm
This article can be found at http://www.jspcmb.jp/

1984, 1992).

There is no report available on the interactions between Japanese birch and *I. obliquus* in relation to defense mechanisms. Some studies have been reported on the wood decay of Japanese birch (Yamaguchi 1989) and several *Betula* species (True et al. 1955; Shigo 1969; Robert 1976) infected with *I. obliquus*. In this study, we observed phenolics deposition and suberized NP formation in birch plantlets infected with *I. obliquus*. The aim of this research was to clarify the defensive mechanisms of Tohoku birch plantlets infected with the *I. obliquus* IO-U1 strain.

Sterile plantlets of a Tohoku clone of Japanese birch were prepared by implanting the nodal explants in a Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 2.5 μM indole-3-butyric acid and 0.1 μM 1-naphthaleneacetic acid. Three plantlets were grown in individual 200 ml conical flasks with 75 ml of the medium. The cultures were maintained under aseptic conditions for three months prior to use in the experiments. Pathogenic cultures of the *I. obliquus* IO-U1 strain were established by subculturing in a potato-dextrose-agar medium.

The third internode from the shoot tip of a 3 month-old plantlet was cut open into a V-shape with a surgical knife. For infection by the fungus, a small plug of mycelium was placed on the entire cut. Intact and wounded sterile plantlets were used as controls. All treated plantlets were further incubated under *in vitro* conditions, and samples were collected after 2 and 4 h and 2, 10, and 30 dpi.

The samples were collected at 2 and 10 dpi and then cut transversely at the desired positions for morphological observation. Slices showing infection or injured sites of fresh samples were observed under a stereomicroscope (SZX 12, Olympus, Japan).

Internodal samples were collected and immediately fixed with an FAA (5 ml formalin, 130 ml ethanol, and 5 ml acetic acid) solution, dehydrated in an alcohol series, and then embedded in paraffin. Embedded materials were sectioned transversely at 10 μm thickness using a rotary microtome (1512, Leitz, Germany) and deparaffinized with xylene. Fungal mycelia were observed in the sections after staining with lactophenol blue (Lewandowski et al. 2006) under a light microscope (BX 51, Olympus, Japan). The sections were prepared from fixed samples at 2 and 10 dpi. For the detection of phenolics deposition, treated plantlets at 2 and 4 h and 2 and 10 dpi were used, and fresh samples from these plantlets were cut into transverse sections (20 μm thick) with a cryostat sliding microtome (Sliding microtome: Yamato Kouki Co., Japan; Cryo system: MA-101 and MA-201, Komatsu Electronics Inc., Japan). The sections were stained with a 0.08% Fast blue BB solution in 50% ethanol for 20 min (Gahan 1984). For histochemical tests to detect lignin, treated plantlets at 2 and 10 dpi were used. Transverse sections of fresh samples were stained with Wiesner and Mäule color reagents (Nakano and Meshitsuka 1992) and observed under a light microscope. The staining method of sections by the Wiesner reagent is as follows: i) dipping the section in a few drops of 1% phloroglucinol solution in 95% ethanol on a glass slide, and ii) adding 1 drop of 35% HCl. With this reagent, lignin containing coniferyl aldehyde structures achieves a purple-red color. The procedure for the Mäule test is as follows: i) immersing the section in 1% $KMnO_4$ for 5 min and washing it with distilled water three times, ii) immersing it in 3% HCl for 1 min and washing one time with distilled water, and iii) immersing it in 25% NH_4OH for 1 min, followed by washing with distilled water. The Mäule reagent stains guaiacyl lignin a dark-brown color and syringyl lignin a pinkish-red color. Non-stained sections were prepared from the fixed samples and observed under a fluorescent microscope (BX 51, Olympus, Japan) to detect lignin. Fresh samples of the treated plantlets at 30 dpi were used to detect suberized NP. Transverse sections of fresh samples were stained with a saturated Sudan III solution in 70% ethanol for 20 min and observed under a light microscope. The suberized periderm was stained a pinkish color (Biggs 1984).

When Japanese birch plantlets were only wounded, no morphological changes were observed in the sample at 2 days after the treatment. However, in the wounded samples after 10 days, plantlets produced calli in the wounded area (Figure 1). A similar tendency for callus formation was also observed in the infected samples. At both the wounded and infected plantlets, calli formed internally in the beginning, and then the callus ridge was found externally on the outside of the bark at 10 dpi. On the other hand, infected samples showed remarkable morphological changes: a brown necrotic portion was observed in xylem of both the samples at 2 and 10 dpi. Regarding the duration of the infection, a brown necrotic portion spread from the bark surface into the xylem area in the plantlets at 10 dpi (Figure 1).

Mycelia were not observed in the xylem and other tissues of the sample at 2 dpi (Figure 2A), while in the sample at 10 dpi, intercellular mycelia were observed in the cortex, ray parenchyma, and pith (Figures 2B–D).

No accumulation of phenolics was observed in intact and wounded plantlets after 2 days of the treatments (Figures 3A, B). Red to dark-brown stained phenolics was first observed in the cut margin at 2 h of infection (Figure 3C). Their deposition was also observed in vessels at 4 h after the infection (Figure 3D). The phenolics deposition was extended to the cortex, pith, and other xylem elements, such as wood fiber, with an increase in the infection period. At 2 dpi, phenolics depositions were observed in many vessels and wood

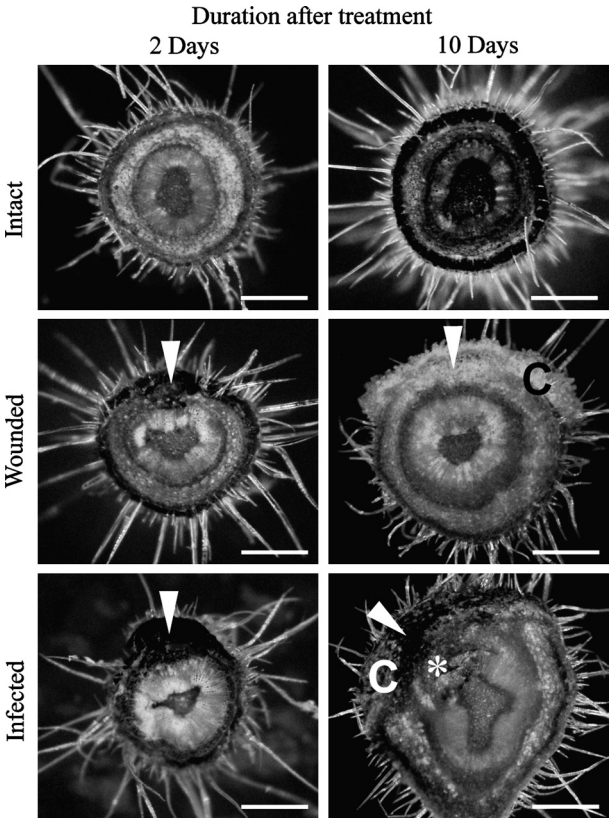

Figure 1. Stereomicroscopic photographs of fresh transverse sections of intact, wounded, and infected Japanese birch plantlets 2 and 10 days after treatment. A callus ridge (C) formed on the bark of wounded and infected internodes and brownish necrotic tissues (asterisk) grew inside the infected sample at 10 dpi. The arrowheads indicate wounded and infection sites. Bar=20 μm.

fibers (Figure 3E). Within 10 days of infection, the phenolics-deposited area extended to the cortex and pith. Large numbers of cells with phenolics deposits were observed in two-thirds of the entire transverse sections of stem (Figure 3F). In the early stage of infection (2 dpi), mycelium growth was not observed in the infected plantlets, while phenolics were deposited in a small part of the xylem at the infection site. On the other hand, at a

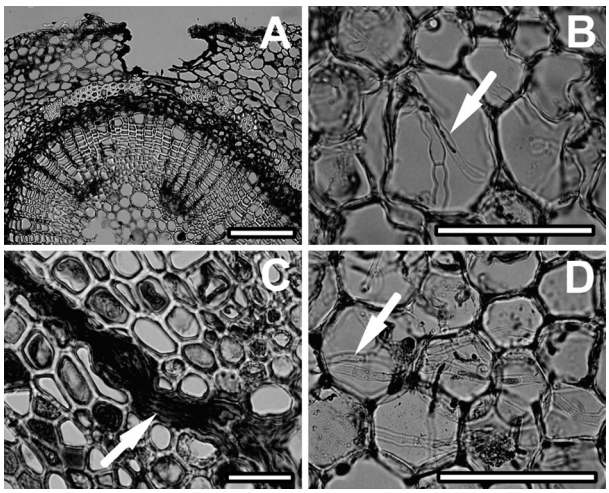

Figure 2. Fungal localization in transverse sections of Japanese birch plantlets infected with *I. obliquus* at 2 and 10 dpi after staining with lactophenol blue. (A) Fungal mycelium was not present in the section at 2 dpi. Bar=100 μm. (B–D) Localization of mycelia in the sections at 10 dpi. Bar=50 μm. (B) Infected cortex area. (C) Infected xylem ray. (D) Infected pith area. The arrows indicate the mycelium. The infection directions are on the upper sides of all photographs.

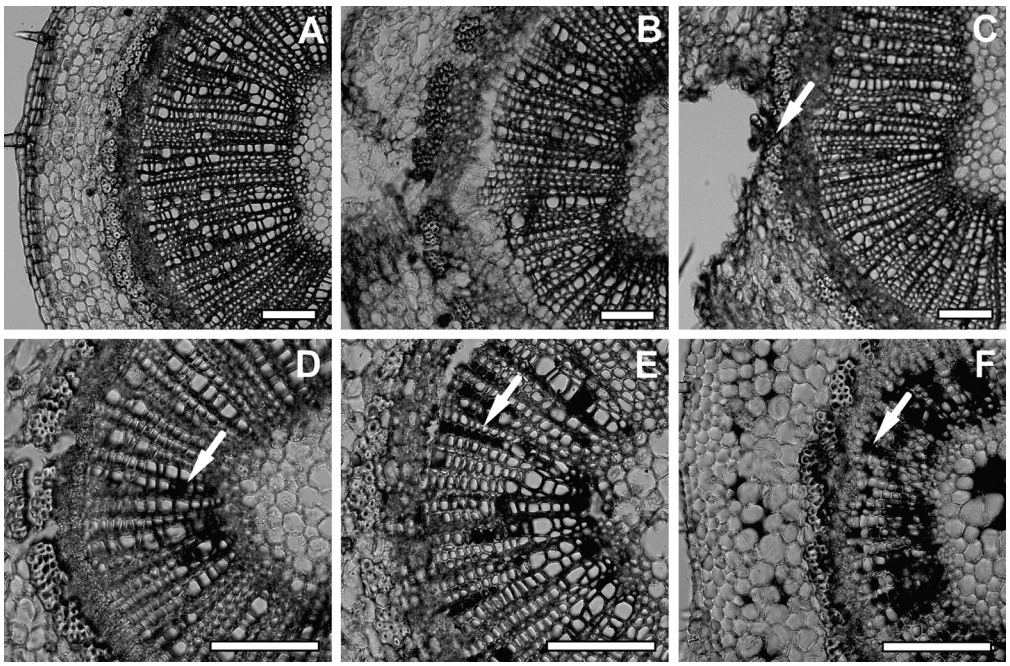

Figure 3. Phenolics localization in transverse sections of intact, wounded, and infected Japanese birch plantlets at 2 h to 10 days after the treatments. (A) Intact sample 2 days after incubation. (B) Wounded sample 2 days after treatment. (C–F) Sections of infected samples collected after 2 h (C), 4 h (D), 2 days (E), and 10 days (F). The arrows indicate cells with phenolics deposits. The wound and infection directions are on the left sides of all photographs. Bar=100 μm.

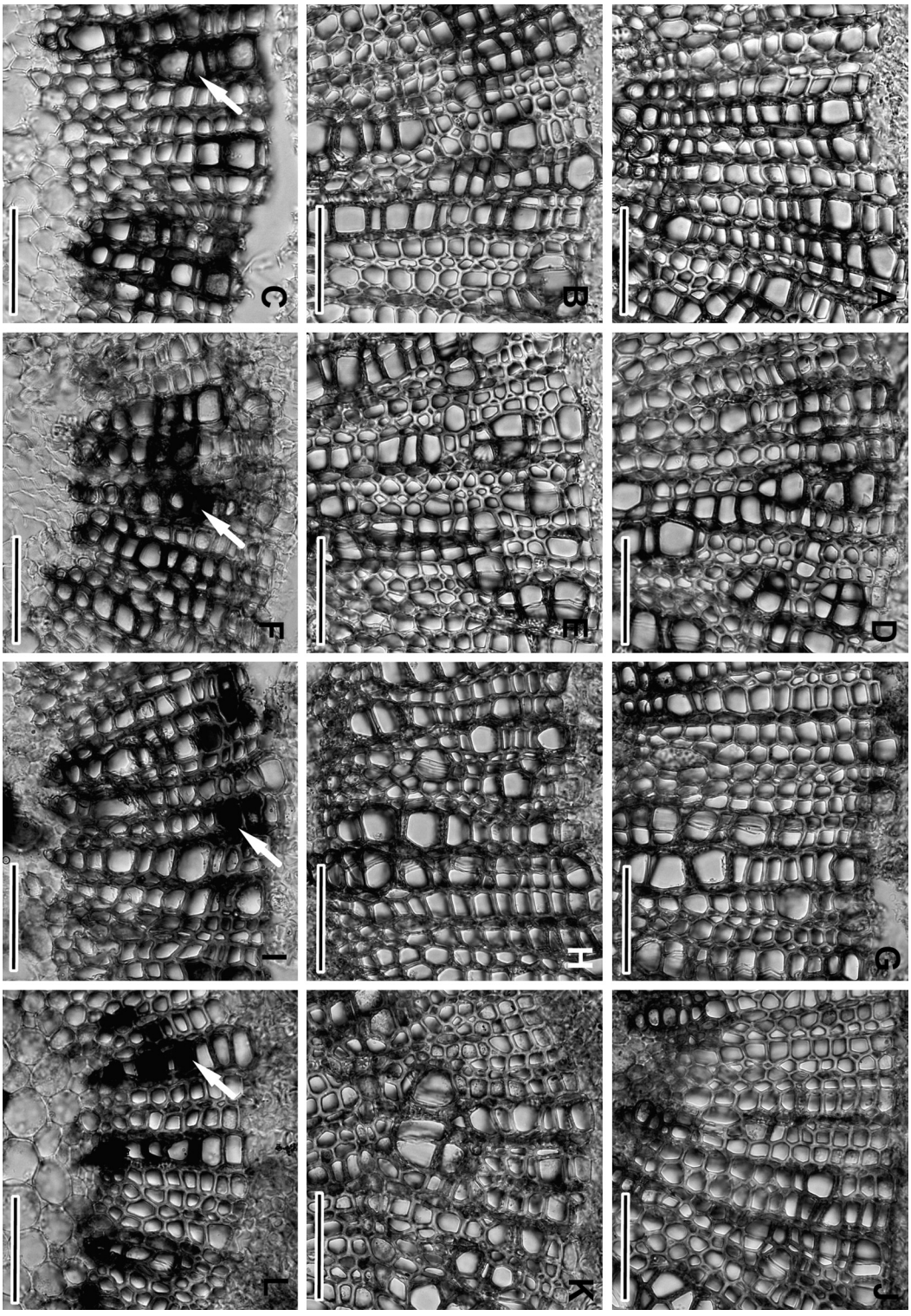

Figure 4. Detection of lignin deposition in transverse sections of treated Japanese birch plantlets. (A–F) Lignin deposition in xylem tissues stained with a Wiesner reagent at 2 and 10 days after the treatments. Lignin deposition in xylem tissues at 2 days in intact (A), wounded (B), and infected (C) samples. Lignin deposition in xylem tissues at 10 days in intact (D), wounded (E), and infected (F) samples. (G–L). Lignin deposition in xylem tissues stained with a Mäule reagent at 2 and 10 days. Lignin deposition in xylem tissues at 2 days in intact (G), wounded (H), and infected (I) samples. Lignin deposition in xylem tissues at 10 days in intact (J), wounded (K), and infected (L) samples. The arrows indicate xylem cells with phenolics deposits. The wound and infection directions are on the upper sides of all photographs. Bar=50 μm.

later stage (10 dpi), the mycelia grew extensively in plantlets and phenolics deposition was observed in most of the part at transverse sections. Hence, the phenolics deposition was enhanced with the development of mycelial growth. Phenolics deposition is considered to be closely related to a defense response against fungal infection (De Vecchi and Matta 1988; Siegrist et al. 1994). The early phenolics deposition at 2 h after fungal infection is quite notable, and the biosynthesis of antifungal phenolics might be triggered in the very early stages of infection. This trend agrees with the results of Graham and Graham (1991), who observed a rapid induction of phenolics within 4 h as a result of an elicitor treatment and maximum deposition after 24 h. It has been reported that an extensive phenolics deposition was found at a stage after fungal infection, and then the phenolics gradually disappeared with the deposition of other compounds (Moran 1998). Beimen et al. (1992) reported that soluble and cell-bound phenols increased in tomato cells as defense-related chemicals with an increase in the post-infection period. We investigated the duration for the first phenolics deposition and its increase with an increase in infection periods. However, we could not determine the duration for the maximal phenolics deposition.

When the sections were stained using Wiesner and Mäule reagents, no remarkable difference was observed at the treated site in the xylem among the intact, wounded, and infected samples at 2 and 10 days after the treatments. In the infected samples at both 2 and 10 dpi, deep-stained cells by Wiesner and Mäule reagents were rich in phenolics deposition (Figure 4). The intensity of autofluorescence from non-stained sections of intact and wounded samples at 2 and 10 days after treatments did not show remarkable differences (Figures 5A, B, D, E). The autofluorescence intensity of some vessel and wood fiber cell walls at 10 dpi was higher than that at 2 dpi (Figures 5C, F). This autofluorescence was derived from the cell walls with phenolics deposits. At 10 dpi, high phenolics deposition was observed in the xylem cell walls as an infection-induced response. In the present study, we did not find fungal growth in infected plantlets at 2 dpi. After 10 dpi, due to the presence of mycelia in the birch plantlet, intense phenolics deposition was observed in the xylem cell walls at the infection site.

Figure 6 shows transverse sections of intact, wounded, and infected plantlets that were stained with Sudan III. The original and NPs were suberized and stained a pinkish color after staining with Sudan III. The suberized original periderm of intact samples was composed of 2–3

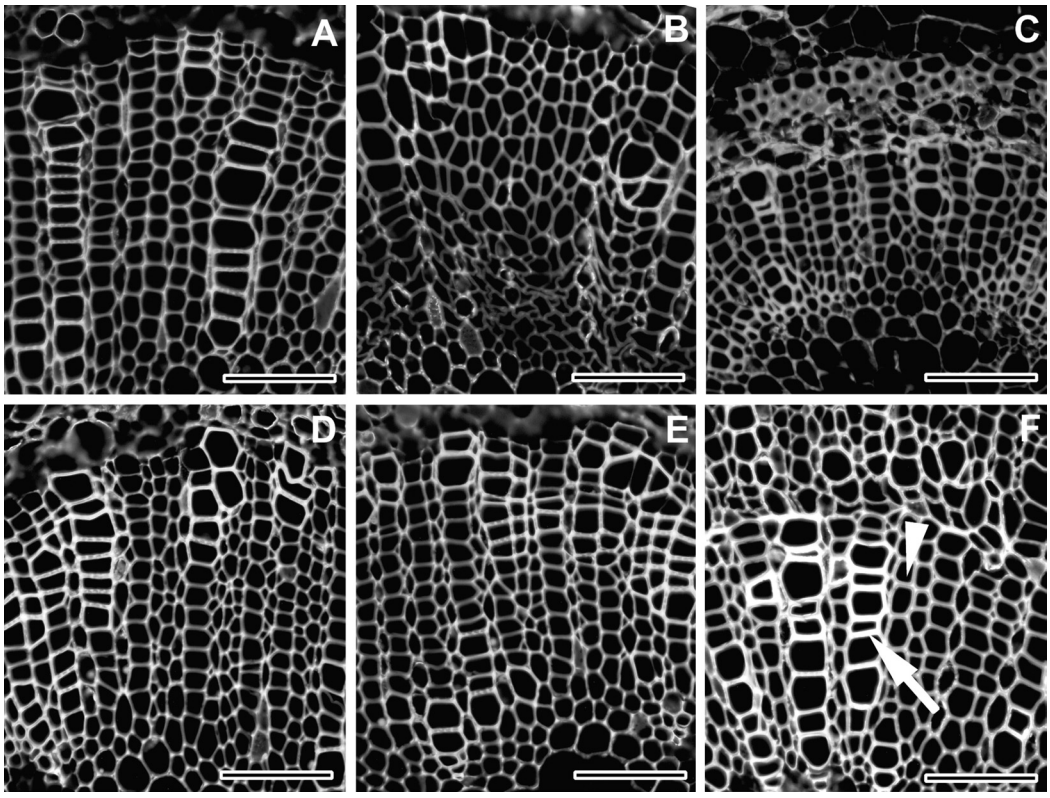

Figure 5. Detection of lignin deposition using fluorescence microscopy in transverse non-stained sections of treated Japanese birch plantlets. (A–C) fluorescence micrographs of non-stained sections of intact (A), wounded (B), and infected (C) samples at 2 days after the treatments. (D–F) fluorescence micrographs of non-stained section of intact (D), wounded (E), and infected (F) samples at 10 days. The arrow indicates a vessel with phenolics deposits, and the arrowhead indicates a wood fiber with phenolics deposits. The wound and infection directions are on the upper sides of all photographs. Bar = 50 μm.

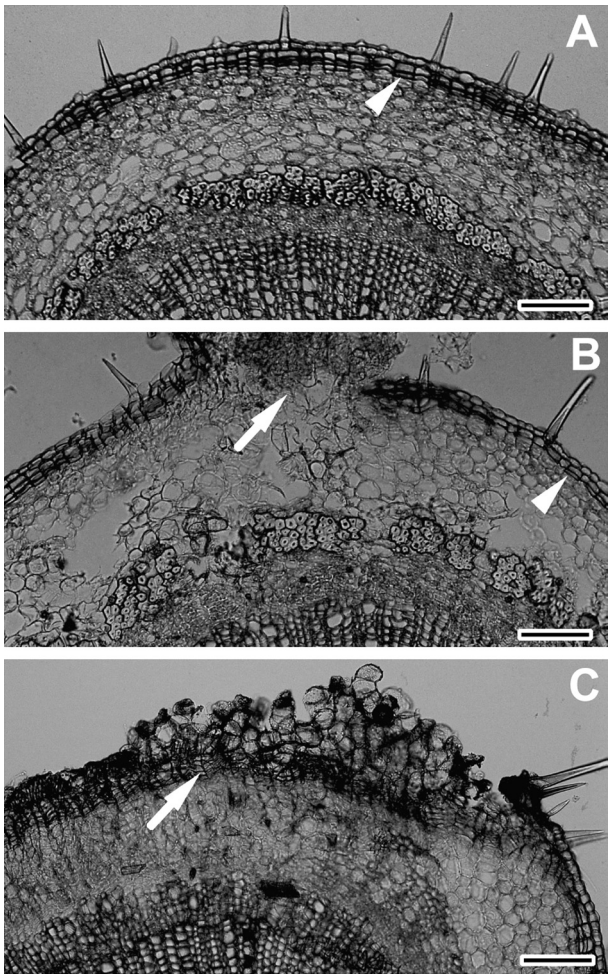

Figure 6. NP formation in the bark of infected Japanese birch plantlet at 30 dpi. Transverse sections were stained with Sudan III. (A) Original periderm (arrowheads) in an intact sample. (B) Discontinuous periderm in wounded sample (the arrow indicates a discontinuous portion, and an arrowhead indicates the original periderm). (C) NP (arrow) in infected sample. The wound and infection directions are on the upper sides of all photographs. Bar＝100 μm.

phellem cell layers (Figure 6A). In wounded samples, a discontinuous periderm layer was observed at the cut portion (Figure 6B). On the other hand, the continuous periderm layer was observed in an infected sample (Figure 6C). In this infected sample, a new NP was observed at the infected site. In addition, the number of phellem cell layers in NP (2–4 layers) at the infected portion was higher than the original periderm (2–3 layers) of intact sample. The NP development occurred as a defense response at a later infection stage against fungal infection. It has been suggested that NP formation is a response to pathogen attacks (Biggs 1984; Robinson et al. 2004). Suberin deposition occurred at the early stage of NP formation. Cells became suberized prior to the formation of phellogens and subsequent regeneration of NP. The process of NP formation might be involved in the suberization of tissues (Biggs 1984).

In conclusion, the results obtained in this study indicate that after infection of Japanese birch Tohoku plantlets with *I. obliquus* IO-U1, some defense responses occurred, such as phenolics deposition and NP formation. The activities of these defense reactions were enhanced with an increase in the infection period. This suggests that Japanese birch Tohoku plantlets possess mechanisms to inhibit the fungal growth of *I. obliquus* by means of defense reactions. It is not yet known whether these mechanisms exist universally throughout the birch plantlet population. However, the ability to show these mechanisms and other defensive responses is beneficial for birch species to overcome *I. obliquus* infection.

Acknowledgements

The Tohoku clones of *B. platyphylla var. japonica* were kindly provided by Professor Hamako Sasamoto (Division of Plant Biotechnology, Faculty of Environment and Information Sciences, Yokohama National University). The authors are also grateful to Professor Minoru Terazawa (Forest Chemistry, Forest Resource Science, Division of Environmental Resources, Graduate School of Agriculture, Hokkaido University) and Associate Professor Yutaka Tamai (Forest Resource Biology, Forest Resource Science, Division of Environmental Resources, Graduate School of Agriculture, Hokkaido University) for kindly providing the IO-U1 strain of *I. obliquus*. This work was supported in part by Grant-in-Aids for Scientific Research (C) from the Japan Society for the Promotion of Science (Nos. 15580119, 19580162).

References

Anetia M, Kojima H, Katoh Y, Uchino E, Izumi T, Hori Y (2000) Time course study of some constituents in sap of Japanese white birch. *Rep Hokka Inst Pub Health* 50: 41–46 (in Japanese)

Beimen A, Bermpohl A, Meletzus D, Eichenlaub R, Barz W (1992) Accumulation of phenolic compounds in leaves of tomato plants after infection with *Clavibacter michiganense* subsp. *Michiganense* strains differing in virulence. *Z Natureforsch* 47: 898–909

Biggs AR (1984) Boundary-zone formation in peach bark in response to wounds and *Cytospora leucostoma* infection. *Can J Bot* 62: 2814–2821

Biggs AR (1992) Anatomical and physiological responses of bark tissues to mechanical injury. In: Blanchette RA, Biggs AR (eds) *Defense Mechanisms of Woody Plants Against Fungus.* Springer-Verlag, Berlin, pp 13–40

De Vecchi L, Matta A (1988) An ultrastructural and cytochemical study of peroxidases, polyphenoloxidases and phenols in xylem of tomato plants infected with *Fusarium oxisporum* f. sp. *Lycopersici or Melonis*. *Caryologia* 42: 103–114

Gahan PB (1984) *Plant Histochemistry and Cytochemistry*. Academic Press, Inc, London

Graham MY, Graham TL (1991) Rapid accumulation of anionic peroxidases and phenolics polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *Glycinea*. *Plant Physiol* 97: 1445–1455

Lagrimini ML (1991) Wound-induced deposition of polyphenols in transgenic plants over expressing peroxidase. *Plant Physiol* 96:

577–583
Lewandowski SM, Bushenell WR, Evans CK (2006) Distribution of mycelial colonies and lesions in field-grown barley inoculated with *Fusarium graminearum.* *Phytopathol* 96: 567–581
Moran PJ (1998) Plant-mediated interactions between insects and a fungal plant pathogen and the role of plant chemical responses to infection. *Oecologia* 115: 523–530
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Plant Physiol* 15: 473–497
Nakano J, Meshitsuka G (1992) The detection of lignin. In: Lin SY, Dence CW (eds) *Methods in Lignin Chemistry.* Springer-Verlag, Berlin, Heidelberg, New York, pp 23–32
Ohara S, Yatagai M, Hayashi Y (1986) Utilization of wood extractives I. extractives from the bark of *Betula platyphylla* Sukatchev var. *japonica* Hara. *Mokuzai Gakkaishi* 32: 266–273 (in Japanese)
Robert AZ (1976) Basidiocarp development in *Inonotus obliquus* and its inhibition by stem treatments. *For Sci* 22: 431–437
Robinson RM, Jensen GD, Morrison DJ (2004) Necrophylactic periderm formation in the roots of western larch and Douglas-fir trees infected with *Armillaria ostoyae.* I. the response to abiotic wounding in non-infected roots. *For Path* 34: 105–118
Shigo AL (1969) How *Poria obliqua* and *Polyporus glomeratus* incite cankers. *Phytopathol* 59: 1164–1165
Siegrist J, Jeblich W, Kauss H (1994) Defense responses in infected and elicited cucumber (*Cucumis sativus* L.) hypocotyl segments exhibiting acquired resistance. *Plant Physiol* 105: 1365–1374
Simard M, Rioux D, Laflamme G (2001) Formation of ligno-suberized tissues in jack pine resistant to the European race of *Gremmeniella abietina.* *Phytopathol* 91: 1128–1140
True RP, Tyron EH, King JF (1955) Cankers and decays of birch associated with two *Poria* species. *J For* 53: 412–415
Vance CP, Kirk TK, Sherwood RT (1980) Lignification as a mechanism of disease resistance. *Ann Rev Phytopathol* 18: 259–288
Yamaguchi T (1989) Decay of *Betulla platyphylla* var. *japonica* caused by *Fuscoporia obliqua.* *Japanese For Hokkaido* 37: 91–93 (in Japanese)