Induced wound response of Norway spruce *Picea abies* P. Karst. after artificial inoculation by imagoes of *Ips typographus* L.

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ABSTRACT: Inoculation experiments were carried out on a set of trees with imagoes of *Ips typographus* L. which origin from the Šumava Mts. and the Křtiny Training Enterprise. The objective of back inoculations was to determine whether species found on the surface of *Ips typographus* imagoes spread after the inoculation also through host tissues. It the vicinity of inoculation by *Ips typographus* imagoes, marked necrotic zones are evident including symptoms of the penetration of vascular pathogens through phloem and sapwood. The most marked reactions were observed in case of inoculation by an untreated *Ips typographus* imago. Treatment of *Ips typographus* imagoes by Ibefungin and Fundazol preparations did not demonstrate expected effects in full scale. The spores of several ophiostomoid fungi like *Ceratocystis polonica* (Siem.) C. Moreau were observed on the surface of bark beetles and at the same time were re-isolated from wounds inoculated by *Ips typographus* imagoes. The other fungi like *Ophiostoma bicolor* Davidson & Wells, *Leptographium* cf. *lundbergii* Lagerberg & Melin., *Pezicula eucrita* Karst., *Phomopsis* sp. and other were found in wounds with the imagoes artificial infection.

Keywords: ophiostomoid fungi; pathogens; vascular tissues; *Ceratocystis polonica*; *Ophiostoma bicolor*; wound reaction; vascular mycosis; *Ips typographus*

Different species of bark beetles are mostly destructive pests of coniferous boreal forests. Bark beetles kill more trees in coniferous boreal forests than all other natural factors. In the 90's, a very important bark beetle mass outbreak affected some areas in the Czech Republic. Spruce forests in the National Park of the Šumava Mts. in the border with Germany were totally disrupted due to infestation by *Ips typographus* L. This outbreak was accompanied by marked blue staining of sapwood around bark beetle galleries which is caused by several kinds of ophiostomoid fungi transmitted by bark beetles as well. *I. typographus* vectors several blue-stain fungi, spores of which are carried both externally and internally (Furniss et al. 1990). The role of these fungi in tree-killing has been discussed for a long time.

Almost all of the bark beetles carry on their skeleton, especially on pronotum and abdomen or in the gut spores of some potentially pathogenic fungi (Beaver 1989). Some of bark beetles transported spores in special mycetangia formed for the preservation of spores. Description of the mycetangia of some so-called ambrosia beetles has been given by several authors (Francke-Grosmann 1956, 1967; Fisher et al. 1953). Although the most destructive bark beetle of spruce stands *Ips typographus* L. is not classed as ambrosia beetle, its skeleton is rich in spores including ophiostomoid fungi and, therefore, this species is classed as semi-ambrosia beetle.

In recent years, in connection with cambioxylophagous insect the role of pathogens of vascular tissues (Mrkva, Jankovský 1996) is increasingly discussed in relation to tree decline. Pathogenic aspects of fungi of the associated mycoflora of *Ips typographus* L. is mentioned e.g. by Christiansen (1985a,b), Christiansen and Solheim (1994), Horntvedt and Solheim (1991), Krokene and Solheim (1997, 1998), Jankovský et al. (1998), Jankovský and Mrkva (1997).

In the Czech Republic, Kotýnková-Sychrová (1966) studied the mycoflora of some bark beetles including *Ips typographus* L. Jankovský and Mrkva (1997) demonstrated the occurrence of spores of ophiostomoid fungi including *Ceratocystis polonica* (Siem.) C. Moreau (= *Ophiostoma polonicum* Siem.) and *Ophiostoma bicolor* Davidson & Wells on the body of *Ips typographus* L. and *Ips duplicatus* Sahlb.

The author is grateful for a support through Ministry of Education, Youth and Sports of the Czech Republic No. MSM 434100005 Projects.

Table 1. The characteristic of examined trees at stand No. odd 628 A3, forest district Podlesí, Communal Forest of Brno City

No.	Social position	d.b.h.	*h*	Buttress breaking
118	dominant	17	18	–
117	dominant	17	21	+
119	dominant	16	20	–
113	dominant	17	19	–
115	dominant	13	19	+
112	dominant	17	23	–
111	dominant	15	19	+
128	dominant	15	18	–
116	dominant	16	20	+
129	dominant	16	20	+
130	subdominant	11	15	+
114	subdominant	12	14	–
122	subdominant	15	16	+
121	subdominant	13	15	–
120	subdominant	12	16	+
123	subdominant	12	14	+
124	subdominant	13	15	–
125	subdominant	13	16	–
126	subdominant	12	17	+
127	subdominant	11	16	–

The aim of the project was to study the response of tissues of a host species in the vicinity of artificial infection of spruce bark beetle as vector of pathogen of vascular tissues and to carry out the back isolation of fungi from the tissues of a host species as well as mycoflora of bark and wood of Norway spruce at experimental back inoculations.

MATERIAL AND METHODS

Presence of spores on the surface of *Ips typographus* L. imagoes

The occurrence of spores was studied on the body surface of newly emerged beetles by means of a scanning electron microscope, magnification 60–6,000×. The spores were best evident under 3,600× magnification. Spores were also determined using a microscope in skims from the body surface of beetles.

Infection experiments and wound reactions of trees

Wound responses of spruce were studied on the set of 28 Norway spruce at the Podlesí near Kuřim locality, about 15 km north of Brno in 1997–1999. The age of stand was about 30 years.

In part of trees, drought stress was simulated breaking one of buttresses. Differences in responses were also studied in dominant and subdominant trees. Newly caught and killed *Ips typographus* L. were used as inocula. In 1997–1998, imagoes origin from the Drahany Highland, in 1999 imagoes from the Šumava Mts. were used. Experiments were conducted from middle August to October. For the purpose of control, imagoes were also treated using fungicide Fundazol and a biological preparation Ibefungin on the basis of *Bacillus subtilis* (Ehrenb.) Cohn.

Imagoes were put into sapwood under a circular hole perforated by a punch 10 mm in diameter in bark and phloem. After two months, bark with phloem was removed in the vicinity of inoculation by means of a punch 50 mm in diameter. Intensity of the reaction was measured according to axial axes from the centre of inoculation.

Re-isolation of fungi from wound

The mycoflora of tissues from re-isolations was studied at two localities in the neighbourhood of Brno in the area of

Table 2. Overview of studied samples

Sample No.	Date	Locality	Material sampled
3	19 Oct. 1999	area near Blansko, a trial plot by Klepačov	6 samples of wood and bark from inoculations
4	12 Nov. 1999	Podlesí near Kuřim, back isolation from tissues in the vicinity of infection	10 samples of wood and bark from inoculations

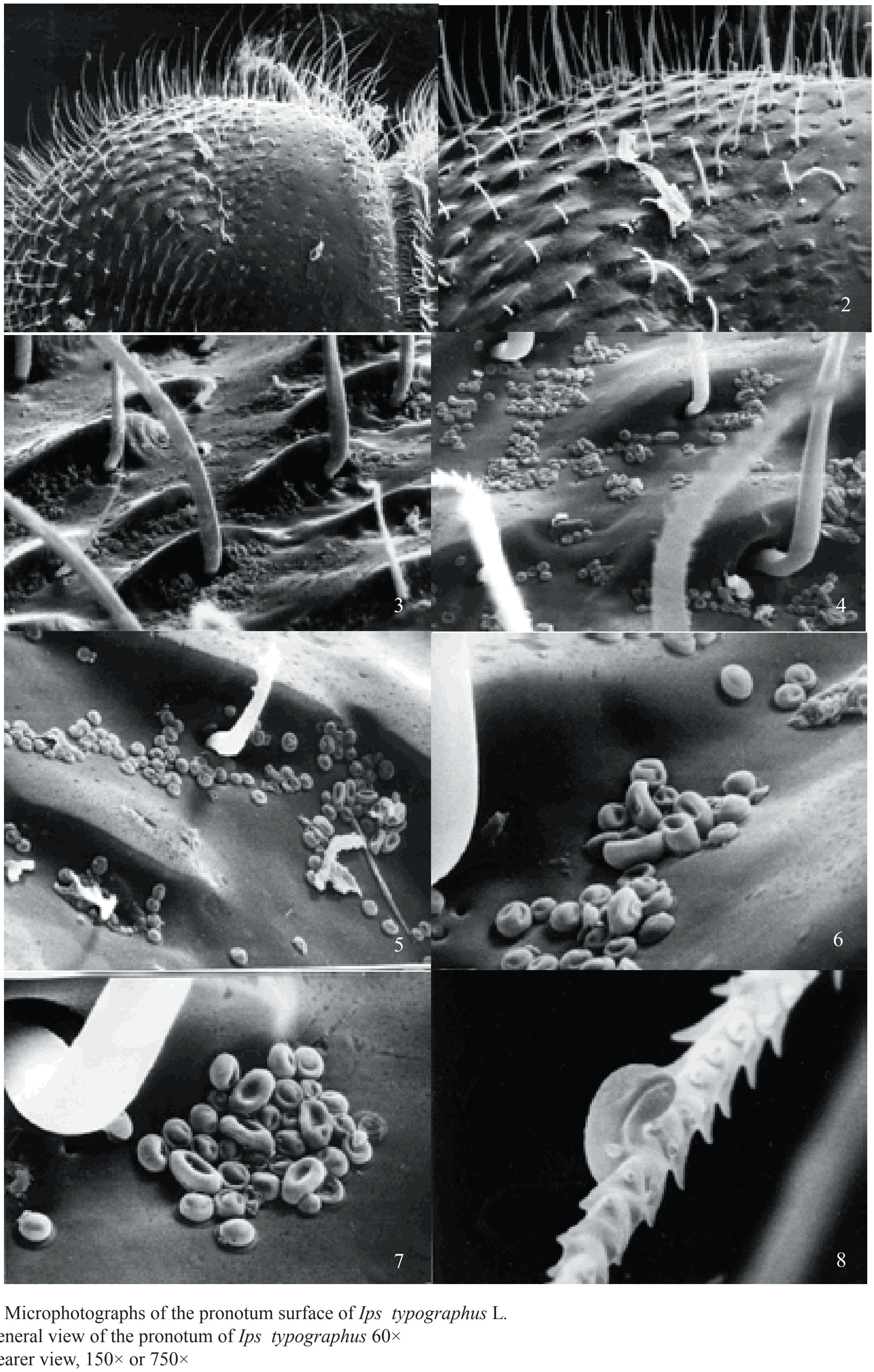


Plate 1. Microphotographs of the pronotum surface of *Ips typographus* L.
1. General view of the pronotum of *Ips typographus* 60×
2, 3. Nearer view, 150× or 750×
4–5. Tiny formations on the surface are ascospores of the genus *Ophiostoma* and conidia of various species, magnification 1,500×
6.–7. Nearer view of the group of spores, oval formations are ascospores of *Ceratocystis polonica* oval more undetermined conidiospores
8. A stuck spore on a seta protruding from the bark beetle body
(Original L. Jankovský, photo J. Lhotecký, 1996)

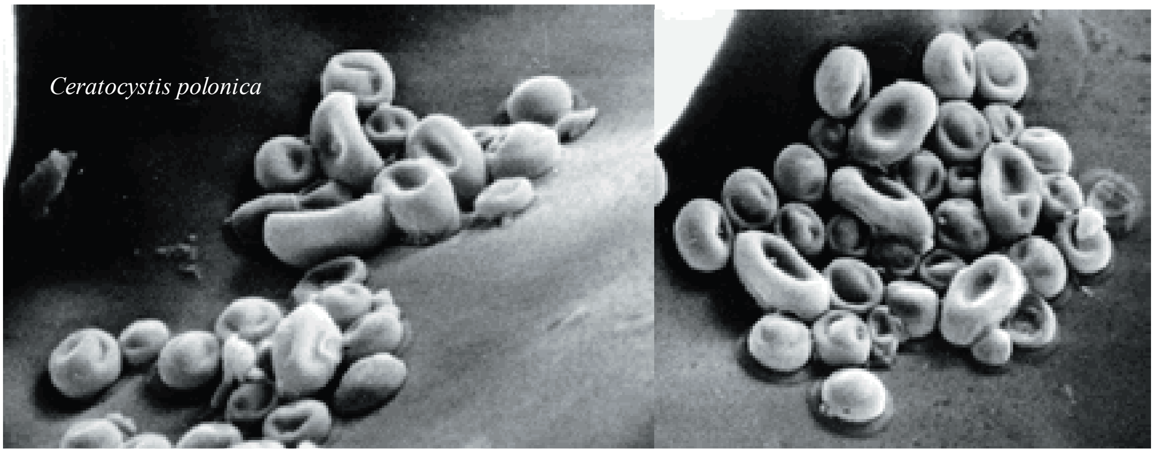


Plate 2. Ascospores of *Ceratocystis polonica* on the pronotum of *Ips typographus*. Magnification 3,600× (Original L. Jankovský, photo J. Lhotecký, 1996)

Drahany Highland at the Křtiny Training Forest Enterprise near town Blansko, 25 km to north from Brno and in spruce stand by Podlesí near Kuřim, 10 km to west from Brno. Split or rollers of wood were taken. Rollers of a diameter of 8 mm and height 2–4 mm were taken using a punch from the vicinity of places of artificial inoculation by spruce bark beetle.

A so-called endophytic method was used during the study when the small piece of wood and phloem of spruce was sterilized (for 1 minute by 96% ethanol, for 3 minutes by NaClO, for 0.5 minutes by 96% ethanol) and cut into split of a size of 3–5 × 3–5 × 1–2 mm. These split were placed at five into 90-mm Petri dishes with 2% malt extract agar (MA2). Incubation took 14–21 days at a temperature of 20–25°C.

RESULTS

Presence of the spores on the surface of imagoes

Electron microscopy photographs corroborated the presence of spores of fungi on the surface of studied *Ips typographus* L. imagoes which is of use as a vector (Plate 1). Pit-type mycetangia known e.g. from the pronotum and wing-cases of ambrosia beetles of the genus *Platypus* were not found. The function of the mycetangia is, however, taken over very well by pits in the vicinity of setae and depressions on the pronotum.

Rounded formations corresponding to conidia were abundant. Elongated oval or finely crooked ascospores of the genus *Ophiostoma* were also commonly present. According to the size of spores and their form, three representatives of the genus *Ophiostoma* were distinguished. *Ceratocystis polonica* (Siem.) C. Moreau was a common species, some spores under study corresponded to *Ophiostoma penicillatum* (Grosmann) Siemaszko and *Ophiostoma bicolor* Davidson & Wells (Plate 2).

In newly emerged beetles, there is the smallest pollution of the body surface by detritus and spores are best evident. There, the possibility of subsequent secondary infection after emergence is also excluded. The pronotum surface is furrowed by pits while setae grow from them. Clusters of spores are just evident in these hollows. Characteristic, finely crooked spores are individually stuck on prickly setae protruded from the pronotum.

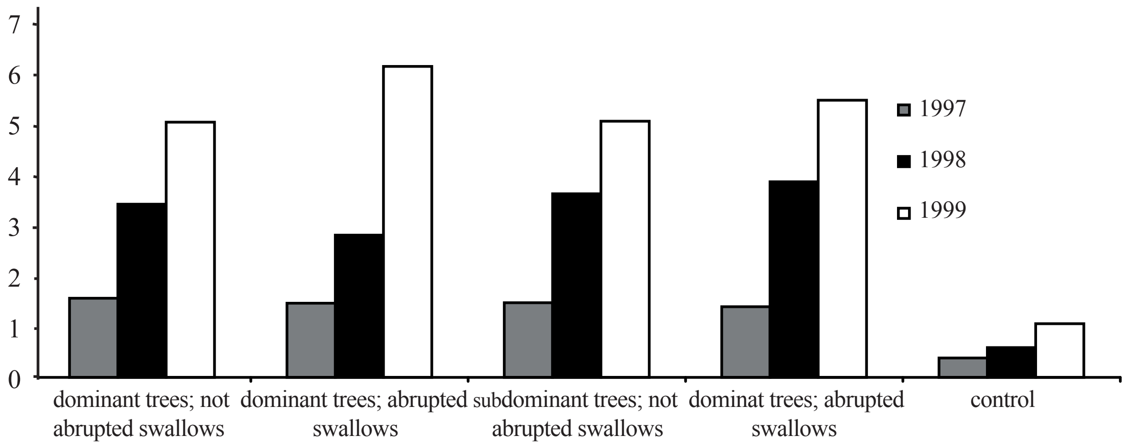


Fig. 1. Graphical of summarized average reactions in 1997, 1998 and 1999

 J. FOR. SCI., *49*, 2003 (9): 403–411

Reaction to infection experiments with *Ips typographus* L. imagoes

In trees inoculated by spruce bark beetle, elliptical brown zones were observed in the vicinity of infection places, i.e. 'wound reactions' elongated in the direction of transpiration and assimilatory flux. In control trees, weak reaction only was observed in the vicinity of wounds. Dark-coloured brown zones spreading in the vicinity of infection even grey-blue colouring of sapwood were not observed.

As compared with control, a marked reaction zone occurred around places of inoculation 1.1–1.5 cm wide (measured from the centre of a wound) and 2.3–3 cm long (Fig. 2). In the control, these values did not differ from the oval slit. In some trees, an extreme reaction was observed associated with blue-staining of sapwood to a depth of 5–12 mm (Plate 3). In this case, *Leptographium* cf. *lundbergii* Lagerberg & Melin. was determined particularly from this type of wound. Thus, it is possible to confirm that the occurrence of *Ips typographus* L. and spores brought in by the beetle is a causal agent of necrotic damages to phloem tissues.

Responses to an artificial infection were variable and responses observed in one tree differed also radically.

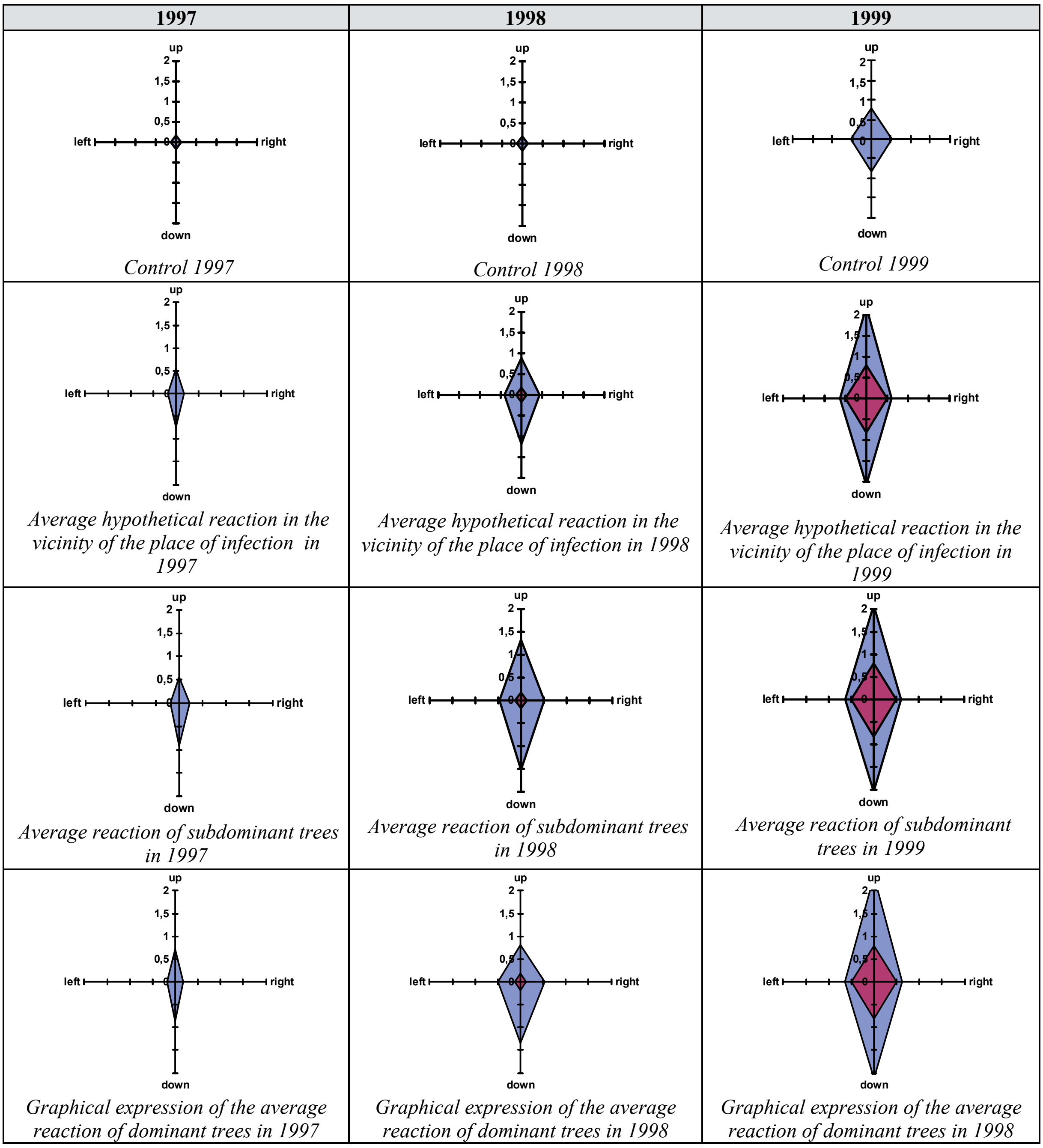
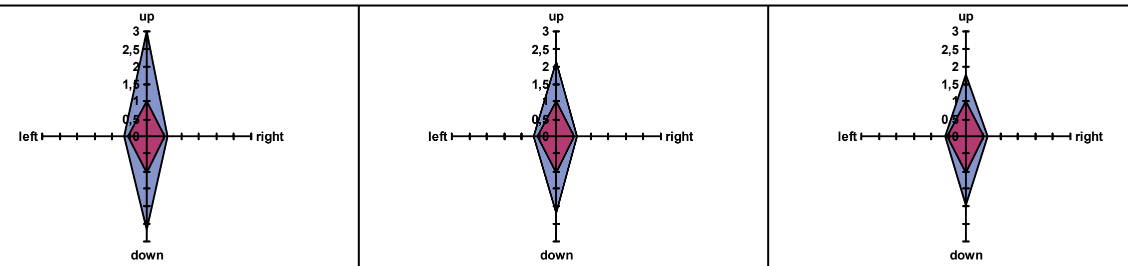


Fig. 2. Graphical expression of a response in the vicinity of infection in selected groups of trees. Note: In 1999, reactions were measured from the centre of wound, in 1997 and 1998, reactions were measured from the border of a hole perforated by a punch



Average reaction in the vicinity of infection by an untreated imago. Control is marked in the centre

Average reaction in the vicinity of infection by imago treated with Fundazol. Control is marked in the centre

Average reaction in the vicinity of infection by imago treated with Ibefungin. Control is marked in the centre

Fig. 3. Graphical expression of an average reaction in the vicinity of infection by differently treated imagoes in 1999

Compared to expectations, the zones spread rather downward which indicates that fungus metabolites spread by assimilatory flux in sieve tubes. The width of zones in particular variants of the experiment arrangement was roughly comparable. On average, marked differences between the subdominant tree variant and dominant tree variant or trees with an interrupted and uninterrupted root system were not found (Fig. 2). A response in dominant trees with the interrupted root system (where compared to the control group different reaction was observed) was an exception. In the direction of transpiration flux ('upwards') the zone was longer and on the contrary in the direction of the flux of assimilates the reaction zone was shortened.

Treatment of *Ips typographus* imagoes by fungicides Ibefungin and Fundazol preparations did not demonstrate expected effects in full scale. Comparing variants when imagoes treated by fungicides were used it is possible to observe decrease in the size of reaction zones in case of inoculations by treated imagoes by several type of fungicide (Fig. 3).

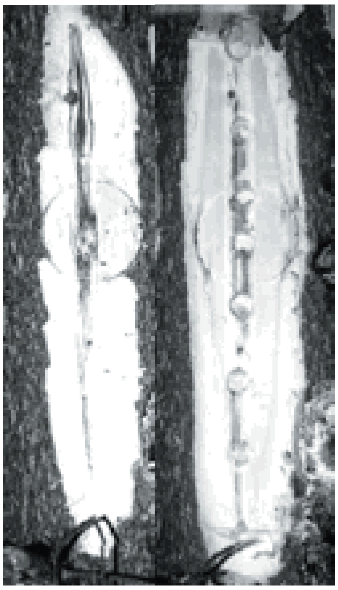


Plate 3. The example of induced wound reaction after artificial inoculation by bark beetle with visible blue staining which was visible 15 mm to the depth of sapwood. On the picture is situation after removing of the bark. On the left side is situation after removing of bark and 3–5 mm of sapwood. The *Leptographium* cf. *lund-bergii* was re-isolated from this wound (Photo L. Jankovský)

The comparison of summarised reactions is visible from Fig. 1. The differences between control wound and wound with inoculum are obvious. They are not significant excesses between group of dominant and subdominant trees and between tress with abrupt and not abrupt buttresses**.**

Re-isolation of fungi from wound

Re-isolation were studied at two localities. At the first of them, mycoflora was determined in six infection experiments and at the second in ten experiments. Results obtained at both localities differ fundamentally.

At the first locality situated in the Drahany Highland, eight species of fungi were determined so far in six samples taken from trees at the locality. The highest frequency of occurrence showed *Leptographium* sp. 1 and basidiomycetes sp. 1. A smaller frequency demonstrated: *Ceratocystis polonica*, *Leptographium* sp. 1, *Leptographium* sp. 2, *Graphium* sp 1., *Ophiostoma bicolor* Davidson & Wells, white sterile mycelium and a dematium-like sterile fungus. A very similar spectrum was found at the study of *Ips typographus* surface mycoflora.

At the second locality Podlesí near Kuřim, 10 samples of wood and bark were taken from experimental inoculations. From 75 samplings 27 samples were sterile. None of determined fungi can be designated as dominant. The spectrum of species found is rather similar to the endophytic mycoflora of spruce than a mycoflora accompanying spruce bark beetle (*Leptographium* cf. *lundbergii* Lagerberg & Melin., *Pezicula eucrita* Karst., *Phomopsis* sp., yeast sp.1, yeast sp. 2, coelomycet sp. 2, brown sterile colony sp. 3, coelomycet sp.1, brown sterile colony sp. 1, coelomycet sp. 3, hyphomycet sp. 1, brown sterile colony sp. 4, brown sterile colony sp. 2, white hyphomycet, brown sterile colony sp. 5. None representative of the genus *Ophiostoma* sp., *Ceratocystis* sp. or *Ceratocystiopsis* sp. was determined so far. There is, however, an exception, viz. *Leptographium* cf. *lundbergii* Lagerberg & Melin. which was isolated from several artificial infections which were characterized by markedly blue staining of the vicinity of infection.

In spite of the infection of tissues by several ophiostomoid fungi as an effect of inoculation by bark beetle imagoes, any symptoms of decline or yellowing were not observed in monitored spruce trees until 2002.

DISCUSSION

Fungal infections appear to be an important factor of the successfulness invasion of cambioxylophagous insect and overcoming defensive reactions of a host tree (LORIO, HODGES 1985).

Spores of fungi are natural part of the surface mycoflora of cambioxylophagous insect including *Ips typographus*. In addition to non-pathogenic species, *Ceratocystis polonica* was noticed which is considered to be a potential pathogen (SOLHEIM 1986, 1988, 1991, 1993; KROKENE, SOLHEIM 1996, 1997, 1998). In Austria, KIRISITS (1998), KIRISITS and ANGLBERGER (1999) and KIRISITS and OFFENTHALER (2000) came to similar conclusions. *Ips typographus* is associated with a number of vascular pathogens, e.g. from the genera *Ophiostoma* and *Ceratocystis* it is *Ceratocystis polonica*, *Ophiostoma penicillatum, O. bicolor, O. piceae, O. minutum, O. tetropii* etc. (SOLHEIM 1988). *C. polonica* appears to be the most important forest species which is able to colonize tissues of healthy sapwood (SOLHEIM 1986). In itself, it is able to kill healthy trees (HORNTVEDT et al. 1983; SOLHEIM 1988). *Ceratocystis polonica* was able to grow for a longer time than other species transmitted by *Ips typographus* under the lack of oxygen which can be considered to be a property characteristic of pathogens of vascular tissues (SOLHEIM 1991).

Ceratocystis polonica is constantly at the advancing front of micro-organisms spreading into *I. typographus* – attacked trees (SOLHEIM 1992). The experiments have also established that *C. polonica* is pathogenic enough to kill trees of various spruce species when artificially inoculated under the bark in adequate doses (e.g., HORNTVEDT et al. 1983; CHRISTIANSEN 1985a). Douglas-fir may also get killed by artificial inoculation of this fungus (CHRISTIANSEN, SOLHEIM 1990).

The pathogenicity of semi ambrosia fungi associated with different bark beetles varies considerably, but all aggressive bark beetles are associated with at least moderately pathogenic blue stain fungi which play probably an important role in tree killing (KROKENE 1996).

In the vicinity of places of tree inoculation by *Ips typographus* L., it is possible to expect the spread of fungi of surface mycoflora and at the same time the response of host tissues (JANKOVSKÝ et al. 1998). SOLHEIM (1988, 1991) considers the size of wound reaction to artificial infection by pure cultures of ophiostomoid fungi to be the manifestation of pathogenicity of the fungi. The most marked reaction was observed in *Ceratocystis polonica*. In the studied wound reaction, however, it is possible to observe both the reaction of host tissues and the activity of fungi. SHIGO (1991) terms as compartmentation a phenomenon when living parenchymatous cells prevent the propagation of necrosis to sides.

CHRISTIANSEN (1992) did not find a difference in the spread of an artificially induced infection by *Ceratocystis polonica* between a stand with artificially simulated water stress and a control stand.

These results can be confirmed through the comparison of wound infections in a set of trees where buttresses were artificially interrupted and in trees without any breakage.

KROKENE and SOLHEIM (1996) corroborated the occurrence of ophiostomoid fungi on the set of 5 beetles and at the same time, they studied wound reactions after inoculation of 5 species of beetles. The most marked necrosis of phloem was observed in *Ips typographus* and *Ips duplicatus*.

The spectrum of fungi, re-isolated from the wound was comparable with this one from the surface of imagoes of *Ips typographus,* including observation of potentially pathogenic *Ceratocystis polonica* (JANKOVSKÝ et al. 2000). Other determined species *Ophiostoma bicolor* Davidson & Wells belongs to the most abundant species of the accompanying mycoflora of spruce bark beetle. In Europe, it was recorded for the first time by KOTÝNKOVÁ-SYCHROVÁ (1966) in galleries of spruce bark beetle from the Šumava Mts.

Although the observed strong wound reaction is assigned to *Ceratocystis polonica* (KROKENE 1996) in some papers, we isolated from similar reactions besides *Ceratocystis polonica* also other species which cause significant blue staining, especially *Leptographium* cf. *lundbergii*.

CONCLUSION

In tissues of examined spruce trees, 7 species of fungi were found out in the course of isolation from experimental inoculations. Most abundant were *Leptographium* sp. 1 and a basidiomycet sp. 1. The *Ceratocystis polonica,* as potentially pathogenic fungi were observed in bark beetles and in re-inoculations from wound artificially inoculated by imagoes of bark beetles.

Tissue necroses and sapwood blue-staining were observed in the vicinity of infection places as the result of artificial inoculation of spruce by imagoes of spruce bark beetle. Back isolations were not quite identical in all cases with the associated mycoflora of *Ips typographus* L. imagoes.

Imagoes of *Ips typographus* from observed areas functions as vectors of potentially pathogenic vascular mycosis, especially *Ceratocystis polonica*. This species were reinoculated from wound reaction after artificial inoculations using imagoes of *Ips typographus.* The other species *Leptographium* cf. *lundbergii* was present in the most visible wound reactions.

Nevertheless, a relationship was demonstrated between spruce bark beetle as the vector of a number of potentially pathogenic fungi and necrotic reactions or blue-staining in the vicinity of gallery systems. Phloem necroses and infections of sapwood and phloem by vascular mycoses appear to be one of the main causes of disturbance of the function of conducting tissues of spruce after the invasion of spruce bark beetle. Even at the unsuccessful invasion of the beetles, vascular mycoses spread in tissues disturbing plant water relations of the tree species. Probability of the successful repeated invasion of *Ips typographus* L. increases with the predisposition of a host species. A similar relationship of the activity of insect and fungi as an invasion agent is probably a common event in nature requiring further research with respect to the necessity to explain various types of decline.

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Received for publication March 3, 2003
Accepted after corrections June 20, 2003

Ranové reakce smrku na umělou inokulaci imagy lýkožrouta smrkového *Ips typographus*

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ABSTRAKT: Na souboru stromů byly provedeny infekční pokusy s imagy lýkožrouta smrkového *Ips typographus* L. původem ze Šumavy a Školního lesního podniku Křtiny. U stejného souboru brouků byla kvalitativně zjišťována povrchová mykoflóra. Cílem zpětných izolací bylo zjistit, zda druhy nalezené na povrchu imag *Ips typographus* se po inokulaci šíří i hostitelskými pletivy. V okolí inokulace imagy *Ips typographus* jsou patrny výrazné nekrotické zóny včetně symptomů pronikání vaskulárních patogenů lýkem i bělí. Nejvýraznější reakce byly pozorovány v případě inokulace neošetřeným imagem lýkožrouta smrkového (*Ips typographus*). Ošetření imag lýkožrouta smrkového přípravky Ibefungin a Fundazol nemělo v plném rozsahu očekávaný efekt. Na povrchu těl brouků byly pozorovány spory potenciálně patogenní houby *Ceratocystis polonica* (Siem.) C. Moreau, která byla zjištěna i ze zpětných izolací z pletiv okolí umělých infekcí lýkožroutem smrkovým. Zde byly zjištěny i některé další druhy jako *Ophiostoma bicolor* Davidson & Wells, *Leptographium* cf. *lundbergii* Lagerberg & Melin., *Pezicula eucrita* Karst., *Phomopsis* sp.

Klíčová slova: ophiostomatální houby; patogeni; vaskulární pletiva; *Ceratocystis polonica; Ophiostoma bicolor*; ranové reakce; vaskulární mykózy; *Ips typographus*

V posledních letech je ve spojitosti s podkorním hmyzem stále více diskutována úloha hub vaskulárního vadnutí, resp. patogenů vaskulárních pletiv (Mrkva, Jankovský 1996) v souvislosti s chřadnutím dřevin. Fytopatogenní aspekty hub doprovodné mykoflóry lýkožrouta smrkového uvádí např. Christiansen (1985a,b), Christiansen a Solheim (1994), Horntvedt a Solheim (1991) a Jankovský et al. (1998).

V okolí míst inokulace stromu lýkožroutem smrkovým lze očekávat rovněž šíření hub povrchové mykoflóry a zároveň reakce hostitelských pletiv (Jankovský et al. 1998). Solheim (1988, 1991) považuje velikost ranové reakce na umělou infekci čistými kulturami ophiostomatálních hub za projev patogenity těchto hub. Nejvýraznější reakci pozoroval u druhu *Ceratocystis polonica* (Siem.) C. Moreau.

V pozorované ranové reakci lze však sledovat jednak reakci hostitelských pletiv, jednak aktivitu hub. Shigo (1991) označuje jev, kdy živé parenchymatické buňky zabraňují šíření odumírání do stran, kompartmentací odumírání stromů.

V pletivech smrků bylo zpětnými izolacemi zjištěno sedm druhů hub. Nejhojnějšími byly druhy *Leptographium* sp. 1 a basidiomycet sp. 1.

Jako důsledky umělé inokulace smrku imagy lýkožrouta smrkového byly pozorovány nekrózy pletiv a zamodrání běli v okolí míst infekce. Zpětné izolace nebyly ve všech případech zcela identické s doprovodnou mykoflórou použitých imag lýkožrouta smrkového. Přesto však byla prokázána spojitost mezi lýkožroutem smrkovým jako vektorem řady potenciálně patogenních hub a nekrotickými reakcemi, resp. zamodráním v okolí požerků. Nekrózy lýka a infekce běli i lýka vaskulárními mykózami jsou jednou z hlavních příčin narušení funkce vodivých elementů smrku po náletu lýkožrouta smrkového. I při neúspěšné invazi brouka se v pletivech šíří vaskulární mykózy, dále narušující vodní režim dřeviny. Pravděpodobnost úspěchu opakovaného náletu lýkožrouta se zvyšuje s predispozicí hostitelské dřeviny. Podobné propojení aktivity hmyzu a hub jako invazního agens je pravděpodobně v přírodě běžným jevem, vyžadujícím další studium s ohledem na potřeby vysvětlení různých typů chřadnutí.

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