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Xylem dysfunction in Yezo spruce (*Picea jezoensis*) after inoculation with the blue-stain fungus *Ceratocystis polonica*

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Summary

The blue-stain fungus *Ceratocystis polonica* is pathogenic to Norway spruce (*Picea abies*) in Europe, as well as to Yezo spruce (*Picea jezoensis*) and Sachalin spruce (*Picea glehnii*) in Japan. The wilting mechanism in *P. jezoensis* saplings after inoculation with *C. polonica* was examined based on anatomical studies of the phloem and xylem of periodically harvested trees. In addition, the course of sap ascent in the trunks was traced by injection of acid fuchsin solution at harvest. As an initial external symptom, needle discolouration was observed. In dye conduction tests, xylem dysfunction in the xylem of inoculated trees became obvious. The dehydrated xylem area (dry zone) had extended more than 20 cm above the inoculation wounds, within 1 month after inoculation. When the sap flow to the branches had nearly stopped, the leaves began to discolour. Hyphae of *C. polonica* colonized the ray tissue around the inoculation wounds, but were absent at the front of the dry zones. Defence reactions occurred in ray parenchyma cells adjacent to the penetrating hyphae. It is suggested that secondary metabolites, which are formed by the ray cells and epithelial cells of resin canals, are involved in the obstruction of sap flow. Limited necrotic lesions of the phloem and cambium were not associated with foliar symptoms. It is proposed that the dry zone formation caused by *C. polonica* is the main mechanism leading to tree death.

1 Introduction

The blue-stain fungus *Ceratocystis polonica* (Siemaszko) C. Moreau, associated with the spruce bark beetle *Ips typographus* L., is known to be pathogenic to Norway spruce [*Picea abies* (L.) Karst.] in Europe (Christiansen and Solheim 1990; Solheim 1992a; Kirisits 1998; Krokene and Solheim 1998b). A very similar pathosystem exists in Japan (Hokkaido), where *C. polonica* occurs on Yezo spruce [*P. jezoensis* (Sieb. et Zucc.) Carr.] and Sachalin spruce [*P. glehnii* (Fr. Schm.) Masters] (Numata 1931; Aoshima and Hayashi 1956) in association with the bark beetle *I. typographus* L. *japonicus* Niijima (Yoshida 1994). Of the 10 species of ophiostomatoid fungi isolated directly from *I. typographus japonicus* and from Yezo spruce trees infested by this insect (Yamaoka et al. 1997), at least two fungi, *C. polonica* and *Ophiostoma penicillatum* (Grosmann) Siemaszko, have the ability to kill Yezo spruce trees whose trunks have been inoculated with high doses of each of these fungi (Yamaoka et al. 2000). In most inoculation trials with *C. polonica*, fungal virulence or levels of tree resistance were assessed based on needle symptoms, tree mortality, extension of blue-stain, desiccation in the sapwood and necrotic lesions of the cambium and phloem. Only a few studies considered physiological effects of infection by *C. polonica* on the host trees (Horntvedt et al. 1983; Kirisits and Offenthaler 2002). In particular, a decrease of xylem sap flow after inoculation with *C. polonica* was reported in Norway spruce (Kirisits and Offenthaler 2002). However, the cytological and physiological processes that cause wilting in infected trees have not been studied so far.

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When microorganisms attack a tree, defence reactions like traumatic resin canal (TRC) formation and synthesis of secondary metabolites, are initiated in the host tissues (Hillis 1987). On the contrary, a series of studies on other wilt diseases of trees, such as pine wilt caused by the nematode *Bursaphelenchus xylophilus* (Stainer and Buhrer) Nickle, and a wilt disease of oak trees in Japan caused by *Raffaelea quercivora* Kubono et Shin. Ito, have provided information on the mechanisms, which induce xylem dysfunction (Kuroda et al. 1988; Kuroda 1991, 2001; Kuroda and Yamada 1996). When a host tree is unable to prevent the spread of a pathogen, the area of defence reaction expands. Dry zone and pathological heartwood that lost the ability of water conduction are known to be formed in sapwood associated with the defence reaction (Hillis 1987). The xylem dysfunction covering a large portion of trunk cross-section is fatal to the tree (Kuroda 2001). In the wilt disease caused by *C. polonica* and other vascular diseases, the size of necrotic lesions in the phloem is not related to the virulence of the causal organisms (Solheim 1988; Krokene and Solheim 1998b; Kuroda 2001).

In the present study, the process of symptom development in Yezo spruce after inoculation with *C. polonica* was studied macroscopically and microscopically with emphasis on the early period of infection. The specific aim was to characterize the mechanisms leading to wilt in Yezo spruce infected by *C. polonica*.

2 Materials and methods

2.1 Inoculation

Ten 7-year-old Yezo spruce saplings, *P. jezoensis* (height: approximately 150 cm, diameter: 30–37 mm at 20 cm above ground level), growing in the nursery of the Hokkaido Research Centre, Forestry and Forest Products Research Institute, Sapporo, Japan, were used in the experiment. A strain of *C. polonica*, YCC-115, isolated by Y. Yamaoka from a gallery wall of *I. typographus japonicus* on *P. jezoensis* in the Tokyo University Forest in Hokkaido (Yamaoka et al. 2000), was used for inoculation of the spruce trees. Prior to inoculation, the isolate was grown on malt extract agar (MEA; 20 g malt extract, 15 g agar, 1000 ml distilled water) in 9 cm Petri dishes at 20°C for 4 weeks. A sterilized bark disc (diameter 10 mm) from Yezo spruce was added to each dish to enhance the sporulation of *C. polonica*. As control inocula, 2% MEA media with bark discs were prepared.

For inoculation, bark discs (diameter 10 mm) were removed with a sterilized cork borer from tree stems at approximately 20 cm above ground level, on 21 July 1999. On each tree stem, two sets of two holes were made on opposite sides of the stem, with a 90° shift between sets of inoculation points (Fig. 1). Inocula, consisting of MEA plugs bearing mycelium and perithecia of *C. polonica*, were inserted into the wounds of eight saplings (nos 1–8; Table 1), and the bark discs were replaced. Control inocula, consisting of sterile agar were inserted into the wounds of two saplings (nos 9 and 10). After inoculation, the wounds were covered with Parafilm to prevent rapid desiccation. Low-density inoculations were given preference over mass inoculations because the aim of the present experiment was not to induce rapid wilt but to characterize the cytological reactions of the host tissues in response to fungal activities.

2.2 Macroscopic and microscopic observations

Development of external symptoms of fungal infection, i.e. needle discolouration on the specimens, was recorded at 3- or 4-day intervals for 8 weeks. Some of the inoculated specimens were harvested 2, 4 or 6 weeks after inoculation (Table 1) in order to examine them for internal symptoms of infection by *C. polonica*. For long-term observation of

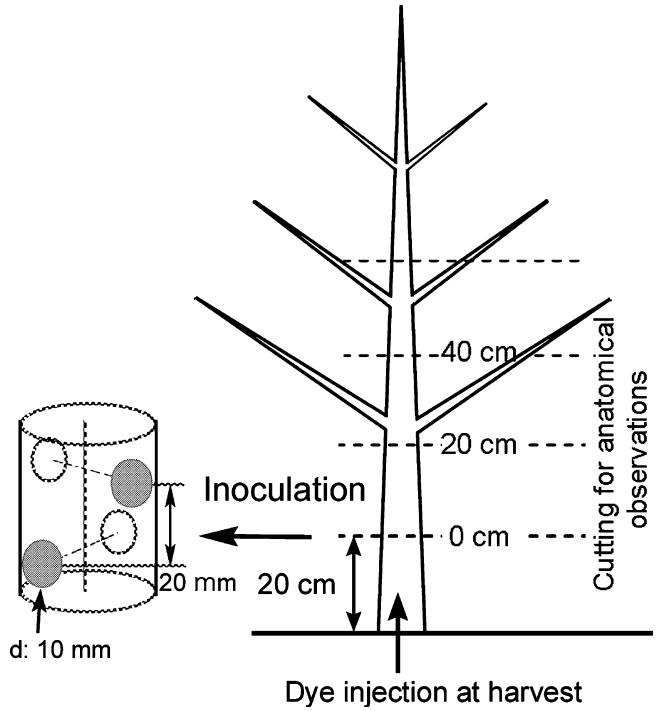


Fig. 1. Schematic diagram showing the arrangement of inoculation points on the test trees, as well as the sampling procedures for the dye injection tests and the anatomical studies on Yezo spruce trees (*Picea jezoensis*) inoculated with *Ceratocystis polonica* or sterile malt extract agar

Table 1. **Harvest schedule and experiments on 7-year-old *Picea jezoensis* saplings inoculated on 21 July 1999 with *Ceratocystis polonica* or with sterile malt extract agar**

Harvest period (date)	Tree number	External symptoms at harvest	Experiments	Figures
Periodical harvest				
2 weeks (4 August)	1	No symptom	Dye injection/anatomy	
	2	No symptom	Dye injection/anatomy	
4 weeks (18 August)	3	Resinosis	Dye injection/anatomy	2c–e,h–i and 3a–e
	4	Needle discolouration	Dye injection/anatomy	2a,f–g and 3f
6 weeks (2 September)	5	Resinosis	Anatomy	
Long-term observation during 2000	6	Shoot dieback	Macroscopic observation	
	7	Normal growth in 2000	Macroscopic observation	
	8	Normal growth in 2000	Macroscopic observation	
Controls[1]				
2 weeks (4 August)	9	No symptom	Dye injection/anatomy	2b
	10	No symptom	Macroscopic observation	

[1]Inoculated with sterile malt extract agar.

external symptoms, one control specimen and three specimens inoculated with *C. polonica* were left intact in the nursery and were checked at irregular intervals until June 2000 (Table 1).

To visualize water conduction in the main stems, a dye injection test was conducted on five specimens at harvest (Table 1). Immediately after being cut at the base, the cut ends of the main stems of the saplings were soaked in 1% aqueous acid fuchsin for 6 h in the open air (Kuroda et al. 1988). Thereafter, the main stems were cut into 20 cm pieces and then divided into shorter sections (Fig. 1). On the cut surfaces, the functional xylem area was checked for red staining with dye, and changes in the host tissue, especially necrosis, resinosis and blue-stain, were observed macroscopically. The term 'dry zone' (Hillis 1987) refers to the dysfunctional and desiccated xylem area that formed in connection with infection and was not dyed red by the acid fuchsin solution.

After macroscopic observation, specimens were processed for anatomical investigations. Stems of all harvested saplings were cut into 3–5-cm long segments and were fixed in FAA (formalin, acetic acid, 50% ethyl alcohol; 5 : 5 : 90, v/v) for 1 week and subsequently washed for 1 day under tap water. Cross-, radial- and tangential-sections, 20–25 μm thick, were made from stem blocks with a sliding microtome (Ruzin 1999). For observation of fungal hyphae and living plant cells in the phloem, cambium and xylem, some of the sections were stained with 0.05% aqueous toluidine blue-O, dehydrated with ethanol and xylene and embedded in Canada balsam on glass slides for microscopic examination (Conn 1977). Toluidine blue-O stained young hyphae, host cell walls and cytoplasm light blue or purple (Kuroda et al. 1988). To detect resinous or oily substances, other sections were stained with 1% aqueous nile blue, which is used to stain fat or fatty acid (Conn 1977), and embedded in aqueous media, Apathy's gum syrup (Arabic gum, sucrose, water; 1 : 1 : 1, w/w). Some sections were mounted onto slides without staining to observe cytological changes. The distribution of hyphae in the xylem and the anatomical reactions of the host tissues in response to fungal infection were then examined with light microscopes (Fluophot, Nikon and BX60, Olympus, Tokyo, Japan) in bright field.

3 Results

3.1 Symptom development and water conduction

As an initial external symptom following inoculation with *C. polonica*, Yezo spruce saplings showed a reddish-brown discolouration of some needles (no. 4; Fig. 2a). It started around 3 weeks after inoculation in two specimens (nos 4 and 6; Table 2). No needle discolouration was observed on the two control trees. Symptom development was not synchronous in all trees inoculated with *C. polonica* (Tables 1 and 2). In some specimens, needles on the lower branches showed yellow discolouration (nos 7 and 8) or slightly yellow-hued green (no. 5) from 3 to 5 weeks after inoculation onwards. At the time of harvest of tree No. 4, brown needle discolouration was restricted to only a portion of the branches (Fig. 2a). Extensive (nos 3 and 7) or slight (no. 5) resinosis from the inoculation wounds occurred in three of the infected trees by about 3 or 4 weeks after inoculation (Table 2). On the cross-sections of all fungus-inoculated specimens, both with and without resinosis from the trunk, a part of the xylem around the inoculation wounds, approximately 4–8 mm inwards from the cambium, was soaked with resinous substances and displayed a dark brown colour (Fig. 2d,g; R). In the control, a resinous soaking was also visible, but it was narrow and extended only approximately 2 mm inwards from the cambium (Fig. 2b).

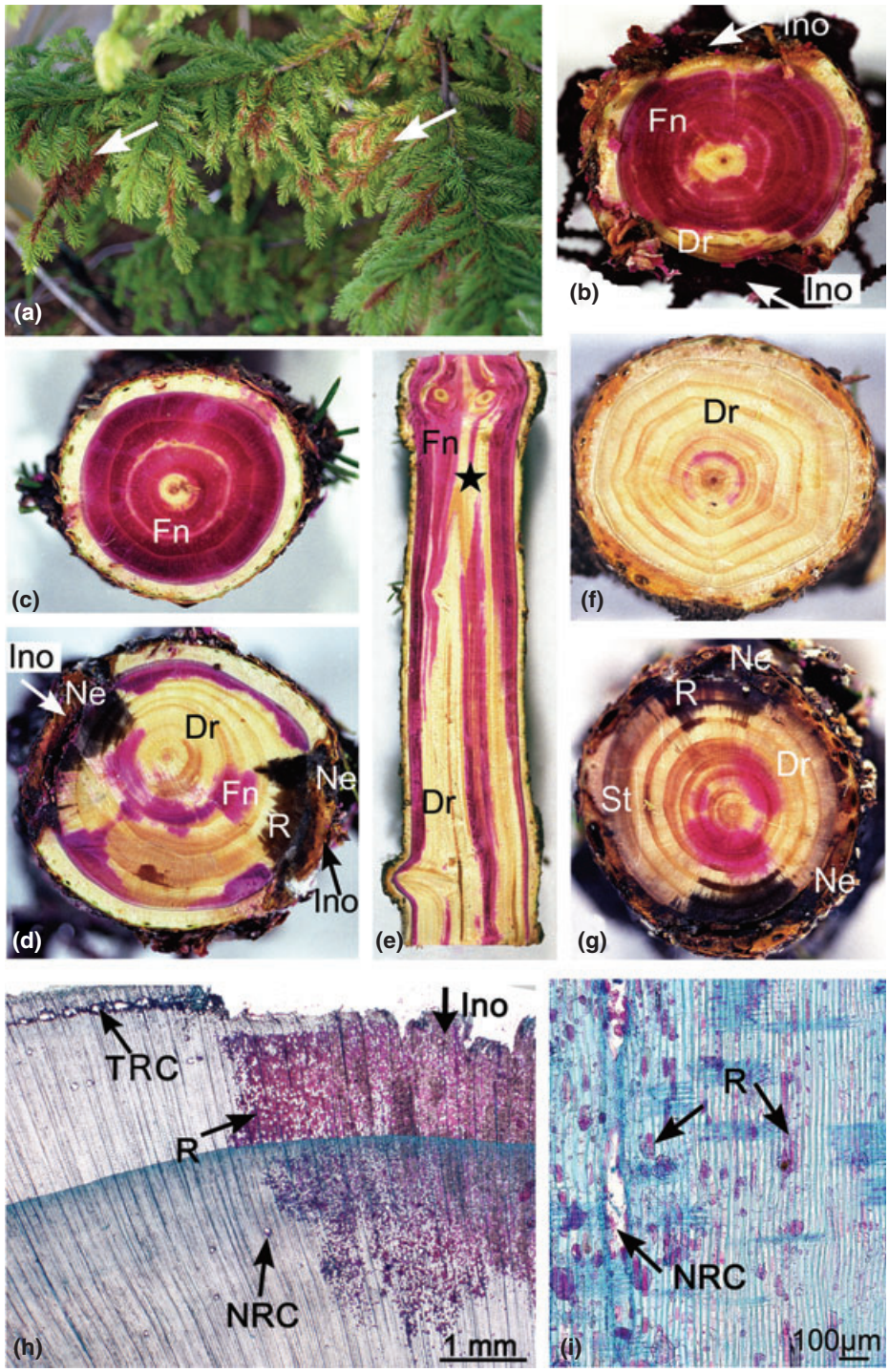
Shoot dieback was detected on May 2000 on one of the three inoculated saplings (no. 6) that had been kept in the nursery for long-term observation. Previously, this sapling had shown reddish-brown needle discolouration in August 1999. The other two *C. polonica*-

inoculated saplings, which had displayed needle yellowing in August 1999, were growing well in 2000 keeping the yellow needles and without additional symptoms. The control tree (no. 10) showed vigorous growth in 2000, too.

In specimens used for dye injection tests (Table 1), the functioning xylem area was dyed red by absorption of the acid fuchsin solution from the trunk bases just after harvest (Fig. 2b–e; Fn). In a control tree (no. 9), most of the xylem was dyed red except in narrow areas around the inoculation wounds (Fig. 2b; Ino). In the stems of specimens inoculated with *C. polonica* (nos 1–3; Tables 1 and 2), white parts unstained with dye solution (Fig. 2d–g; Dr) were noticeably wide and long before needle discolouration had occurred. Such xylem areas without dyeing were dehydrated and desiccated. The dry zone, i.e. the dehydrated xylem, extended 20–40 cm above the inoculation wounds in the specimens in which no needle discolouration had been visible by the harvest at 2 or 4 weeks after the inoculation (Fig. 2e; Dr), and the water-conducting area on the cross-sections of these saplings was reduced to less than half in the lower trunk. In these specimens, however, most parts of the cross-sectional area were dyed red in stem sections taken more than 20 cm (no. 3; Fig. 2c,e) or 40 cm (nos 1 and 2) above the inoculation wounds, and the tracheids in upper stems and branches were functional. This finding indicates that sap flow continued through the narrow, still functional zones of xylem at the inoculation heights and water was supplied to the upper branches.

In the specimen no. 4 which was harvested 4 weeks after inoculation and in which leaf discolouration had started 1 week before harvest, dyed red xylem was observed only in a very narrow area in the centre of the stem at the height of the inoculation points (Fig. 2g), and the xylem of the current annual ring was completely dysfunctional. In upper stem sections of this tree, the zone of dyed red xylem was much narrower (Fig. 2f) than in the other three saplings (nos 1–3; Fig. 2c,e), which had not displayed needle discolouration at the time of harvest. The dye was not detected more than 30 cm above the inoculation sites, which indicates that sap flow had become extremely reduced and the water supply to the branches had stopped almost completely.

Fig. 2. External and internal symptoms of fungal infection on 7-year-old Yezo spruce saplings inoculated with *Ceratocystis polonica* or sterile malt extract agar. (a) Reddish-brown discolouration of needles (arrow) 4 weeks after the inoculation on tree no. 4. (b) Functional xylem (Fn) in a control specimen (no. 9) demonstrated by red dyeing of the xylem with acid fuchsin solution except a small area of desiccation (Dr) close to the inoculation wounds (Ino). (c–e) Blockage of sap ascent indicated by the pattern of dye absorption in the main stem of tree no. 3, harvested 4 weeks after inoculation, before needle discolouration occurred. (c) Functional xylem, 40 cm above the inoculation wounds, showing normal water conduction as indicated by red dying of the xylem. (d) Wide zone of dehydrated xylem (Dr), resinosis (R) and phloem necrosis (Ne) in a stem section at the height of the inoculation sites. (e) Longitudinal extension of the dehydrated area, 'the dry zone', up to 20 cm above the inoculation wounds, as indicated by the area unstained with dye. (f and g) Extensive xylem dysfunction on tree no. 4, which showed needle discolouration at harvest, 4 weeks after inoculation (see 'a'). (f) Almost total dysfunction of the xylem (Dr) 15 cm above the inoculation site. (g) Complete xylem dysfunction (Dr) in the outer annual rings, phloem necrosis (Ne), resinosis (R) and blue-staining (St) caused by *C. polonica* at the height of the inoculation wounds. (h and i) Microscopic views of the xylem tissues around an inoculation wound on tree no. 3. (h) Crosscut section from site R, shown in (d). Resinous substances (R) which are stained purple with nile blue are visible in tracheids. Traumatic resin canals (TRC) occur in the current annual ring, in addition to preformed, 'normal', vertical resin canals (NRC) which are scattered in the xylem. (i) Enlarged radial view of the xylem with resinous substances (R) occurring in tracheids in the second annual ring from the cambium. Dr, dehydrated xylem (dry zone); Fn, functional xylem; Ino (arrow), inoculation wound; Ne, necrotic region; NRC, 'normal' vertical resin canals; R, resinous substances; St, stain caused by *C. polonica*; TRC, traumatic resin canals

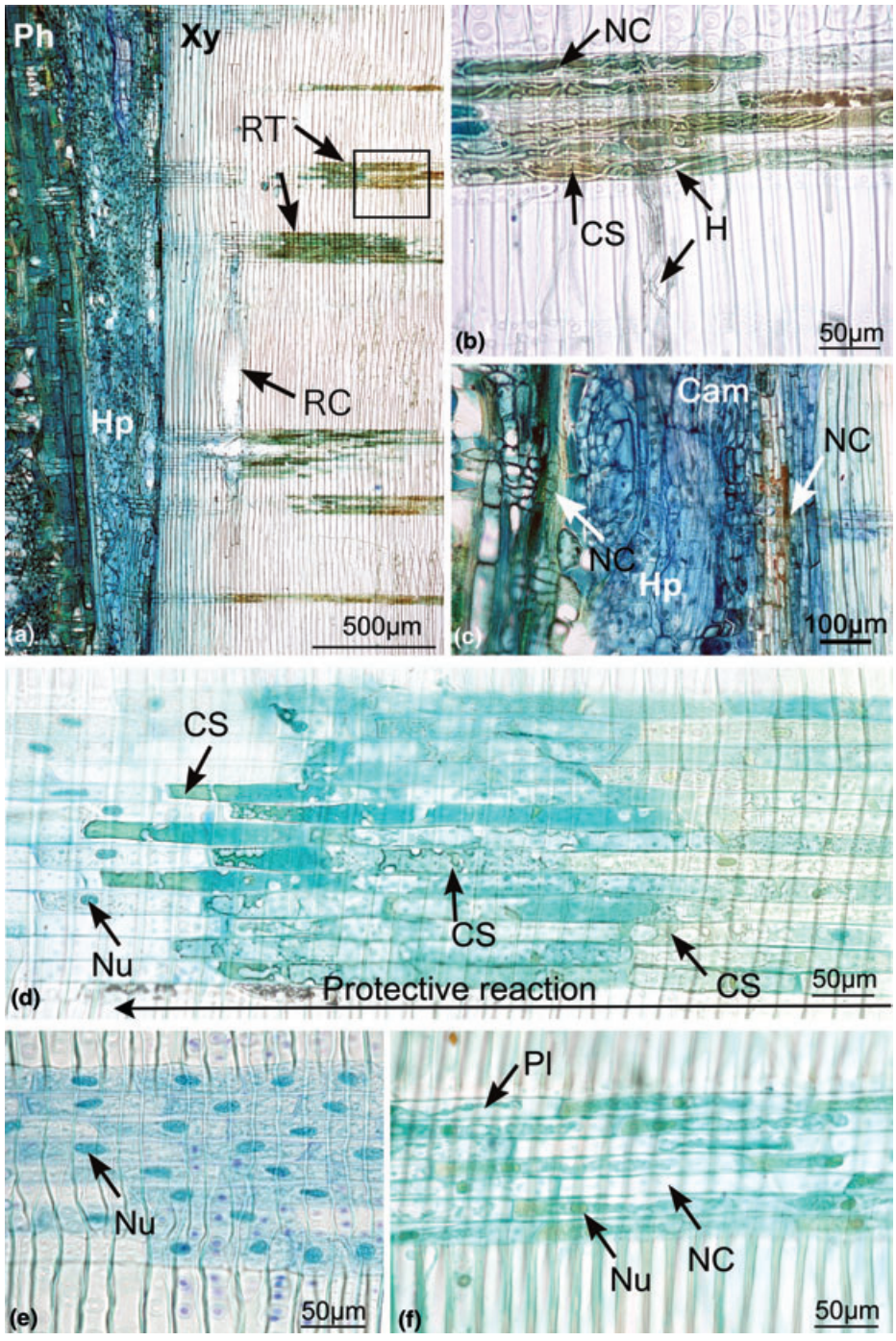

(a)
Ino
Fn
Dr
(b)
Ino
Fn
(c)
Fn
Dr
(f)
Ino
Ne
Dr
Fn
Ne
R
Ino
(d)
Fn
Dr
(e)
Ne
R
Dr
St
Ne
(g)
Ino
TRC
R
NRC
(h)
1 mm
R
NRC
(i)
100µm

3.2 Anatomical observations

Under the light microscope, TRCs were found in the xylem near the cambium in both fungus-inoculated and control specimens (Fig. 2h; TRC). They had recently formed as a defence reaction of the trees to fungal infection or wounding. In addition, 'normal', preformed vertical resin canals scattering in the xylem is characteristic to the spruce species (Fig. 2h,i; NRC). In the area soaked by resinous substances (Fig. 2d; R), oily droplets were stained purple with nile blue (Fig. 2h; R) and they filled tracheids or abundantly adhered to the tracheid walls (Fig. 2i). In the control specimen only the surfaces of inoculation wounds indicated slight resinous occlusions. This substance was water-insoluble but was removed from sections during dehydration with ethanol and xylene. Areas with resinous occlusion roughly overlapped with zones in which hyphae were present. In contrast, resinous occlusion was not associated with newly formed TRCs near the cambium. Before external symptoms had occurred, necrotic cells (NC) that looked yellowish-brown in the unstained sections, were observed in the phloem and immature xylem (Fig. 3c; NC) just around the inoculation wounds (Fig. 2d; Ne), but most of the cambial cells were living, and hyperplasia was observed (Fig. 3a,c; HP). This reaction indicates the resistance of cambial initials against the fungal hyphae.

Fungal hyphae were abundant in cross- and longitudinal-sections just around the inoculation wounds inoculated with *C. polonica*. Hyphae had penetrated radially into the xylem through ray tissues (Fig. 3a; RT) and killed ray parenchyma cells (Fig. 3b; NC). Ray cells in the control specimen except those adjacent to the inoculation wounds and those in the functional xylem of inoculated specimens (Fig. 2c–e; Fn) contained oval nuclei (Fig. 3e) and were clearly healthy and unaffected. Despite the common distribution of hyphae around the inoculation wounds, radial and vertical extension of the fungus was very limited. Before needle discolouration had occurred, in the specimens harvested 2 and 4 weeks after inoculation, the hyphae extended radially only to a depth of 4–6 mm from the cambium. In the specimen (no. 4; Fig. 2f,g) that showed needle discolouration and was harvested 4 weeks after inoculation, fungal hyphae had penetrated radially a little deeper, approximately 8 mm inwards the cambium at the inoculation height. The hyphae spread in vertical direction through tracheids and resin canals (Fig. 3b; H). During the early period of infection, 2–4 weeks after inoculation, few or no hyphae were observed beyond a distance of 20 mm above the inoculation wound. The distribution of hyphae in xylem became sparse and infrequent with the distance from inoculation wounds during this early

Fig. 3. Microscopic views of the phloem and xylem tissues highlighting the defence reactions in the ray tissues and the cambial region around the inoculation wounds of Yezo spruce saplings infected with *Ceratocystis polonica*. Radial–longitudinal sections from specimens harvested 4 weeks after inoculation, tree no. 3 without foliar symptoms (a–e) and no. 4 displaying needle discolouration (f). Sections stained with toluidine blue-O. (a) Necrosis of ray parenchyma cells (RT) invaded by fungal hyphae and hyperplasia (HP) in the cambial zone in sections sampled 12 mm above the inoculation wound. (b) Higher magnification of the area enclosed by the box in (a). Ray cells were invaded by fungal hyphae (H) and were necrotic (NC). A yellow-coloured substance (CS) was synthesized as part of the defence reactions of spruce trees against the invading hyphae. (c) Necrotic cells (NC) in the phloem and immature xylem that were coloured brown. Active cell division of cambial initials resulted in hyperplasia (Hp). Section sampled just above an inoculation wound. (d) Ray tissue in a section taken 20 mm above the inoculation wound and at the front of the elongating hyphae (from the right side of the photograph). Adjacent to the reacting cells producing secondary metabolites (CS), 'normal' cells with oval nuclei (Nu) occur in the cambial side (left side of the photograph) of the outermost annual ring. (e) Healthy ray parenchyma cells with normal oval nuclei (Nu) and cytoplasm in a section taken 25 mm above inoculation wounds; ray tissues looked identical to those occurring in control specimens. (f) Necrotic ray cells (NC) showing plasmolysis (Pl) and shrunken nuclei (Nu) in sections sampled within the dry zone, 15 mm above an inoculation wound, where no fungal hyphae were seen. Cam, cambial zone; CS, yellow-coloured substance; H, hyphae; Hp, hyperplasia; NC, necrotic cells; NRC, 'normal' vertical resin canals; Nu, nucleus; Pl, plasmolysis; RT, ray tissue; Xy, xylem


Ph
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CS
CS
Nu
Protective reaction
CS
50μm
(d)
Nu
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(e)
Pl
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NC
50μm
(f)

Table 2. **Symptom development on *Picea jezoensis* saplings inoculated with *Ceratocystis polonica***

Days	Date	Tree number: Inoculated No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	Control No.9	No.10
0	21.Jul	H	H	H	H	H	H	H	H	H	H
3	24.Jul										
7	28.Jul										
10	31.Jul										
13	4.Aug	(NS)	(NS)		NS					(NS)	
18	9.Aug				Di						
20	11.Aug						NS	NS			
23	14.Aug			NS		NS	Di	Y/R			
27	18.Aug			(R)	(Di)	R			NS		
32	23.Aug								Y		
41	2.Sep					(R)					
47	8.Sep										
54	15.Sep						Di	Y/R			
281	2.May						SD	Y	Y		NS

H: Healthy, NS: No external symptoms, Di: Brown discolouration of needles, Y: Needle yellowing, R: Resinosis from trunk, SD: Shoot dieback.
Encircled specimens: Harvested during the experiment period in 1999.

period. Around the NCs invaded by the hyphae (right side of Fig. 3d), ray parenchyma cells exhibited alterations of their contents (Fig. 3d; CS). These cells contained dense, yellowish cytoplasm or many droplets. Based on the shrunken nuclei, it was assumed that some of the cells were dead. Adjacent to these reacting cells, other ray cells and cambial cells contained normal oval nuclei (Fig. 3d; Nu). Under the microscope, such reactions were not seen in living cells of stem sections taken 25 mm or higher above the inoculation wounds (Fig. 3e). In the specimen harvested 6 weeks after inoculation, the vertical distribution of hyphae did not extend beyond 50 mm from the inoculation wounds. The area of fungal distribution in xylem was roughly overlapped with the area of resinous occlusion. In the saplings that had been harvested within 4 and 6 weeks after inoculation, hyphae were not observed near the end of the dry zone, the dehydrated xylem (Fig. 2c; star). Plasmolysis occurred in most ray cells in the dehydrated xylem areas that were not colonized by fungal hyphae (Fig. 3f; Pl).

In the inoculated specimens, longitudinal spread of the resinous smears did not extend more than 50 mm above and below the inoculation sites, and the area of extension was much smaller than that of the above-mentioned dehydrated xylem area. Following desiccation of the xylem and as external symptoms progressed, necrosis of the cambium and phloem expanded in tangential direction around the inoculation sites. Blue-stain caused by hyphae of *C. polonica* spread circumferentially and covered throughout the current annual ring, as indicated in Fig. 2g (St). In saplings harvested within 6 weeks after inoculation, the necrotic lesions in the phloem, however, did not extend longitudinally beyond 50 mm from the inoculation wounds.

4 Discussion

In the present study, the pathogenicity of *C. polonica* to Yezo spruce, which has previously been shown by Yamaoka et al. (2000), was demonstrated by the development of needle

discolouration on saplings after inoculation with this fungus. The symptom development was less intensive compared with the results by Yamaoka et al. (2000). This was probably due to the lower inoculum density in the present experiment, which had to be chosen to avoid too rapid symptom development and to make the three-dimensional analysis of the host response possible. The dye injection tests and anatomical studies indicated that sap ascent of the saplings that had been inoculated with *C. polonica* was considerably reduced or even blocked prior to the appearance of visible foliar symptom and resinosis from inoculation wounds. The rapid enlargement of the desiccated xylem in horizontal and longitudinal directions within 4 weeks after inoculation confirms the observations of Krokene and Solheim (1997) on Norway spruce. The extension of the dysfunctional xylem observed in the present experiment was similar to that reported by Yamaoka et al. (2000) after 5 or 8 weeks after the inoculation of mature Yezo spruce trees with *C. polonica*.

During the early stages of infection prior to the appearance of visible symptoms, water was supplied to the shoots of the inoculated saplings as judged from the complete dyeing of xylem on upper stem and branches. Sap flow, however, must have diminished because the water-conducting xylem area had decreased dramatically in the lower stems where the ascending water had to pass through a bottleneck of still functional sapwood. Then, a sharp decrease of the water supply to branches occurred as a result of the expansion of the dysfunctional area in the lower stems. This view is supported by sap flow measurements on Norway spruce trees that have been mass inoculated with *C. polonica*, in which sap flow in the outer sapwood decreased abruptly within the first weeks after inoculation until no sap flow was measurable 4–6 weeks after inoculation (Kirisits and Offenthaler 2002). Internal physiological processes seemed to progress similarly in young Yezo spruce and mature Norway spruce. However, on mature trees of Yezo spruce and Norway spruce, visible external symptoms occurred in various periods from 6 weeks to 1 year after the inoculation (Solheim 1992a; Yamaoka et al. 2000; Kirisits and Offenthaler 2002). The expression of foliar symptoms seems to be influenced by the dimensions of trees, inoculation densities and the environmental conditions as suggested by Krokene and Solheim (1998b). In the mature trees, it is reasonable that the foliar symptoms are delayed because needles can use water kept in the branches and a part of the thick trunk for a while after the sap flow had stopped.

Among the anatomical changes occurred in the host tissues after inoculation with *C. polonica*, resinous occlusion of the xylem, formation of wound resin canals and necrosis of cells are commonly known in spruce and pine trees infected by blue-stain fungi (Ballard et al. 1982; Christiansen 1985; Nagy et al. 2000; Krokene et al. 2003). It is also known that hyphae of blue-stain fungi use ray parenchyma tissues for their spread in the radial direction, and extend in tracheids for vertical direction as observed on lodgepole pine (Ballard et al. 1982, 1984). In the present experiment, the distribution of fungal hyphae was very restricted during the early period of infection until appearance of foliar symptoms. Anatomical investigations somewhat later (Ballard et al. 1982, 1984) give the false impression that the blue-stain fungi spread extensively in the xylem already during the initial stage of infection. The finding that fungal hyphae are not present in the margin of the dry zones at the initiation of needle discolouration is in agreement with Hobson et al. (1994) and Krokene and Solheim (1997). The present results clearly indicate that dry (desiccated) zones in the sapwood of Yezo spruce develop in advance of extensive colonization of the xylem tissues by blue-stain fungi. This lag of fungal colonization by blue-stain fungi behind the enlargement of desiccated area is detectable only by the observation of specimens during the initial stage of infection.

The presence of yellowish material and droplets in the ray parenchyma cells and in adjacent tracheids at the hyphal front suggested an accumulation of secondary metabolites. These reacting cells did not always form a continuous 'reaction zone' (Hillis 1987) but

were scattered as patches in the sapwood in the early period of infection adjacent to fungal hyphae which occurred in low densities in the host tissues. This suggests that the hyphae trigger only a localized host response. Plasmolysis of ray cells observed in the dry zone indicated that cell necrosis in xylem was also promoted by desiccation of tissues. Based on the pattern of distribution of a resinous substance in the xylem that is not associated with TRCs and is not always in contact with preformed vertical resin canals, it is suspected that ray parenchyma cells might contribute to the synthesis of the exudates in addition to epithelial cells of resin canals.

The rapid vertical extension of a dehydrated area over 20 cm within 2 weeks after inoculation suggests an embolism (cavitation) process in tracheids similar as in pine wilt disease (Kuroda 1991). A similar effect is assumed for Norway spruce infected by *C. polonica* (Krokene and Solheim 1997). According to the cohesion theory, xylem sap in water conduits is kept under strong tension during transpiration; therefore, the water columns are vulnerable, and cavitation is a likely result of any kind of disturbance in water conduits (Zimmermann 1983; Sperry and Tyree 1988). For instance, materials with a surface tension lower than water are known to reduce the resistance of xylem sap and promote embolism (Zimmermann 1983; Sperry and Tyree 1988).

Based on the present study, the following hypothesis is suggested to explain the wilting process in Yezo spruce trees caused by *C. polonica*: secondary metabolites that are synthesized in epithelium and ray cells close to the infection sites as part of a defence reaction against *C. polonica*, are released into tracheids. A resinous substance that plugs tracheids (Mathre 1964; Ballard et al. 1982) and other water-insoluble materials will become a physical obstacle to sap flow. The presence of dry zones that are wider than the areas with resinous occlusion suggest the contribution of other materials that promote cavitation in the sapwood.

Necrotic lesions in the phloem around inoculation wounds are clearly not the direct cause of needle discolouration and shoot dieback on spruce trees after infection by *C. polonica* (Krokene and Solheim 1997). The natural infection with *C. polonica* originates from numerous wounds initiated in the course of mass attacks of spruce trees by *I. typographus* (Christiansen and Solheim 1990; Solheim 1992b). At high inoculation densities, blockage of the sap flow is accelerated by the merging of dehydrated xylem areas (Horntvedt et al. 1983; Christiansen and Solheim 1990; Krokene and Solheim 1998b; Yamaoka et al. 2000; Kirisits and Offenthaler 2002). Also in other wilt diseases, the complete blockage of sap flow occurs by the mass infection of the pathogen associated with the vector beetle's mass attack (Kuroda and Yamada 1996).

Ceratocystis polonica forms larger dry zones in spruce sapwood than other, less virulent ophiostomatoid fungi (Krokene and Solheim 1997, 1998a,b; Yamaoka et al. 2000), in spite of the limited fungal distribution and necrotic lesion around the inoculation wounds during the early stage of infection. The process of wilt disease development should be interpreted by separating the main processes that actually induce the wilt of the host from other phenomena that do not contribute symptom development. In the interaction between spruce trees and *C. polonica*, the dry zone formation in the sapwood is the main process inducing the wilt symptom. As recommended by Krokene and Solheim (1997, 1998a,b) and demonstrated by the present results, the pathogenicity/virulence of blue-stain fungi should not be assessed by the length of necrotic lesions in the phloem, but by the amount of sapwood desiccation.

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Résumé

Disfonctionnement du xylème de l'Epicéa Yezo (Picea jezoensis) après inoculation avec le champignon de bleuissement Ceratocystis polonica

Le champignon de bleuissement *Ceratocystis polonica* est pathogène de l'Epicéa commun (*Picea abies*) en Europe et des Epicéas Yezo (*P. jezoensis*) et Sachalin (*P. glehnii*) au Japon. Le mécanisme de flétrissement de plants de *P. jezoensis* après inoculation de *C. polonica* a été étudié par des examens anatomiques du phloème et du xylème d'arbres prélevés à différentes dates. De plus, le trajet ascendant de la sève dans les troncs a été suivi par injection d'une solution acide de fuschine au moment du prélèvement. La décoloration des aiguilles est le premier symptôme externe observé. Les tests de conduction de colorant montrent de façon claire un disfonctionnement du xylème des arbres inoculés. La surface de xylème déshydraté (zone sèche) s'étend à plus de 20 cm au-dessus du point d'inoculation un mois après l'inoculation. Quand le flux de sève vers les branches s'interrompt, les aiguilles commencent à se décolorer. Les hyphes de *C. polonica* envahissent les rayons ligneux autour de la zone d'inoculation mais sont absents à la marge de la zone sèche. Des réactions de défense se produisent dans le parenchyme des rayons ligneux au contact des hyphes. Des métabolites secondaires formés dans les cellules des rayons et les cellules épithéliales des canaux résinifères pourraient être impliqués dans l'obstruction du flux de sève. Des petites lésions nécrotiques du phloème et du cambium ne sont pas associés aux symptômes foliaires. La formation de la zone sèche causée par *C. polonica* serait le principal mécanisme conduisant à la mort des arbres.

Zusammenfassung

Störung der Xylemfunktion bei Picea jezoensis nach Inokulation mit dem Bläuepilz Ceratocystis polonica

Der Bläuepilz *Ceratocystis polonica* ist pathogen an *Picea abies* in Europa sowie an *P. jezoensis* und *P. glehnii* in Japan. Zur Aufklärung des Welkemechanismus wurde das Phloem und das Xylem von mit *C. polonica* inokulierten *P. jezoensis* -Sämlingen zu verschiedenen Zeitpunkten anatomisch untersucht. Zusätzlich wurde der Saftfluss in den Stämmen mit der Injektion einer Fuchsinlösung dargestellt. Als erstes äusserliches Symptom wurde eine Verfärbung der Nadeln beobachtet. Mit den Färbeversuchen wurde eine Blockade des Xylems nachgewiesen. Der dehydrierte Bereich des Xylems (Trockenzone) hatte sich innerhalb eines Monats nach der Inokulation bis über 20 cm oberhalb der Inokulationswunde ausgebreitet. Wenn der Saftfluss zu den Zweigen fast vollständig unterbrochen war, begannen sich die Nadeln zu verfärben. Die Hyphen von *C. polonica* besiedelten das Holzstrahlgewebe in der Umgebung der Inokulationsstellen, waren aber an der Peripherie der Trockenzone nicht vorhanden. An der Blockade des Saftflusses sind wahrscheinlich Sekundärstoffe beteiligt, die in den Holzstrahlzellen und im Epithel der Harzkanäle synthetisiert werden. Bei begrenzten Nekrosen des Phloems und Kambiums traten keine Blattsymptome auf. Aus den Ergebnissen wird gefolgert, dass die durch *C. polonica* induzierte Bildung einer Trockenzone der zentrale Mechanismus ist, welcher zum Tod des Baumes führt.

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