Pathogenicity of three blue-stain fungi associated with the bark beetle *Ips typographus* to Norway spruce in Austria

THOMAS KIRISITS, e-mail: KIRISITS@edv1.boku.ac.at
Institute of Forest Entomology, Forest Pathology and Forest Protection
University of Agricultural Sciences Vienna
Hasenauerstraße 38
A-1190 Vienna, Austria

Received July 6, 1998

Key words: *Ascomycetes*, ophiostomatoid blue-stain fungi, *Ceratocystis polonica*, *Ophiostoma bicolor*, *Ophiostoma europhioides*, *Ips typographus*, *Picea abies*. - Pathogenicity, virulence.

Abstract: In an inoculation experiment the pathogenicity of *Ceratocystis polonica*, *Ophiostoma bicolor* and *O. europhioides*, associated with the spruce bark beetle *Ips typographus* was evaluated. All three fungi gave rise to lesions on the inoculated Norway spruce trees. However, only *C. polonica* caused obvious discoloration of the sapwood and it also killed one tree. *Ophiostoma europhioides* and *O. bicolor* displayed also some levels of virulence. These species killed considerable portions of the phloem, but did not cause discoloration of the sapwood. Thus, *C. polonica* appears to play an important role in the tree killing process following attack by *I. typographus*. The role of the other fungi associated with this insect remains questionable. This is the first report of pathogenicity of ophiostomatoid fungi associated with *Ips typographus* outside Scandinavia.

Zusammenfassung: In einem Inokulationsversuch wurde die Pathogenität der Bläuepilze *Ceratocystis polonica*, *Ophiostoma bicolor* und *O. europhioides*, die mit dem Fichtenborkenkäfer *Ips typographus* assoziiert sind, getestet. Alle drei Pilze riefen pathologische Veränderungen an den inokulierten Fichten hervor. *Ceratocystis polonica* verursachte als einzige der drei Arten eine starke Blauverfärbung des Splintholzes und brachte einen Baum zum Absterben. *Ophiostoma europhioides* und *O. bicolor* zeigten ebenfalls gewisse pathogene Eigenschaften. Diese Arten brachten beträchtliche Teile des Bastes zum Absterben, verursachten aber keine Verfärbung des Splintholzes. Aufgrund der Ergebnisse und der bisherigen Befunde in der Literatur wird angenommen, daß *C. polonica* eine große Rolle bei den Absterbevorgängen nach dem Befall durch den Buchdrucker zukommt. Ein entscheidender Beitrag der anderen Arten bei der Überwindung der Widerstandskraft der Wirtsbäume scheint dagegen eher fraglich. Diese Arbeit ist der erste Bericht über die Pathogenität ophiostomatoider Pilze, die mit *Ips typographus* assoziiert sind, außerhalb Skandinaviens.

Blue-stain fungi belonging to the genera *Ceratocystis* and *Ophiostoma*, also known as ophiostomatoid fungi, are commonly associated with bark beetles (*Coleoptera*: *Scolytidae*) (see WINGFIELD & al. 1993 for a comprehensive view). The association of bark beetles with blue-stain fungi has been known for almost a century (MÜNCH 1907). In aggressive bark beetle species, ophiostomatoid fungi are suspected to aid beetles in overwhelming defense systems of the host trees (CHRISTIANSEN & al. 1987, KROKENE 1996). This view is supported by the high virulence displayed by some of the fungal associates. Pathogenicity of bark beetle associated blue-stain fungi was first demonstrated by NELSON & BEAL (1929) and has later been shown for a variety of bark beetle/blue-stain fungal-systems (e.g. NELSON 1934, BRAMBLE & HOLST 1940, BASHAM

1970, HORNTVEDT & al. 1983, SOLHEIM & LÅNGSTRÖM 1991, YAMAOKA & al. 1995). However, the precise role of the fungi in the ecology of bark beetles is still unknown and is the source of much debate (PAINE & al. 1997).

The European spruce bark beetle, *Ips typographus* L., is a major pest of Norway spruce, *Picea abies* (L.) KARST. During outbreaks, this insect can kill millions of trees (POSTNER 1974, CHRISTIANSEN & BAKKE 1988, FÜHRER 1996). *Ips typographus* is associated with several species of blue-stain fungi (GROSMANN 1931; SIEMASZKO 1939; MATHIESEN-KÄÄRIK 1953; SOLHEIM 1986, 1992a, b, 1993a; FURNISS & al. 1990; HARDING 1989a; VIIRI & WEISSENBERG 1995; KIRISITS 1996; KROKENE & SOLHEIM 1996; YAMAOKA & al. 1997). Among the transmitted species, *Ceratocystis polonica* (SIEMASZKO) MOREAU is highly pathogenic to Norway spruce. This fungus was originally described as *Ophiostoma polonicum* by SIEMASZKO (1939) and was known as an *Ophiostoma* species until VISSER & al. (1995) recognized it as a member of *Ceratocystis* sensu stricto.

In inoculation experiments, *C. polonica* was able to kill trees in absence of the beetles (CHRISTIANSEN & HORNTVEDT 1983, HORNTVEDT & al. 1983). Other species, such as *Ophiostoma bicolor* DAVIDSON & WELLS and *O. penicillatum* (GROSMANN) SIEMASZKO, exhibited only weak virulence (CHRISTIANSEN & HORNTVEDT 1983, HORNTVEDT & al. 1983, SOLHEIM 1988, HARDING 1989b). Thus, *C. polonica* is regarded as the most important associate of *I. typographus*.

In Austria, a fungal flora similar to that in other parts of Europe is associated with *Ips typographus*. The purpose of the present study was to evaluate the relative virulence of *C. polonica*, *O. bicolor* and *O. europhioides* (WRIGHT & CAIN) SOLHEIM, which are amongst the six most frequent fungal associates of *I. typoghraphus* in Austria (KIRISITS 1996).

Materials and methods

Trees for inoculation: An inoculation experiment was performed in the experimental forest of the University of Agricultural Sciences Vienna (Austrian Federal Forests, Forest District Wr. Neustadt) 50 km south of Vienna. In a pole stand of Norway spruce mixed with European larch (*Larix decidua* MILL.) eight trees were selected for inoculation. Details of tree age and size are given in Table 1.

Fungal isolates and inoculation: The following fungi were used for the pathogenicity tests on living spruce trees: *Ceratocystis polonica* (ATCC 90965), *Ophiostoma bicolor* (CBS 436.94 = IMI 363172) and *O. europhioides* (CBS 437.94 = IMI 363174). Ten days prior to inoculation fungal isolates were transferred to plates containing malt extract agar (MEA, Sigma 2% malt extract: 1.6% agar) to produce inoculum.

On May 21st 1992, two trees each were inoculated with one of the test fungi or designated as control (Table 1). Bark plugs were removed with a cork borer (diameter 7 mm) and pieces of agar bearing fungal mycelium were inserted into the wounds before the bark plugs were put back. In the case of control trees bark plugs were put back without adding fungal inocula. Multiple inoculations were applied to each tree, following the mass inoculation technique of SOLHEIM (1988). Trees were inoculated in six rings approximately at breast heigh with rings 10 cm apart. Within the rings the inocula were placed with their edges 2 cm apart from each other. This corresponds to a dose of 4 inoculations per dm^2 bark surface.

Assessment of virulence: Five and a half months after inoculation the experimental trees were harvested. A stem section (1 to 2 m long) containing the inoculation zone was cut from each tree and transported to the laboratory. The intensity of resin outflow from the inoculation points was estimated by counting the inoculation points with resin exudation. The outer bark of the stem sections was removed and the extent of dead phloem estimated on a scale from 1 to 5. At the height of the inoculation rings, thin discs (5 mm thick) were cut from the stem sections. In addition, discs were cut above and below the inoculation belt 10 cm apart from each other in order to estimate the full extent of sapwood discoloration. Based on translucency, the sapwood/heartwood border, the desiccated areas and the blue-stained areas were marked on each stem disc. Desiccated, blue-stained and apparently fresh (translucent) sapwood was then measured using a digital planimeter. From the six discs within the inoculation belt, the one with the highest degree of desiccation or blue-staining was selected to characterize the damage caused by the fungi.

Table 1. Characteristics of the experimental trees and inoculation treatments. *Dbh* diameter at breast heigh.

Tree no.	Age	Dbh (cm)	Tree height (m)	Inoculum	Number of inoculations
1	23	9	14.5	*O. europhioides*	67
2	20	8	11.5	*O. bicolor*	58
3	26	12	15.5	*O. polonica*	90
4	31	16	16.5	Control	119
5	20	12	16.5	*O. europhioides*	88
6	20	11	12.5	*O. polonica*	78
7	18	7	11.5	Control	52
8	19	9	12.0	*O. bicolor*	69

Table 2. Average moisture content (% water/dry weight) in the outer sapwood of the experimental trees (2 measurements per tree).

Tree-no.*	Treatment	Moisture content (%)
1	*O. europhioides*	71.8 ± 6.9
2	*O. bicolor*	69.6 ± 5.2
3	*C. polonica*	29.3 ± 1.6
4	Control	141.5 ± 3.1
5	*O. europhioides*	97.5 ± 28.0
7	Control	131.9 ± 12.9
8	*O. bicolor*	62.1 ± 3.2

* Tree no. 6 died during the course of the experiment and was, therefore, not included

Between the first and the second and between the fifth and the sixth inoculation rings, stem discs (approximately 3 cm thick) were cut for re-isolation of fungi. Small pieces of wood were axenically taken from the discs and placed on MEA. Between the third and the fourth inoculation ring, an additional disc (approximately 3 cm thick) was taken. On each disc, wood blocks (2 x 1 x 3 cm) were split along a radius in order to determine the moisture content of the wood, gravimetrically. Fresh wood blocks were dried for 72 hours at 103° C and re-weighed. The moisture content was then calculated as percentage of dry weight.

Results

At the time of harvest one experimental tree (no. 6) inoculated with *C. polonica* had died. On the basis of foliage condition and colour, the remaining trees appeared to be healthy. Resin production was a common response to inoculation with the three blue-stain fungi and obvious differences between treatments were recorded (Fig. 1A). Trees inoculated with *O. europhioides* and *O. bicolor* responded more heavily than those inoculated with *C. polonica*. The response to the control treatment appeared to be stronger than the inoculation with *C. polonica*.

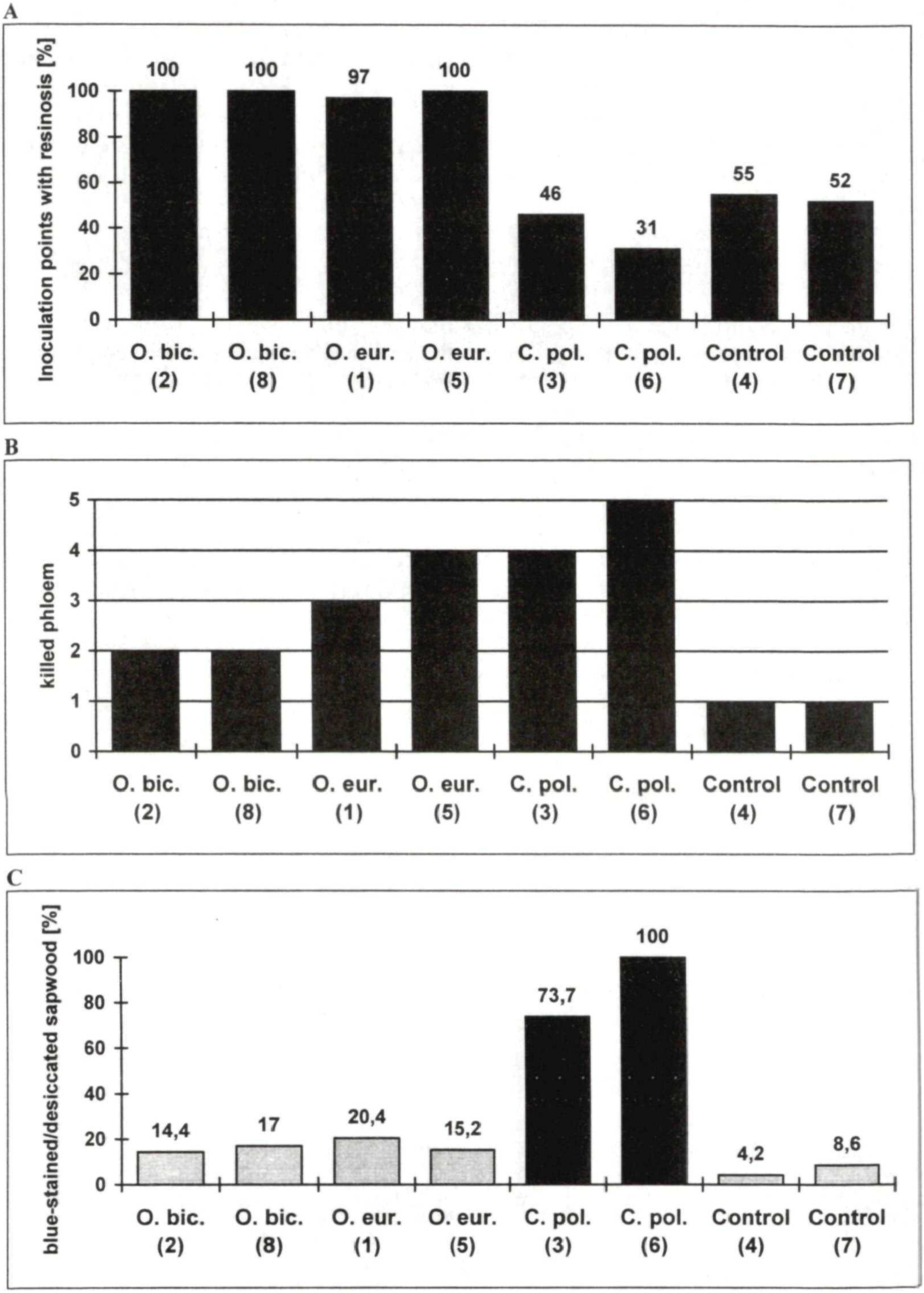
Resin-soaked wound reaction zones developed in the phloem around the inoculation points (Fig. 2). Obvious differences could be detected in the case of the phloem lesions (Fig. 1B). Wound reaction zones associated with *C. polonica* and *O. europhioides* frequently coalesced both longitudinally and tangentially and thus in trees no. 3 and 5 inoculated with *C. polonica* and *O. europhioides*, respectively, almost complete girdling of the phloem occurred. Wound reaction zones caused by *O. bicolor* were discrete and deliminated from each other. The control treatment gave rise to only small necrotic areas, restricted to a few rows of cells around the inoculation points. The differences in the wound reaction amongst treatments are clearly reflected by the amount of phloem killed within the inoculation belts (Fig. 1B).

Only *C. polonica* caused blue-stain in the wood of the experimental trees (Figs. 1C, 3). The sapwood of the killed tree (no. 6) was completely blue-stained, whereas in the second tree (no. 3) inoculated with *C. polonica*, 74% of the sapwood was discoloured. At some inoculation points on tree no. 3, resin impregnation of the sapwood was noted that might reflect a level of tree defense (Fig. 3). At other inoculation points wedge-shaped blue-stained areas developed. However, from the majority of the inoculation points the blue-stained areas had coalesced (Fig. 3). *Ceratocystis polonica* also penetrated into the sapwood in the longitudinal direction and caused substantial blue-stain below and above the inoculation belts of the trees (Fig. 4).

Ophiostoma europhioides caused only slight desiccation and did not penetrate deeply into the sapwood (Fig. 1C). The presence of *O. bicolor* in the sapwood was difficult to evaluate due to the fact that this fungus has a hyaline mycelium and does not cause obvious discoloration. A small part of the sapwood of the trees inoculated with *O. bicolor* showed signs of light brown staining and/or desiccation (Fig. 1C). The control treatment also caused a small amount of desiccation (Fig. 1C). None of the inoculations with *O. bicolor*, *O. europhioides* or the control caused desiccation or blue-staining in the sapwood beyond the inoculation belts.

All three blue-stain fungi were successfully re-isolated from the trees to which they had been inoculated. A variety of other fungi, predominantely saprophytes such as *Penicillium* spp., *Aspergillus* spp. and *Cladosporium* spp. were re-isolated from the inoculated as well as from the control trees. From tree no. 6 inoculated with *C. polonica*, which was affected by a stem rot, an unidentified basidiomycete was frequently isolated.

Fig. 1. Response of the trees inoculated with *Ips typographus* associated blue-stain fungi. *A* resinosis from inoculation points (%); *B* killed phloem within inoculation belt: 1 small necroses only, 2 less than 1/3 killed, 3 1/3 to 2/3, 4 more than 2/3, 5 all phloem killed; *C* blue-stained (black) and desiccated (gray) sapwood area. *O. bic.*, *O. eur.*, *C. pol.* Inoculation with *Ophiostoma bicolor*, *Ophiostoma europhioides* and *Ceratocystis polonica*, respectively (density: ca. four inoculations per dm^2 bark surface). *Control* Control inoculation. Numbers in parentheses refer to individual tree numbers.


A
Inoculation points with resinosis [%]
100
80
60
40
20
0
100
100
97
100
46
31
55
52
O. bic. (2)
O. bic. (8)
O. eur. (1)
O. eur. (5)
C. pol. (3)
C. pol. (6)
Control (4)
Control (7)
B
killed phloem
5
4
3
2
1
0
O. bic. (2)
O. bic. (8)
O. eur. (1)
O. eur. (5)
C. pol. (3)
C. pol. (6)
Control (4)
Control (7)
C
blue-stained/desiccated sapwood [%]
100
80
60
40
20
0
14,4
17
20,4
15,2
73,7
100
4,2
8,6
O. bic. (2)
O. bic. (8)
O. eur. (1)
O. eur. (5)
C. pol. (3)
C. pol. (6)
Control (4)
Control (7)

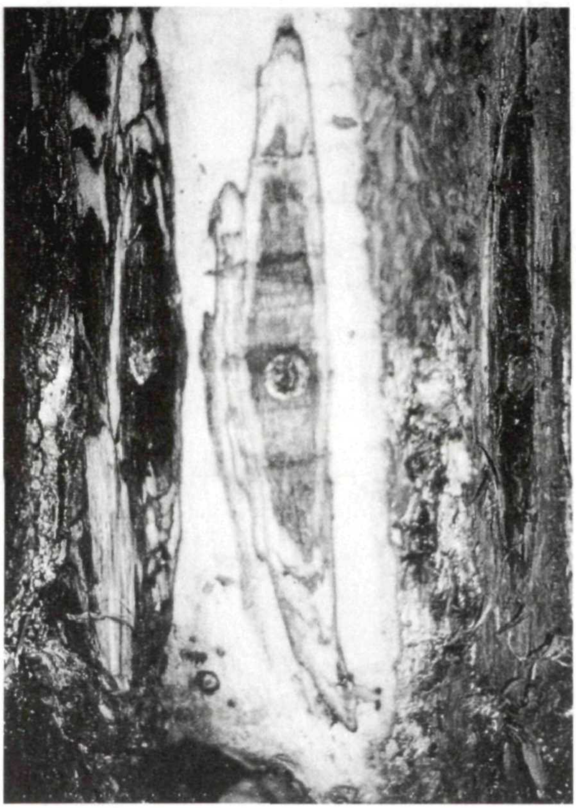
The moisture content of the outer sapwood of the trees inoculated with the blue-stain fungi was considerably reduced (Table 2). In tree no. 3 inoculated with *C. polonica* the moisture content was as low as that found in the heartwood. In this tree, the moisture content of the inner sapwood was also reduced to levels similar to the heartwood (approximately 30%). Inoculation with *O. bicolor* and *O. europhioides* caused a reduction of the moisture content only of the outer sapwood (Table 2). The control treatment caused a reduction of the moisture content of the outer sapwood, but to a lesser extent than for trees inoculated.

Discussion

The three blue-stain fungi tested in this experiment displayed varying degrees of virulence. All three species caused reactions similar to those previously described in similar inoculation experiments (CHRISTIANSEN & HORNTVEDT 1983, HORNTVEDT & al. 1983, SOLHEIM 1988, HARDING 1989b, CHRISTIANSEN & SOLHEIM 1990, 1994). Norway spruce, like many other conifers, has two lines of defense against phloem feeding insects and their associated microorganisms. Defense mechanisms can be divided in a constitutive and hypersensitive response (CHRISTIANSEN & HORNTVEDT 1983). Norway spruce possesses a well developed resin duct system in the phloem and in the wood (POLLAK 1993). When phloem feeding insects attempt to establish galleries, resin exudes from the resin ducts to stop beetle attack. If resin exudation fails to prevent beetle establishment, the tree responds with a hypersensitive wound reaction. In the tissue surrounding beetle infestations, large amounts of resin are deposited in order to arrest and kill the insects and associated fungi. Both defense systems appear to be dose dependant, and thus increasing number of attacks deplete defenses of the trees (CHRISTIANSEN 1985a, b).

When blue-stain fungi associated with bark beetles are artificially inoculated into trees, both defense reactions (constitutive and hypersensitive) of the trees are activated. In this study, inoculated trees responded with resin outflow and hypersensitive wound reaction which was a predictable reaction. However, only *C. polonica* proved to be able to cause sapwood discoloration and tree death. The second tree inoculated with *C. polonica*, which was still alive at the end of the experiment, would presumably have died, since a considerable percentage of the sapwood was disfunctional. The other two fungi killed varying portions of the phloem, but caused only slight desiccation of the sapwood. Similar to *C. polonica*, *O. europhioides* gave rise to long and broad wound reaction zones, which frequently coalesced. Tree no. 5 inoculated with *O. europhioides* was nearly girdled at various inoculation rings and would presumably have died soon after completion of this study. *O. bicolor* displayed some signs of virulence, but trees inoculated with this fungus would presumably have survived the inoculation, if the experiment had been prolonged.

The results of this study are consistent with most of the inoculation experiments performed in Scandinavia with similar fungi (CHRISTIANSEN & HORNTVEDT 1983, HORNTVEDT & al. 1983, SOLHEIM 1988, HARDING 1989b, CHRISTIANSEN & SOLHEIM 1990, 1994). This experiment confirms that *C. polonica* is apparently the only fungal associate of *I. typographus* able to cause severe discoloration and tree decline due to sapwood occlusion when artificially inoculated to Norway spruce (HORNTVEDT & al. 1983, SOLHEIM 1988). *Ceratocystis polonica* is not only pathogenic to Norway spruce, but also to other spruce species and to Douglas fir (CHRISTIANSEN & SOLHEIM 1990, 1994). As in this experiment other *I. typographus* associated blue-stain fungi, such as *O. penicillatum*, *O. bicolor* and *Graphium* spec. have not been found to be highly pathogenic to Norway spruce or Douglas fir (CHRISTIANSEN & HORNTVEDT 1983, HORNTVEDT & al. 1983, SOLHEIM 1988, CHRISTIANSEN & SOLHEIM 1990, 1994).


2

3

Fig. 2. Wound reaction zone in the phloem of Norway spruce caused by *Ceratocystis polonica*. Fig. 3. Stem disc of tree no. 3, inoculated with *Ceratocystis polonica*. Most of the sapwood is disfunctional due to the influence of the fungus inoculated. *h* heartwood, *b* blue-stained sapwood, *r* resinous reaction zone, *s* water-holdig (fresh) sapwood.

Ophiostoma europhioides displayed similar virulence to that of *C. polonica* in an inoculation experiment in Denmark (HARDING 1989b). This was, however, in contrast to the results of the present study. The results of the present study indicate, that *O. europhioides* is a relatively strong long-term pathogen of Norway spruce due to its ability to cause long and broad lesions in the phloem. In contrast to HARDING (1989b) *O. europhioides* did, however, not cause discoloration of the wood and caused only slight desiccation of the sapwood. Differences in virulence between Danish and Austrian strains of *O. europhioides* might be responsible for the conflicting results in the respective inoculation experiments.

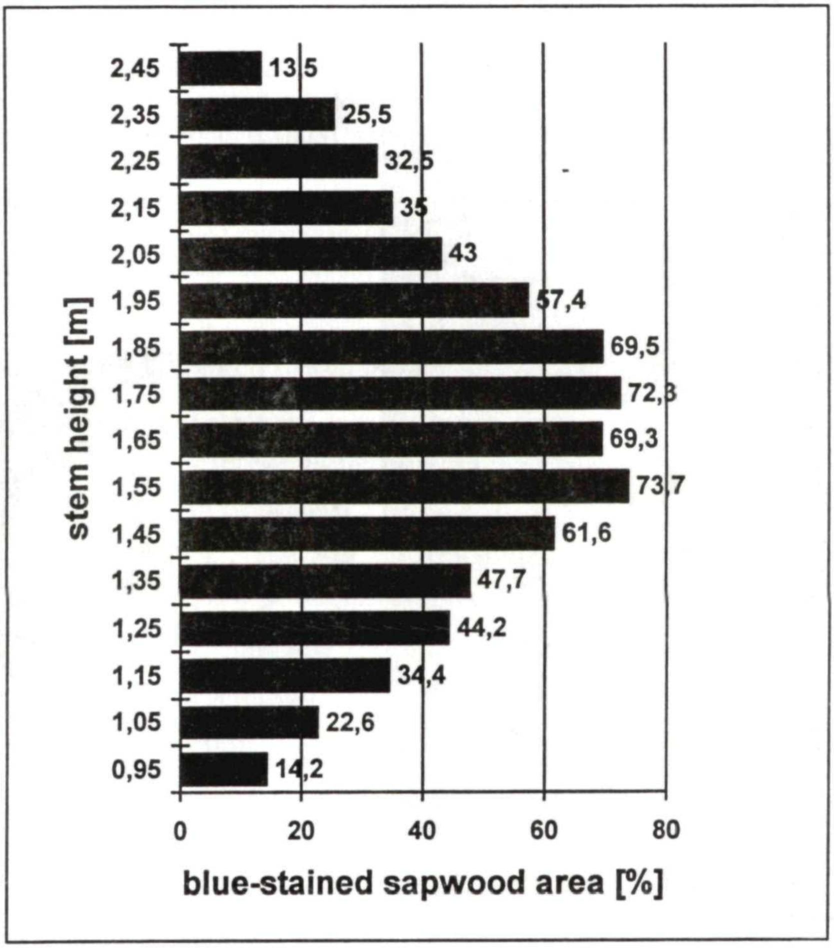


Fig. 4. Blue-stained sapwood area below, within and above the inoculation belt in tree no. 3 inoculated with *C. polonica*. The tree was inoculated at six rings ranging from 1.45 -1.95 m heigh at the stem.

Varying levels of virulence displayed by *I. typographus*-associated blue-stain fungi appear to be connected to their ability to tolerate low oxygen levels, that are found in fresh sapwood of living trees attacked by bark beetles (SOLHEIM 1991, 1993b). *Ceratocystis polonica* is well adapted to grow under conditions of oxygen deficiency, whereas other species associated with *I. typographus* (*Graphium* spec., *O. bicolor* and *O. penicillatum*) cease growth under such conditions after a few days (SOLHEIM 1991, 1993b). This enables *C. polonica* to be the primary invader of Norway spruce sapwood following attack by *I. typographus*, e.g., in south-eastern Norway (SOLHEIM 1992a, b). Apart from *C. polonica*, *O. europhioides* is not very much affected by low oxygen levels (SOLHEIM 1993b), which may explain the relatively high level of virulence reported by HARDING (1989b), and that observed in this study.

In mass inoculation experiments, fungal success is related to the inoculation dose (CHRISTIANSEN 1985a, b). In Norway, 50% of inoculated trees were killed by *C. polonica* when treated with four inoculations per square decimeter bark surface at a 60 cm long belt at the stem (CHRISTIANSEN 1985a). In the same experiment, a dose of eight inoculations per square decimeter killed all experimental trees. In the present study, trees were inoculated in six rings, which corresponds to a density of four inoculations per square decimeter bark surface within the inoculation belt. It is therefore possible that *O. bicolor* and *O. europhioides* might have displayed a higher level of virulence at higher inoculation dosages. However, the results of various inoculation experiments by a range of authors (e.g., HORNTVEDT & al. 1983, CHRISTIANSEN & HORNTVEDT 1983, SOLHEIM 1988) indicate, that *C. polonica* is the only fungal associate of *I. typographus* involved in tree killing.

It has been stated that pathogenic blue-stain fungi play an important role in tree killing by aggressive bark beetles and that they assist beetles in overwhelming host resistance (WHITNEY 1982, CHRISTIANSEN & al. 1987, KROKENE 1996). However, in the case of *I. typographus* and associated fungi, many aspects of this relationship remain more or less enigmatic. *Ceratocystis polonica*, which is the only associate of *I. typographus* that is highly pathogenic to Norway spruce, is not found in high frequency throughout the distribution range of this insect, having been reported only as a frequent associate in Poland (SIEMASZKO 1939) and Norway (SOLHEIM 1986, 1992a, b, 1993a). In other investigations in Denmark, Sweden and Finland, *C. polonica* was a minor component of the mycoflora of *I. typographus* (MATHIESEN-KÄÄRIK 1953, HARDING 1989a, VIIRI & WEISSENBERG 1995). In Austria, *C. polonica* was only frequent at one area in the western part of the country, whereas it was rarely isolated at four localities in eastern Austria (KIRISITS 1996). HARDING (1989a) and SOLHEIM (1993a) give a possible explanation for this phenomenon stating that *C. polonica* might be a minor component of the fungal flora of *I. typographus* in endemic occurrences of the insect. During severe outbreaks, when apparently healthy trees are colonized, its frequency is thought to increase. Several parts of Austria have been suffering from a severe outbreak of *I. typographus* since 1992. Currently, the fungal flora of *I. typographus* is investigated at various locations in Austria with contrasting epidemiological situations in spruce stands within and outside the natural distribution range of Norway spruce in order to examine the above mentioned hypothesis of HARDING (1989a) and SOLHEIM (1993a). These investigations as well as others in various parts of the distribution range of *I. typographus* might help to assess the possible role of associated blue-stain fungi in the ecology of this insect.

This study was part of a diploma thesis at the Institute of Forest Entomology, Forest Pathology and Forest Protection of the University of Agricultural Sciences in Vienna. I thank Prof. Dr ERWIN FÜHRER for valuable discussions and for critically reading the manuscript. I also wish to express my gratitude to Prof. Dr HALVOR SOLHEIM, Prof. Dr ERIK CHRISTIANSEN (Norwegian Forest Research Institute, Ås, Norway) and Prof. Dr MICHAEL J. WINGFIELD (Forestry and Agricultural Biotechnological Institute, Pretoria, South Africa) for inspiring and cheerful cooperation and for sharing their ideas with me. Prof. Dr MICHAEL WINGFIELD critically read the manuscript and helped to improve the language. Dipl.-Ing. CHRISTIAN AMON and PETER ZABRANSKY gave technical assistance in the field and SUSANNE KRUMBÖCK assisted in the laboratory. The manuscript was written during my employment within the Special Research Program "Forest Ecosystem Restoration" (SF008), funded by the Austrian Science Foundation and the Ministry of Agriculture and Forestry.

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